In-silico optimization and molecular validation of putative anti-HIV antimicrobial peptides for therapeutic purpose

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Philosophiae Doctor

In the Department of Biotechnology

Faculty of Life Science, University of the Western Cape

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DECLARATION

I declare that "*In-silico* **optimization and molecular validation of putative anti-HIV antimicrobial peptides for therapeutic purpose**" is my own work, that it has not been submitted for degree or examination at any other university, and that all the resources I have used or quoted, and all work which was the result of joint effort, have been indicated and acknowledged by complete references.

Marius Belmondo Tincho

December 2016

Signed

ABSTRACT

In-silico optimization and molecular validation of putative anti-HIV antimicrobial peptides for therapeutic purpose

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AIDS is considered a pandemic causing millions of deaths worldwide and a cure for this disease is still not available. Failure to implement early treatments due to the poor diagnostic methods and ineffective therapeutic regimens to treat HIV patients to achieve complete viral eradication from the human body has encouraged the escalation of this disease at an exponential rate. Though the current treatment regimens (High Active Antiretroviral Therapy) have aided in increasing the lifespan of HIV patients, it still suffers from some shortcomings such as adverse side effects and non-eradication of the virus. Thus, there is a need for a non-toxic therapeutic regimen to stop further infection of HIV-infected patients. Antimicrobial Peptides (AMPs) are naturally occurring peptides which are components of the first line of defence of many organisms against infections and have been proven to be promising therapeutic agents against HIV. The use of AMPs as anti-microbial agents is due to the fact that most AMPs have a net positive charge and are mostly hydrophobic molecules. These features allow AMPs to be site directed electro-statistically to the mostly negatively charged pathogens. In a previous study, a number of novel anti-HIV AMPs was identified using a predictive algorithm Profile Hidden Markov Models (HMMER). The AMP's three-dimensional structures were predicted using an _in-silico_ modelling tool I-TASSER and an _in-silico_ protein-peptide interaction study of the AMPs to HIV protein gp120 was performed using PatchDock. Five AMPs were identified to bind gp120, at the site where gp120 interacts with CD4 to prevent HIV invasion and HIV replication. Therefore, the aims of this research

were to perform *in-silico* site-directed mutation on the parental anti-HIV AMPs to increase their binding affinity to the gp120 protein, validate the anti-HIV activity of these peptides and confirm the exclusivity of this activity by testing possible anti-bacterial and anti-cancer activities of the AMPs.

Firstly, the five parental anti-HIV AMPs were used to generate mutated AMPs through *in-silico* site-directed mutagenesis. The AMPs 3-D structures were determined using I-TASSER and the modelled AMPs were docked against the HIV protein gp120 using PatchDock. Secondly, an "in house" Lateral Flow Device (LFD) tool developed by our industrial partner, Medical Diagnostech (Pty) Ltd, was utilised to confirm the in-silico docking results. Furthermore, the ability of these AMPs to inhibit HIV-1 replication was demonstrated and additional biological activities of the peptides were shown on bacteria and cancer cell lines.

In an effort to identify AMPs with increased binding affinity, the *in-silico* results showed that two mutated AMPs Molecule 1.1 and Molecule 8.1 bind gp120 with high affinity, at the point where gp120 bind with CD4. The molecular binding however showed that only Molecule 3 and Molecule 7 could prevent the interaction of gp120 protein and CD4 surface protein of human cells, in a competitive binding assay. Additionally, the testing of the anti-HIV activity of the AMPs showed that Molecule 7, Molecule 8 and Molecule 8.1 could inhibit HIV-1 NL4-3 with maximal effective concentration (EC_{50}) values of 37.5 µg/ml and 93.75 µg/ml respectively. The EC_{50} of Molecule 8.1 was determined to be around 12.5 µg/ml. This result looks promising since 150 µg/ml of the AMPs could not achieve 80% toxicity of the human T cells, thus high Therapeutics Index (TI) might be obtained if 50% cytotoxic concentration (CC_{50}) is established. Further biological activity demonstrates that Molecule 3 and Molecule 7 inhibited *P. aeruginosa* completely after 24 hours treatment with peptide concentrations ranging from 0.5 mg/ml to 0.03125 mg/ml. Nevertheless, moderate inhibition was observed when CHO, HeLa, MCF-7 and HT-29 were treated with these peptides at peptides concentration of 100 µg/ml.

The ability of these AMPs to block the entrance of HIV via the binding to CD4 of the host cells is a good concept since they pave the way for the design of anti-HIV peptide-based drugs Entry Inhibitors (FIs) or can be exploited in the production microbicide gels/films to suppress the propagation of the virus.

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LIST OF ABBREVIATIONS

XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrozlium-5-carboxanilide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
APS	Ammonium Persulfate
ARV	Anti-Retroviral therapy
APD	Antimicrobial Peptide Database
AMPs	Antimicrobial Peptides
ACE	Atomic Contact Energy
BLI	Bio-Layer Interferometry
CA	Capsid protein
CD	Circular Dichroism
CRFs	Circulating Recombinant Forms
CLSI	Clinical and Laboratory Standards Institute
CD4	Cluster of Differentiation 4
cDNA	Complementary DNA
C-score	Confidence Score
CTL	Cytotoxic CD8+ T lymphocytes

CC	Cytotoxic Concentration
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
DPI	Dual Polarization Interferometry
DMEM	Dulbecco's Modified Eagle's medium
DPBS	Dulbecco's Phosphate Buffer Saline
EC	Effective concentrations
EIs	Entry Inhibitors
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FRET	Fluorescence Resonance Energy Transfer
FDA	Food and Drug Administration
FIs	Fusion Inhibitors
GLAM2	Gap Local Alignment of Motifs 2
GST	Glutathione-S-Transferases
AuNPs	Gold Nanoparticles
Ham-12	Ham F-12 Nutrient Mixture
HPLC	High-Performance Liquid Chromatography
HAART	Highly Active Anti-Retroviral Therapy
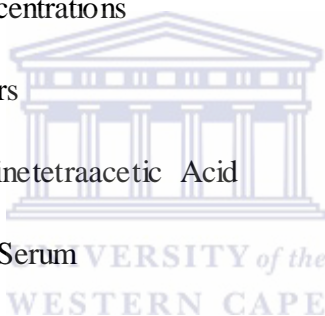
HIV	Human Immunodeficiency Virus
HLA	Human Leukocytes Antigen
HPV	Human Papillomavirus
IFA	Immunofluorescence Assay
IN	Integrase
INSTIs	Integrase Stand-Transfer Inhibitors
ICAM-3	Intercellular Adhesion Molecule 3
INT	Iodonitrotetrazolium Chloride
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
I-TASSER	Iterative Threading ASSEmbly Refinement
kDa	Kilo Dalton
KFC	Knowledge-based FADE and Contacts
LFD	Lateral Flow Device
LD	Linear Discriminant Analysis
LTR	Long terminal repeat
LTNP	Long-term non-progressors
LB	Luria Broth
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin Resistance *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MST	Microscale Thermophoresis

MIC	Minimum Inhibition concentration
MHC	Multiple Histocompatibility Complex
MAC	Mycobacterium avium complex
nm	Nanometer
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NC	Nucleocapsid
NRTIs	Nucleoside Reverse–Transcriptase Inhibitors
O.D	Optical Density
ORFs	Origin Reading Frames
PRPs	Pathogen Recognition Receptors
PAMP	Pathogen-associated Molecular Pattern
PBS	Phosphate-Buffered Saline
HMMER	profile Hidden Markow Models
PML	Progressive multifocal leukoencephalopathy
PR	Protease
PIs	Protease Inhibitors
PDB	Protein Data Bank
QSAR	Quantitative Structure–Activity Relationship
RF	Random Forest
RUs	Resonance Units
RT	Reverse Transcriptase
RPM	Revolutions Per Minute

RNA	Ribonucleic acid
RMSD	Root Mean Square Deviation
RPMI	Roswell Park Memorial Institute medium
SW	Sliding Window
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for the Social Sciences
SSA	Sub-Saharan Africa
SVM	Support Vector Machine
SU	Surface glycoprotein
SPR	Surface Plasmon Resonance
TEMED	Tetramethylethylenediamine
UNAIDS	The Joint United Nations Programme on HIV/AIDS
3-D	Three-Dimensional
TM	Transmembrane glycoprotein
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
UN	United Nations
MA	Viral Matrix
WHO	World Health Organization
INCI	N-lauroylsarcosine or sodium lauryl sarcosinate

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DEDICATION

I dedicate this work to mother and father, my entire family and close friends for their endless supports.

In memory

of

Sigue Takou Jerome

A mentor and a friend
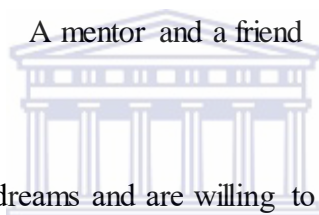
"Blessed are those who dream dreams and are willing to pay the price for their dreams to become true"

CHAPTER ONE: LITERATURE REVIEW

1.1. Introduction

The world has witness deathly pathogens, starting from the mid 19th century to the earlier 21st century. Some of these pathogens included Cholera, Malaria parasite, Poliovirus, Ebola virus, Chikungunya virus, Viral hepatitis, *Mycobacterium tuberculosis*, Smallpox, Typhus, Herpes simplex virus and last but not least, the Human Immunodeficiency Virus (HIV), just to name a few (Pacini, 1854; Pike, 2007; Gallagher, 1990; Kuhnke, c1990; Conlon, (n.d.); Bruns, 2000; Marr, and Cathey, 2013; Khaled, 1993; Miller, 2005; WHO, 2008; UNAIDS, 2010). Though these pathogens have caused many fatalities amongst human populations, researchers have managed to develop advanced techniques and treatment regimens that have enabled most of these pathogens to be kept under control. Moreover, some of these parasitic microbes have been eradicated in certain geographic areas (Baxby, 1999; Stern, and Markel, 2005; Pillay *et al.*, 2009). Nonetheless, the Human Immunodeficiency Virus (HIV) is one of the few pathogens cited above which has caused many deaths on earth and is still of a major clinical concern in certain areas of the world, particularly in Sub-Saharan Africa (SSA) (Kendall, 2012).

Human Immunodeficiency Virus (HIV) causes the condition named Acquired Immune deficiency Syndrome (AIDS). This condition is mainly termed because of the fact that the infection by any type of HIV (HIV-1 and HIV-2) affects the individual's immune system. Later on, the immune system of the infected individual deteriorates as the virus gets into healthy human cells. In the long run, the human immune system cannot withstand other elementary bacterial infections hence the establishment of opportunistic diseases. The situation at which the human immune system collapses thus gives the denomination Acquired Immune deficiency Syndrome (AIDS).

The deterioration of the immune system is mostly affected since the primary cells that are responsible for the body defence system, namely monocytes, macrophages, dendritic cells and T lymphocytes, are targeted by HIV (Chan *et al.*, 1997). This

advantage is possible as HIV utilises the CD4 receptor on the surface of macrophages, monocytes and T lymphocytes to gain entrance into these cells. Furthermore, the virus utilises the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) receptor on macrophages and dendritic cells to mediate their entrance into healthy CD4+ T cells (Geijtenbeek *et al.*, 2000b; Pope and Haase, 2003; Su *et al.*, 2004). Since the CD4 molecule is at the centre of the HIV infection regardless of the initial route of invasion, a decrease in the body CD4 molecule count is observed as the condition of the patient becomes chronic and the immune system progressively deteriorates. The decrease of CD4 is as a result of depletion of the above-mentioned cells.

The inability of the human body to fight back HIV invasion, the collapse of the human immune system as a result HIV infection and the rate at which people are infected, make HIV accounts for the infection with largest number of individuals living with an infectious disease in the modern world. As a result of the abovementioned, AIDS was declared an epidemic by UNAIDS (Kallings, 2008). Judging from the current situation and even though some efforts have been made to combat the virus and to reduce HIV infection, the number of HIV-infected people has not decreased substantially. As from 2013, UNAIDS global statistics report declares that 35 million peoples were still living with HIV, with about 2.1 million new infections in the same year. In addition, this epidemic has caused an average of 1.5 million AIDS-related deaths as from 2013. Looking at the epidemic since its inception in 1981, 39 million people have died from AIDS-related illnesses and 78 million people have become infected with this virus (UNAIDS fact sheet, 2014).

Whilst tremendous efforts have been made to develop drugs that could stop the AIDS condition, it has been impossible to achieve the goal. Thus, there is neither a cure to destroy the virus nor an HIV vaccine to date, to prevent viral infection. The only potential treatment developed so far consists of Highly Active Anti-Retroviral Therapy (HAART) or Anti-Retroviral therapy (ARV). This treatment regimen can only help manage the AIDS condition, so as to prevent further deterioration of the body's immune system, by slowing the progression of HIV and increase the life span of the patient (Dybul *et al.*, 2002; Burgoyne and Tan, 2008). However, the treatment regimen is accompanied with some side effects, which include: hyperlactatemia, hepatotoxicity, hyperglycemia, osteonecrosis, osteopenia and osteoporosis,

lipodystrophy syndrome, dyslipidaemia (Montessori *et al.*, 2004), hyperlipidaemia and an increased risk of cardiovascular disease (Volberding and Deeks, 2010).

While the current treatment regimens can improve the disease condition and increase the life span of the patient, further solutions for HIV treatments ought to be addressed. Therefore, there is a need for additional therapeutic molecules besides the existing HAART drugs, which can prevent gp120 attachment to CD4+ T cells and the penetration of HIV into human cells. This approach would limit further contamination of healthy human cells and prevent spreading of the HI Virus within infected individuals. Antimicrobial Peptides (AMPs) have been put forward as a potential class of molecule that can be utilised to prevent HIV contamination in human cells (Wang *et al.*, 2010; Chen *et al.*, 2012).

AMPs are considered to be the first line of defence of many prokaryotes and eukaryotes and have shown to have a wide range of activity against gram-negative and gram-positive bacteria, fungi, cancer cells, protozoa as well as viruses (Andreu and Rivas, 1998). Experimental data have also shown that AMPs could inhibit HIV proliferation and replication (Wang *et al.*, 2010), and would, therefore, be a likely candidate for the design of future peptide-based drugs. Although these AMPs display promising therapeutic activity, their unique physicochemical properties and qualities could encourage their application as potent microbicides against HIV (Lalezari *et al.*, 2003; Dwyer *et al.*, 2007). These AMPs might be useful as microbicides in gels, creams, films, or suppositories to prevent infection of HIV and if possible other Sexually Transmitted Infections (STIs). Additionally, they could be enhanced into intravenous solution, to be administered to HIV-infected individual, to prevent further binding of HIV to the patient CD4+ T cells. An example of such a fusion inhibitor Enfuvirtide, the first HAART which backbone has an AMP origin, and which was FDA approved in 2003 (Lalezari *et al.*, 2003). This AMP based drug could stop or reduce viral replication in human cells and therefore suppress spreading of the virus in infected patients.

The first section of this review will focus on the HIV-1 genome, its structure, and life cycle. The human immune system during HIV-1 infection, the evasion of this immune system by the HI Virus will be discussed in the later part of this section. The second section will elaborate on the ability of AMPs to counter HIV invasion, based on the

virus structures and its mode to evade the human immune system. Finally, the review will look at the various approaches to applying these AMPs as new molecules to produce HAART or ARVs.

1.2. HIV/AIDS

Human Immunodeficiency Virus (HIV) is a retrovirus, which belongs to the *Retroviridae* family. It has a well-developed and advanced structure as compared to other single cell microorganisms, especially for a virus. Such structure has enabled the HI Virus to establish a perfect defence mechanism to invade the host strategic cells such as the macrophages and dendritic cells. These cells favour HIV invasion because the virus uses the surface receptor CD4+ molecule to get entrance into these host cells. Additionally, these mechanisms are able to evade the human immune system because the virus can bypass these defences (Kamp *et al.*, 2000).

1.2.1. The structure and life cycle of HIV

1.2.1.1. HIV genome and structural organisation

A mature HIV virion is spherical in shape and has a size of 100 to 120 nm in diameter. It is made up of a lipid bilayer that encapsulates its cytoplasmic contents. This lipid bilayer membrane is constituted of viral *Env* glycoproteins and viral proteins (Sierra *et al.*, 2005). Attach to this lipid bilayer are the viral glycoproteins, the surface membrane protein glycoprotein gp120, and the transmembrane glycoprotein gp41. Just beneath the viral membrane are the matrix antigen protein p17, followed by the capsid core protein p24, which enclose the two unspliced genomic RNAs of the virus, forming a ribonucleoprotein complex. The two complete genomic RNA strands could explain the reason whilst HIV is called a diploid virus. However, the genetic material of HIV within infected cells is kept in the form of a proviral double-stranded DNA. Besides the RNA genome, integrase, protease and reverse transcriptase are found in the capsid core. Additional proteins included in the capsid core are the accessory proteins *rev*, *tat*, *vpr*, *vpu* and *nef* (Figure 1.1).
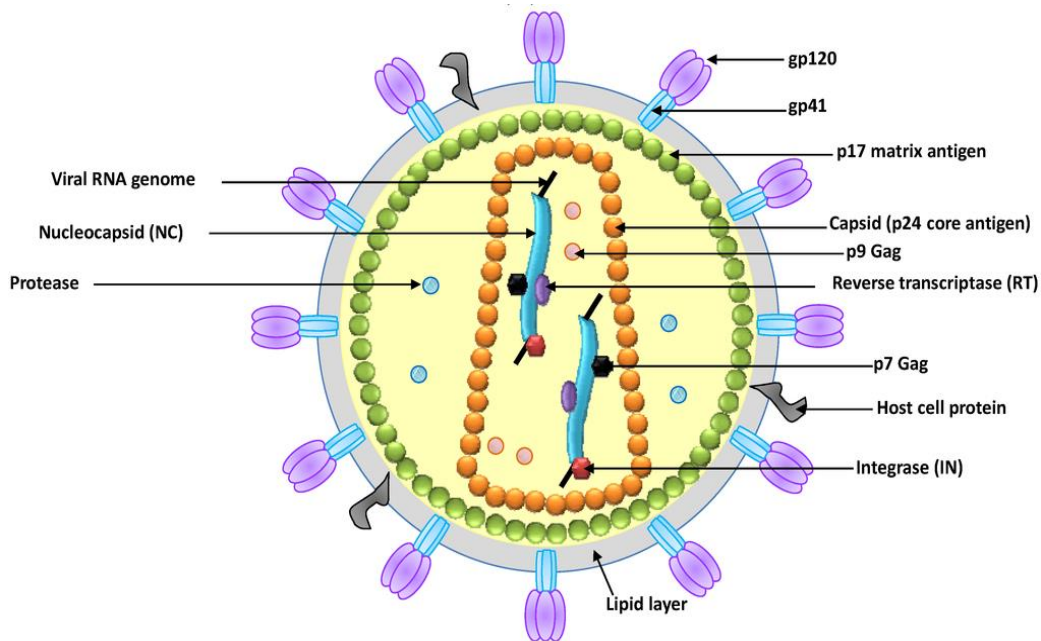
Figure 1.1: Physiological arrangement of an HIV virion. Figure adapted from Shum *et al.*, 2013.

A mature viral genome is made up of a single-stranded, positively charged RNA, roughly 9,7 Kb in length (Figure 1.2). The HIV RNA genome is structurally arranged with seven genetic elements: Long Terminal Repeat (LTR), Trans-activation Response Element (TAR), HIV Rev Response Element (RRE), PE, SLIP, Cis-acting nuclear Retention Sequences (CRS) and INS. These genetic elements serve as the baseline for the future genetic material for either reverse transcription and/or proteins synthesis. Once the fusion of HIV with the host cell has occurred and reverse transcription has happened, a double-stranded viral DNA genome is formed.

The viral DNA consists of nine Open Reading Frames (ORFs), namely *gag*, *pol*, *vif*, *vpr*, *vpu*, *tat*, *rev*, *env* and *nef*. Occasionally, a 10th gene named *tev* (p28) is part of the genomic constituent of HIV (Kuiken *et al.*, 2008). Each gene will be responsible for the rebuilding of the new mature HI Virus to be produced at the end of the life cycle. Hence, the *gag* gene encodes for the viral matrix (p17), capsid (p24) and nucleocapsid (p7). The *pol* gene encodes for protease (p10), reverse transcriptase (p66/p51), integrase (p32) and RNase H. The *env* encodes for the surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41). The *vif* is associated with HIV infectivity, while *rev* has a role of expressing the viral protein. *Vpr*, *vpu*, *tat,* and *nef* encode respectively for the viral protein R, viral protein U, transactivator protein and the negative regulator protein (Suzuki and Suzuki, 2011).
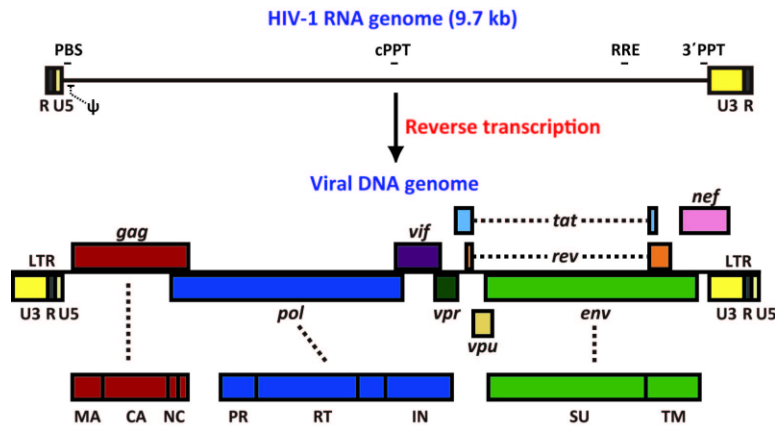
Figure 1.2: The genomic arrangement of HIV. The various colours are used to differentiate the segments on HIV genome. The numbering of each position in HIV is made according to HXB2CG. The positions of the major genomic segments are shown. LTR: Long terminal repeat, MA: viral matrix, CA: capsid protein, NC: nucleocapsid, PR: protease, RT: reverse transcriptase, IN: integrase, SU: surface glycoprotein and the TM: transmembrane glycoprotein. HIV accessory genes: *vif*, *vpr*, *tat*, *rev*, *nef* and *vpu* (for HIV-1) or *vpx* (for HIV-2). The figure was adapted from Suzuki and Suzuki, 2011.

1.2.1.2. HIV classification and genetic variability

HIV-1 is the most mentioned HIV type in literature though another type of this virus exists: HIV-2. The emphasis is mostly placed on HIV-1 because this type of the virus is more virulent and more infective as compared to its counterpart HIV-2. Thus, HIV-1 accounts for the major pathogenic infection of this virus worldwide, while the low infectivity of HIV-2 enabled fewer people to be infected with the virus when exposed to it. The other major difference between HIV-1 and HIV-2 is that HIV-2 infected individuals are majorly located in West Africa while HIV-1 is distributed across the world (Reeves and Doms, 2002; Gilbert *et al*., 2003).

The differences between HIV-1 and HIV-2 infectivity may also be due to their genetic variability. Although HIV-2 is a stand-alone and a distinct type of HIV, HIV-1, however, shows some variability within the sequence of this type; hence various groups of HIV-1 have emerged to separate this diverse organism. The sequence variability arises even within the entire genome amongst various samples obtained from different patients as well as different samples obtained from the same patient (Alizon *et al*., 1986; Saag *et al*., 1988). This may be as a result of error attributed to the reverse transcriptase of HIV-1 RNA that initiates mutations within the HIV genome, during the process of reverse transcription (Mansky and Temin, 1995;

Preston *et al*., 1988). Another explanations may either be the replication rate of the virus, which is high and fast (Perelson *et al*., 1996), or the recombination of different HI Viral genomes in the host (Robertson *et al*., 1995; Burke, 1997). And lastly, the genetic variability within the same patient may be due to multiple variants of the virus, as a result of the patient being exposed to the virus multiple times. However, this aspect of recombination is rarely observed (Artenstein *et al*., 1995, Redd *et al*., 2011).

Due to the mutations and variability observed within the genome of various samples collected from HIV patients, HIV-1 could be divided into four separate groups. These groups are comprised of the "major" group M, the "outlier" group O and two groups, N and P. Group M of HIV-1 is the so-called major group due to fact that it accounts for the majority of HIV-1 infected patients around the world. The M group is subdivided into nine subtypes or clades represented by the alphabetic letters A, B, C, D, F, G, H, J and K; whilst subtype C is the most prevalent strain among HIV-1 subtypes (Gaschen, *et al*., 2002; WHO, 2011). These various subtypes were found when sequencing the genome of the virus obtained from patients, and observation was made that these sequences exhibit around 20% differences in their *env* nucleotide sequence. Furthermore, the variability between various HIV-1 groups was about 35% in the nucleotide sequence of the *env* gene (Gaschen, *et al*., 2002).

Besides the subtypes mentioned above, which belong to the M group of HIV-1, an additional subtype is said to appear in this group and is due to the combination of the various genome subtypes found within the M group, therefore the name Circulating Recombinant Forms (CRFs) (Figure 1.3). This subtype is divided again into four sub-subtypes, namely CRF01_AE, CRF02_AG, CRF03_AB, and CRF04_cpx. An example to illustrate the recombination of this sub-subtype could be CRF03_AB, which genome is majorly constituted with the genome of subtypes A and B (Robertson *et al*., 2000). The phenomena of recombination observed in HIV-1 could be explained in the case where a patient is infected with two subtypes of group M virus, the two genetic materials are mix and create a hybrid virus. This is done with a process similar to sexual reproduction, also called "viral sex" (Burke, 1997).

The geographical distribution of HIV is also diverse. While group O is restricted to West and central Africa, group N, on the other hand, is located in Cameroon as it was

discovered in this country in 1998. The group P, which is related to gorilla simian immunodeficiency, was discovered in a Cameroonian woman (Plantier *et al.*, 2009). However, group M is highly distributed in regions such as Central, South, East and West Africa, Europe, the Americas, Japan and Australia (Buonaguro *et al.*, 2007).
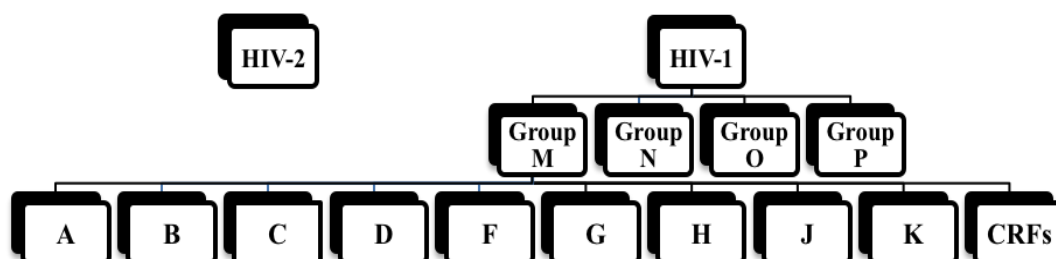
Figure 1.3: Classification of the various HIV types, groups, and sub-types. The diagram was adopted from and modified from WHO 2011.

1.2.1.3. HIV life cycle

The HI Virus life cycle only begins after it has attached its surface membrane, gp120 to the CD4 receptor molecules of macrophages, monocytes, dendritic cells and T lymphocytes (Berger *et al.*, 1999). The CD4 molecules on the surface of these cells act as the primary receptor of HIV. Hence, once the virus gp120 has attached to the CD4 surface molecule, the HIV-1 membrane protein gp120 undergoes a conformational change, allowing the exposure of the chemokine receptors (CCR5 or CXCR4) to bind to the glycoprotein gp120. The chemokine receptors play the role of co-receptors in the process of HIV infection and their attachment to gp120 enable a more stable complex formation between the virus and the human cells (Arrildt *et al.*, 2012).

Following the complex formation between HIV membrane protein and human cells, the fusion of membranes occurs, and the virus releases its contents into the human cells and the capsid membrane is sheared through an uncoating process. This procedure allows the release of viral genetic material and the associated protein into the cytoplasm of the infected human cell. Immediately after, the virus RNA undergoes reverse transcription to generate a double-stranded complementary DNA (cDNA) molecule. This step is followed by the integration of the HI Virus DNA with the human DNA, with the help of the viral integrase protein. The integrated DNA is

then transcribed into mRNA, which will then be spliced into many small segments. The various spliced mRNA will be carried from the cellular nucleoplasm to the cytoplasm where the translation of the mRNA will take place.

The translation enables the formation of accessory proteins such as *rev*, *tat,* and *nef* as these proteins are crucial for the virus assembly and reconstitution. The *tat* protein will encourage the formation of the new virus to be produced in future; whilst the *rev* protein will enable the transportation of the unspliced recombinant genetic material from the cellular nucleus to the cellular cytoplasm, where they are spliced. Also, other components of HIV such as the *gag* and *env* proteins are produced after the translation from the mRNA. The genetic material is also formed here and all the formed viral components are packaged into the future virions. As the assembly is proceeding to completion, a budding process is initiated on the human infected cell membranes and the protease cleaves the viral polyproteins to yield a functional HI Virus during the maturation process. These mature virions are now capable of infecting other human cells (Turner and Summers, 1999; Trkola, 2004).
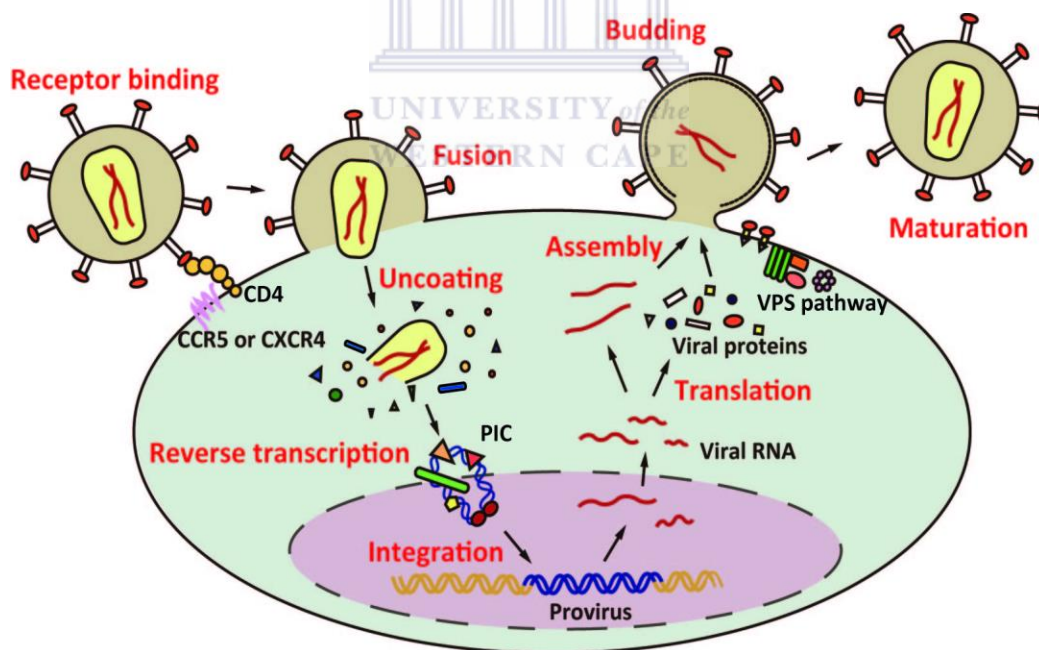
Figure 1.4: Life cycle of HIV. The figure was adapted and modified from Engelman and Cherepanov, 2013.

1.2.2. Infectivity of the disease and the principal role of gp120 in HIV infection

1.2.2.1. HIV infection and pathophysiology

HIV major routes of infection still remain the contact with HIV infected blood, mostly during blood transfusion, infection from mother-to-child during pregnancy, delivery and during breastfeeding, through sexual intercourse with an infected partner, and sharing a needle during drug injection with an infected person (Markowitz, 2007; Coutsoudis *et al.*, 2010). Once in the human body, the virus gets into contact with CD4 related cells such as macrophages, monocytes, dendritic cells and T lymphocytes, so as to gain entry into these cells (Chan *et al.*, 1997; Pope and Haase, 2003; Su *et al.*, 2004). The entrance of the HI Virus to the patient cells following the infection of a human by the virus is the initiation for the HIV/AIDS disease condition. The disease condition is characterized by two major phases, which include an acute phase and a chronic phase that might lead to AIDS, with the appearance of opportunistic illnesses. Though the initial phase of HIV infection is said to be asymptomatic and is not of a major clinical concern to the patients health, the infection however is manifested by some signs of influenza-like illness, temperature increase, headaches, inflammation of the lymph nodes and some viral infection related symptoms (Kahn and Walker, 1998). These symptoms are not of major importance since many infectious pathogens have these manifestations.

Once the macrophages, monocytes, dendritic cells and T cells have encountered the presence of the HI Virus, there is activation of the cellular and humoral immune systems via the macrophages and the dendritic cells, to inactive the HI Virus within the circulatory system. Though a rise of CD4 molecules is observed at the beginning of the HI Virus invasion, there is still a drop in the CD4 molecule count as the infection progresses with time. Such a decrease in the CD4 count in the human body is synonymous to HIV destroying the cells that are responsible for the body defence mechanisms. On the other hand, the decrease in CD4 counts correlates with an exponential increase in the viral load of HIV as the virus infects new human cells (Cadogan and Dalgleish, 2008). The decrease in CD4 molecules might also be due to the fact that CD8+ T cells destroy HIV-infected cells, as the cytotoxic T cells have to control the viral levels or by undergoing apoptosis (Pillay *et al.*, 2009). Following the depletion of CD4 molecules within a few weeks of HIV infection, there is a

progression in the immune system response, with the production of immunoglobulin G (IgG) and immunoglobulin M (IgM); hence a slow rise in CD4 counts and a reduction in viral load. These antibodies also aid in the diagnosis of HIV and give an idea of the stage of the infection (Hauck *et al.*, 2010; Wang *et al.*, 2010).

Whilst there is a gradual maintenance in the CD4+ T cells to fight the HI Virus, the viral load is still increasing as the immune system in ineffective and there is an inability to produce new T cells. This period is marked as a time of clinical latency and may persist up to a period of four to five years. This period is irreversible and the situation is progressing towards a chronic phase of the condition since there is more depletion of the mucosal CD4+CCR5+ T cells. This is aimed at preventing the infection of more T cells with the help of CCR5 co-receptor (Brenchley *et al.*, 2004). At this point, the patient ought to be placed on treatment to boost the immune system by reducing the viral load and increase the CD4 molecule count.

Though the body seems to be at a stable stage, the immune system and the defence mechanism of the body are at their maximum level of protection but cannot do much to avoid further cells contamination, as both CD4+ and CD8+ T cells are critically low. Consequently, there is a breakdown of the immune system and the installation of opportunistic illnesses, which may lead to AIDS. The main problem affecting the collapse of the immune system is due to the fact that the very same cells responsible for eliminating HIV seem to play deleterious roles by driving chronic immune activation, thus the failure of the patient defence mechanism and a progression to AIDS (Quaranta *et al.*, 2012).

1.2.2.2. Mechanism of HIV entry into host cells: the prime role of gp120

The fundamental principle of drug screening and design for a particular disease is to study the disease at a detail level, its route of infection and the different molecules involved in its propagation or its life cycle within the human cells. In the context of HIV, the entry of the virus is a multi-step process, of which many drugs have been designed to act at a particular level of the HI Virus life cycle. Regardless of these therapeutic regimens, new cells are still infected. Due to the fact that gp120 glycoprotein is a crucial HIV protein, with its role well established and found to be a key component in the process of human cells infection by the HI Virus. Reviewing its

composition, structure and role in HIV infection will help shed light on the importance of this protein in the process of viral infection. Additionally, It may illustrate some suggestions to counteract this viral infection to other healthy human cells (Kamp *et al.*, 2000; Briz *et al.*, 2006).

1.2.2.2.1. The envelope glycoprotein of HIV: the surface membrane protein gp120

The HI Virus envelope membrane is an assembly of polypeptide moieties forming the glycoprotein gp160. The splicing of gp160 generates the surface membrane protein gp120 and the transmembrane protein gp41 by cellular proteases. The surface membrane protein is anchored externally to the virus lipid bilayer and is linked to the transmembrane protein gp41 with non-covalent interactions. Both proteins are compiled in a trimer of heterodimer molecules (Dowbenko *et al.*, 1988; Finzi *et al.*, 1999). Since the gp120 forms part of the outer membrane receptor, it is the most important of both proteins as it enables the infection of human cells, by attaching to CD4 molecules of macrophages, monocytes, T lymphocytes and dendritic cells. Thus, it plays a key role in the host attachment to the HI Virus. Though the transmembrane protein gp41 is not of major importance at the initial stage of infection, it contains the fusion protein, the main role of which is to mediate host cell-HIV-1 membrane attachment, and also helps the formation of a linker for the delivery of the viral genetic material to the host cell (Pope and Haase, 2003; Su *et al.*, 2004).

A close analysis of the entire organization of the membrane protein reveals that this protein is made of segments of conserved regions in addition with extremely variable regions. It is said to exist of 6 conserved regions and they are represented as C1-C6. Nonetheless, only 5 conserved regions are recognized and designated as C1-C5 and form the core of the membrane protein. The variable regions, on the other hand, are labelled as V1-V5 and they formed the surface of the protein (Modrow *et al.*, 1987). The gp120 contains complete conserved cysteine amino acids that enable the formation of disulphide bonds; of which 9 highly conserved disulphide bonds are present on this protein (Wiley *et al.*, 1986; Leonard *et al.*, 1990).

The protein is also made up of highly glycosylated regions, which accounts for about half of the protein's molecular weight. The glycosylated moiety is attributed to the presence of roughly 24 N-linked glycans being found on gp120 (Matthews *et al.*,

1987; Leonard *et al*., 1990). Though the glycosylation enables the proper folding and final conformation of gp120 protein so as to mediate an exposed CD4-binding site (Li *et al*., 1993), additional function of the HI Virus N-linked sugars moiety is their vital role in helping the virus to escape attack from neutralization antibodies (Wei *et al*., 2003).
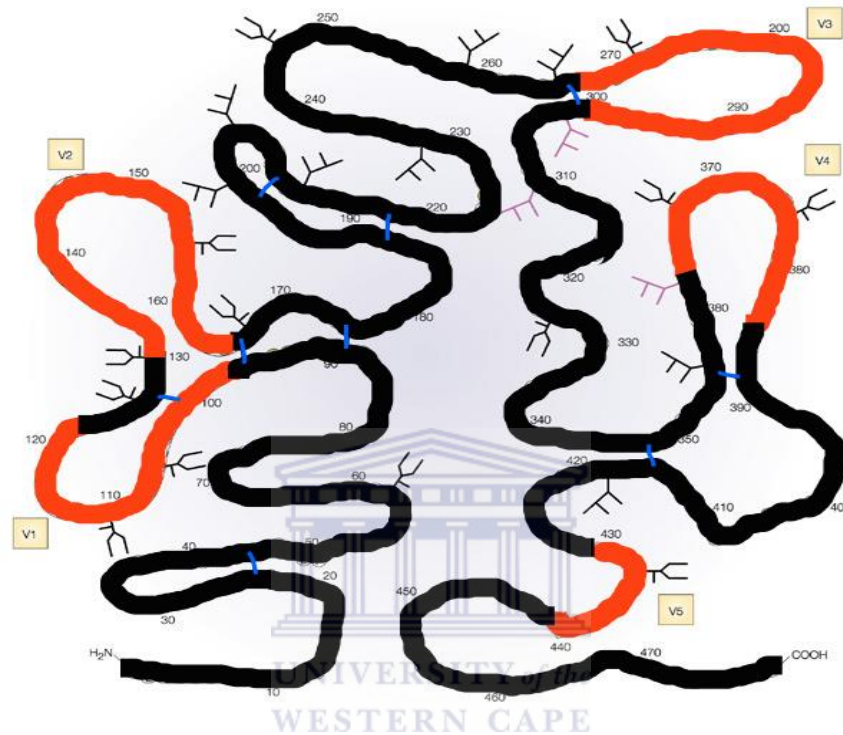
Figure 1.5: Structural representation of gp120 sequence. The variable regions are coloured orange and are labelled V1-V5, and the protein backbone is coloured black. The high-mannose, hybrid-type glycans and complex glycans are shown in black and purple. The disulfide bridges are designated in blue. This picture was taken from and modified from Zolla-Pazner, 2004.

1.2.2.2.2. The structure of gp120

Since the surface membrane gp120 is said to be the point of contact with macrophages, monocytes, T cells via CD4 molecules, thus mediating the entrance of the virus through gp120-CD4 binding. Furthermore, knowing that the adhesion of any pathogen to the host cell surface may prevent any clearance (Cossart and Sansonetti, 2004; Pizzaro-Cerda and Cossart, 2006a), there is a need to prevent gp120-CD4 interaction, thus studying gp120 structure may help develop a proper competitive inhibitor of CD4 binding.

With respect to the complexity depicted by gp120 protein, the availability of the glycosylated regions within this protein has not made it easy to obtain the protein crystal structure at its final conformation. Though the importance of these N-linked glycans have been questioned previously during the gp120-CD4 interaction, a report has however suggested that the glycosylation does not really affect gp120-CD4 interaction but the glycosylation rather enables gp120 to generate a proper conformation required for CD4 binding (Li *et al.*, 1993). Putting the above finding into context, the first crystal structure of gp120 was obtained by removing the sugars moieties found on the protein through deglycosylation and by conserving the flexible variable loops of the protein (Kwong *et al.*, 1998). The glycoprotein gp120 structure was solved in conjunction with the complex it formed between and CD4, and the antigen-binding fragment (Fab) of the anti-gp120 antibody 17b (Kwong *et al.*, 1998; Kwong *et al.*, 2000). An examination of the structure generated by Kwong *et al.*, 1998 reveals that gp120 is made up of 25 β- and 5 α-helical loops (Figure 1.6). It was also noticed that the structure is folded into two major domains. The inner domain of gp120 extends from the N- and C-termini to the V1/V2 loops, whereas the outer domain consists of the V4 and V5 loops. The two domains are connected together by a four-stranded bridging sheet (Figure 1.6).
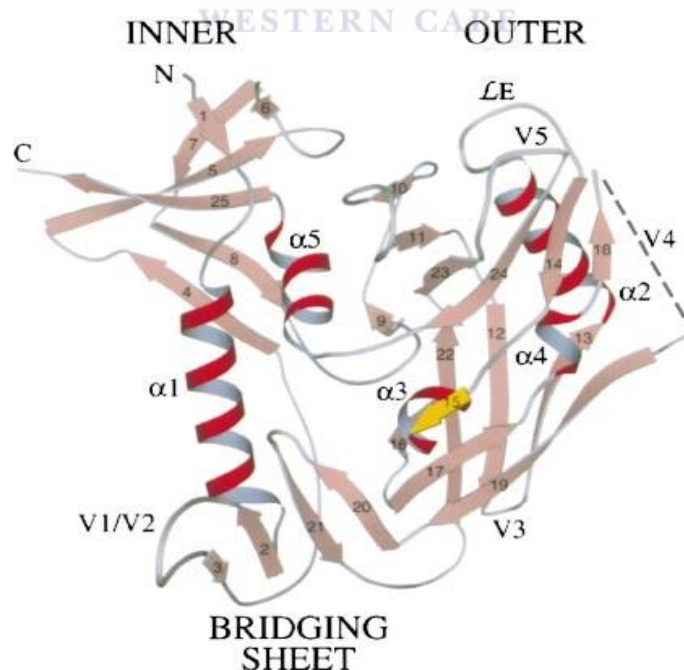
Figure 1.6: Image depicting the carton representation of gp120 conformation in its 3-Dimensional structure with the number of β- and α-helical loops. The structure is ready to bind the CD4 molecule of the host cells. The two domains are clearly visible with the bridging sheet. Adapted from Kwong *et al.*, 1998.

The complex formed by gp120, CD4 and 17b clearly illustrates the events going on during HIV infection. The complex elucidated by Kwong and colleagues in 1998 indicates that CD4 binds to gp120 at the interface of the inner and outer domains and the bridging sheet, with the strong mediation of Phe43 on CD4 (Figure 1.7).

A further study conducted by Huang *et al.*, 2004 with the aim to obtain the main residues involved in gp120-CD4 interaction and to neutralize this binding, noticed that Asp368 on gp120 and Arg59 on CD4 enable the bonds to stay together (Huang *et al.*, 2004). Conversely, the removal of Phe43 and Arg59 residues from CD4 disable the ability of gp120 interacting with the CD4 receptor (Moebius *et al.*, 1992; Ryu *et al.*, 1994). The relevance of these results permitted the design of polypeptides mimicking CD4 domain of interaction with gp120, which could stop gp120 from binding to CD4 in a competitive binding manner (Meier *et al.*, 2012).
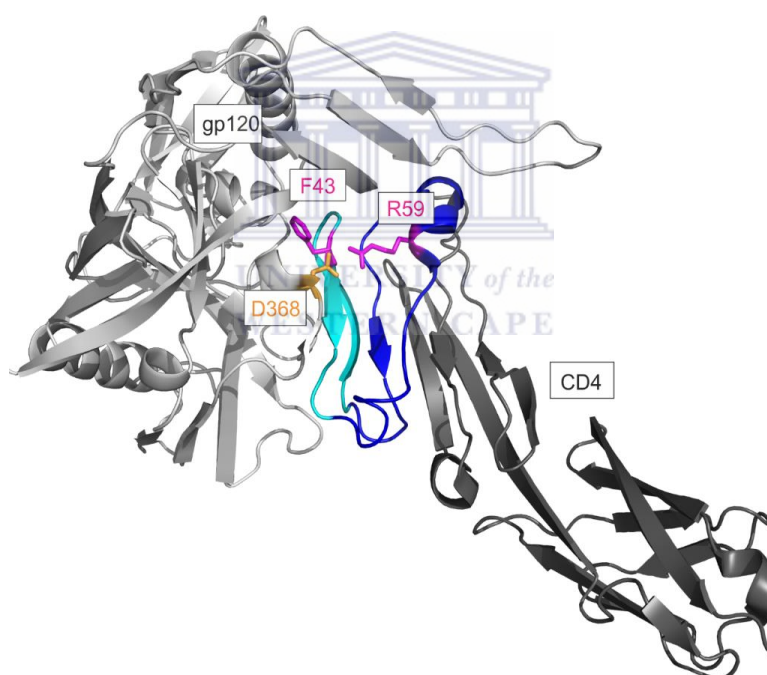
Figure 1.7: Carton representation of gp120 and CD4 interaction during HIV invasion of the host cells. The glycoprotein is in grey while CD4 receptor is in black colour. CD4 domains interacting with gp120 are in green and blue and the important residues are numbered Phe43 and Arg59. The image was adapted from Meier *et al.*, 2012.

1.2.2.2.3. The role of gp120 in HIV infection

Though the HIV tropism infection will dictate the type of chemokine co-receptors to be used in the virus transition to gain entrance to the human cells via gp120, the

chemokine can be of M-tropism if CCR5 co-receptor is used to mediate the entrance of HIV to macrophages and CD4+ T cells. Such mediation involving CCR5 HIV-1 gp120 is called the CD4-dependent as well as CCR5 dependent intracellular signals. However, the chemokine can be a T-tropism if CD4 T cells, as well as macrophages, are infected with the mediation of CXCR4 co-receptor in a CD4-independent manner (Cicala *et al.*, 1999; Iyengar *et al.*, 1999; Moser, 1998). However, it is reported that early HIV infection utilises CCR5 as co-receptor whereas CXCR4 co-receptor is frequently used at the later stage of viral infection in certain patients (Connor *et al.*, 1997; Feng *et al.*, 1996).

Regardless of the differences in co-receptors used during HIV infection, it is known that this virus life cycle only begins once the viral membrane protein gp120 is attached to a CD4 molecule and a co-receptor of macrophages, monocytes, T cells and dendritic cells is utilized (Moser, 1998). Thus, gp120 could become the centre of attention to prevent HIV infection to human cells. The main purpose of gp120 binding to CD4 molecule is to mediate the conformational change of the protein at its V3 loop (Sattentau and Moore, 1991), hence contributing to the exposure of the co-receptor-binding site. Furthermore, this binding also permits the dissociation of gp120 from gp41 by the resulting conformational changes. As such, reports have shown that the deletion of the V3 loop, gp120 was unable to bind the CCR5 chemokine receptor (Lapham *et al.*, 1996; Trkola *et al.*, 1996).

Besides the conformational change observed at the V3 loop after gp120 binds to CD4, the binding of gp120 to CD4 also mediates major alterations of the variable loops V1 and V2, therefore enabling the exposure of protected residues (Morikita *et al.*, 1997). The changes of these loops enable the exposure of the essential domain required for the binding of co-receptor linked with CCR5 and CXCR4 (Alkhatib *et al.*, 1996; Rizzuto *et al.*, 1998; Kwong *et al.*, 1998). Unexpectedly, CD4 has also been reported to exhibit minor conformational changes after binding to gp120 (Kwong *et al.*, 1998).

1.2.2.3. Immunology of HIV/AIDS and the evasion of the human immune system

Once the virus gets into contact with the human system, the pathogen-associated molecular pattern (PAMP) is triggered, which in return actives the pathogen recognition receptors (PRRs) of the body. These cascades of events will then active

the cellular and the humoral immunity of the hosts, which will participate in the clearance of the HI Virus in the system. However, despite the fact that multiple defence systems are put in place by the human body to face such an invasion, the HI Virus infection still progresses to the stage of AIDS due to the virus ability of evading these defence mechanisms.

1.2.2.3.1. Cellular immune response

The ability of the innate immune system to react to a microbe invasion is due to a well-coordinated cellular immune response, through the dendritic cells and the macrophages. These cells are activated by the secreted PAMPs and while the macrophages would attempt to remove the virus by phagocytosis and inflammatory molecules, the dendritic cells will produce cytokines to activate the leukocyte membrane-associated proteins or major histocompatibility complex type 1 and 2 (MHC-1 and MHC-2). These MHCs are termed human leukocytes antigens (HLA) (Mogensen, 2009; Akira, 2009).

Dendritic Cells Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DS-SIGN) is one such molecule that the dendritic cell produces to stimulate the T cells defence mechanism. It is activated through the multiple histocompatibility complex type 2 (MHC-2), which will activate the naïve CD4+ T cells by binding to the Intercellular Adhesion Molecule 3 (ICAM-3). Although the T cells will mature and differentiate to generate different T cells (T helpers, T follicular, and T regulator), the naïve CD4+ T cells through the T-cell receptor (TCR) will bind to the B7 molecule of B cells and activate antibodies production, which will attempt to neutralize the propagation of the HI Virus (Look *et al.*, 2010). The T helpers are the most HIV-specific CD4+ T cells and will help to reduce viral reproduction and control the level of viremia in an infected person (Kalams *et al.*, 1999; Brander and Walker, 1999; Goulder and Walker, 1999).

Regardless of the tremendous works accomplished by the CD4+ T cells to eradicate the virus, the real viral battle is nonetheless accomplished by the HIV-1 specific cytotoxic CD8+ T lymphocytes (CTL) through specific mechanisms. These cells are activated via the multiple histocompatibility complex type 1 (MHC-1). The MHC-1 presents the B7 molecule on dendritic cells to bind to CD28 T-cell receptor of CD8+ T cells and this binding triggers the activation of CD8+ T cells. This complex CTL-

TCR binds the HI Virus peptides that are related to the MHC-1 molecules expressed on the surface of the infected cells. The binding stimulates the production of proteases to kill the infected cells (McMichael and Rowland-Jones, 2001). The activity of HIV-1 specific cytotoxic CD8+ T lymphocytes (CTL) has been shown to play a crucial role at the beginning of HIV infection and has proven to be efficient to control HI Virus replication (Ogg *et al.*, 1998; Brander and Walker, 1999; Goulder and Walker, 1999).

Another mechanism of CTL neutralizing HIV-1 is made possible with the interaction of the Fas ligand (FasL) on the CTL membrane surface with the Fas molecule on the HI Virus. This contact causes the infected cells to lyse through the process of apoptosis (Hadida *et al.*, 1999). The presence of high amounts of HIV-1 specific CTL has been detected in the blood of HIV-seronegative individuals, but who were previously exposed to the virus (Bernard *et al.*, 1999).

The ability of the HIV-1 specific cytotoxic CD8+ T lymphocytes (CTL) to reduced HIV-1 replication is determined by the presence of various alleles of HLA-1 and HLA-2. This argument is advanced as observations have been made that long-term non-progressors (LTNP) harboured certain type of HLA alleles, which have been revealed to possess qualitative and quantitative CTL responses (Migueles *et al.*, 2002; Betts *et al.*, 1999; Pontesilli *et al.*, 1998). The varieties and importance of such alleles will be discussed properly in 1.2.2.3.3.1..

1.2.2.3.2. Humoral immune response

The ideal humoral response of the human defence system during HIV invasion ought to be the secretion of neutralizing antibodies to inhibit the viral replication and if possible eradicate the virus in the patient circulatory system. However, it has been shown that the specific humoral immune response during HIV invasion contributes very little during HIV infection and its responsibility to stop the HI Virus replication and/or to eliminate the virus into the patient bloodstream is limited (Sierra *et al.*, 2005).

During the HIV humoral immune response, the neutralizing antibodies are supposed to bind the virus and mediate the virus incorporation and eradication by phagocytosis. Nevertheless, conclusive reports have indicated that the sera of HIV-1 infected

individuals were unable to reduce a considerable amount of HIV-1 in an *in-vitro* experiment (Kostrikis *et al.*, 1996). The problem of such inefficacy could be explained by the fact that the reduction of HIV replication by antibodies is mediated with an amount of total neutralizing antibodies in the system as oppose to the fact that HIV suppression and eradication would have been possible with the intervention of total HIV-1 specific neutralizing antibodies (Sierra *et al.*, 2005).

Additionally, an observation was made on the ability of HIV-1 to overcome the neutralizing antibodies directed against HIV-1 glycoprotein gp120, thus the neutralization-sensitive HIV-1 were replaced by immune escaping HIV-1 variants. It was achieved by either mutating the residues involved in the conformational changes of gp120 (Wrin *et al.*, 1994); and/or by changing the glycosylation arrangement of gp120 (Wei *et al.*, 2003). With respect to all the mechanisms put in place by the immune system to combat HIV and therapeutic regimens, there is still a sense that these tactics are not enough, as the HIV infection would still progress to the chronic stage of AIDS. While this is due partially to the ability of the virus to mutate and/or change as different strategies are used, it is also due to some genetic variability amongst human genomes.

1.2.2.3.3. HIV evasion of the human immune system and opportunistic diseases

The effort made by the immune surveillance in response to the HI Virus invasion though well coordinated and organized face some challenges, either due to the difficulty to generate a strong response or due to the capacity of the virus to bypass these defence mechanisms by using their own molecules.

1.2.2.3.3.1. The host genetic factor for HIV infection progression

Many observations have shown that the time of disease progression is associated with the genetic variations of the human leukocytes antigen (HLA) haplotypes (Kamp *et al.*, 2000). Studies have detailed that long-term non-progressors (LTNP) infected with HIV-1 possessed a high amount of specific HLA class 1, namely HLA-A1, HLA-A2, HLA-B14, HLA-B17 and HLA-B27. The HIV-1 infected LTNP patients with HLA-B27 allele were mostly found to be heterozygous for this class of loci (Louie *et al.*, 1991; Kamp *et al.*, 2000).

On the other hand, reports have also indicated that HIV-1 infected individuals harbouring the HLA-B35 allele were presenting the possibility to be rapid progressing patients for the disease. This allele was mainly found to be responsible for the rapid development of AIDS in Caucasians (Carrington *et al.*, 1999b). While the HLA-B35 allele was also found in HIV-negative female Gambian prostitutes, however, they possessed cytotoxic T lymphocytes (CTLs) capable to vigorously act against four HIV-1 and HIV-2 cross-reactive peptides epitopes (Rowland-Jones *et al.*, 1995). The same observation of genetic variations associated with HIV progression was also reported for HLA class 2, where HIV-1 infected individuals LTNP exhibit HLA-DR5 and HLA-DR6 alleles, while on the other hand, rapid disease progressing patients have the HLA-DR1, HLA-DR3 and HLA-DQ1 alleles (Magierowska *et al.*, 1999; Kamp *et al.*, 2000).

Besides the genetic predisposition of MHC that facilitates the progression of HIV infection to AIDS, scientific evidences on the genetic variability of HIV-1 co-receptors have also contributed to progression to AIDS. *In vivo* experiments have proven that the natural ligands of co-receptor CCR5 (MIP-1 alpha, MIP-1 beta, and RANTES) and co-receptor CXCR4 (SDF-1) were able to inhibit T cell tropic or monocyte tropic HIV-1 replication (Cocchi *et al.*, 1995; Oberlin *et al.*, 1996). Since it is known that CCR5 co-receptor (R5 strains) initiates HIV-1 infection and that CXCR4 (X4 strains) only appear at the later stage of infection, therefore the appearance of X4 viruses could be associated with the progression to AIDS (Connor *et al.*, 1997; Kupfer *et al.*, 1998). Additionally, different genetic variations within CCR5 gene were identified and they play a vital role in the progression of HIV-1 infection (Carrington *et al.*, 1999a). Nonetheless, total protection against HIV-1 infection and/or reduce progression to AIDS was accomplished when a deletion of a 32 bp was performed within the CCR5 gene (CCR5-delta32) (Dean *et al.*, 1996; Michael *et al.*, 1997).

1.2.2.3.3.2. The virus strategy contributing to HIV infection progression

The inability of the immune system to properly prevent the progression of HIV infection to AIDS could not only be justified by the genetic variability of MHC and HIV co-receptors. Such immune escape could also be explained by the role played by the HI Virus *Tat*, *Vpu,* and *Nef*. Many proofs of the down-regulation of MHC-1 after

HIV infection have been put forward (Scheppler *et al.*, 1989) and as such, early results have confirmed the initial hypothesis and have shown that the down-regulation was due to the viral *Tat* protein (Howcroft *et al.*, 1993).

Another viral protein involved in the down-regulation of MHC-1 is the *Vpu*, even though not initially implicated in this process. However involved in the proteolytic degradation of newly synthesized CD4 in the endoplasmic reticulum (Willey *et al.*, 1992a; Willey *et al.*, 1992b), *Vpu* was designated to be involved in the down-regulation of cell surface expression of MHC-1 molecules by inducing the rapid loss of synthesized endogenous MHC-1 α-chains (Kerkau *et al.*, 1997). The same role was also attributed to *Nef* where mediating the down-regulation of MHC-1, targets the CD4 degradation (Schwartz *et al.*, 1996; La Gall *et al.*, 1998).

The other major contributor for the evasion of the defence immunity by HIV and the deterioration of the human immune system is the presence of CD4 molecule on the surface of the main cells involved in the defence system, namely macrophages/monocytes, dendritic cells and T lymphocytes. The HI Virus entrance into T cells is mediated by the attachment of virus surface glycoprotein gp120 to CD4 of the cells cited above (Chan *et al.*, 1997). This attachment enables the gp120 to undergo conformational changes and contribute to gp120 binding to HIV co-receptors CCR5 and CXCR4; hence the virus can commence its cycle and survive in the human system.

Likewise the use of CD4, the virus also utilises the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) receptor on macrophages and dendritic cells to enable its entrance into healthy CD4+ T cells (Geijtenbeek *et al.*, 2000b; Pope and Haase, 2003). Conclusive reports have shown that this is possible because the viral gp120 protein has a stronger binding affinity to DC-SIGN receptor of dendritic cells, thus preventing the DC-SIGN to bind to the Intercellular Adhesion Molecule 3 (ICAM-3) (Su *et al.*, 2004). The consequence of such action will be the inability of the dendritic cells to activate the DC-mediated T cells (Geijtenbeek *et al.*, 2000b; Geijtenbeek *et al.*, 2002).

1.2.3. Management and treatment regimens of HIV/AIDS

Despite the research efforts made in the past decades towards the process of inhibiting the HI Virus replication within an infected patient, four main classes of drugs standout as standard regimens, used to manage the progression of the AIDS condition. Although scientific researches had advanced, the current regimens only attempt to reduce the HI Virus replication. The treatment regimens could neither prevent transmission of the HI Virus to healthy individual nor eradicate the virus in an infected individual, thus these medications cannot cure the infection. These drugs are generally termed High Active Antiretroviral Therapy (HAART) or Antiretroviral Therapy (ARV) class of treatment regimens. They are comprised of the Reverse Transcriptase inhibitors (RTIs), Integrase Stand-Transfer Inhibitors (INSTIs), Protease Inhibitors (PIs) and Fusion or Entry Inhibitors (FIs). The treatment can be given as a single class or a combination of more than one class of HAART (Volberding and Deeks, 2010).

1.2.3.1. Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTIs)

This sub-class of HAART is grouped under the reverse transcriptase inhibitors (RTIs) class of HIV treatments. The main role of this therapeutic regimen was to produce molecules that are similar to nucleoside or nucleotide bases, which are essential in the composition of the genetic material (RNA) of HIV. The intake of such molecules by the virus will inactive the reverse transcriptase that stimulate the manufacturing of cDNA from the HIV RNA. The Reverse transcription of the virus RNA will take place using these molecules as it will be preferentially incorporated into the newly formed cDNA; hence the introduction of NRTIs into the new DNA. The HI Virus cDNA will not fuse with the patient's genetic material since the synthesized cDNA would be destroyed. This class of HAART was the first therapeutic regimen used to manage the infection of HIV patient. The first available and Food and Drug Administration (FDA) approved NRTI was Zidovudine (AZT). Other FDA approved drugs includes Didanosine (ddI), Dideoxycytidine (ddC), Stavudine (d4T), Lamivudine (3TC), Abacavir (ABC), Enteric coated ddI, Tenofovir (TDF) and Emtricitabine (FTC). Besides the single composition, other NRTIs can be given as a combination of two or three NRTIs. Examples include 3TC plus AZT, ABC plus 3TC plus AZT, TDF plus FTC, ABC plus 3TC (Bean, 2005; Volberding and Deeks, 2010).

1.2.3.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) belong to the RTIs class of HAART. Unlike their counterpart the NRTIs, these molecules are not similar to nucleotide or nucleoside bases that contribute to the building blocks of virus cDNA and RNA. Hence, these molecules are structurally different from NRTIs and their mode of action is made possible by acting directly on the reverse transcriptase enzyme. This is achieved by downregulating the catalytic activity of the enzyme at its active site. Examples of FDA approved NNRTIs include Nevirapine (NVP), Delavirdine (DLV), Efavirenz (EFV) (Bean, 2005; Pang *et al.*, 2009).

The NNRTIs are very good regimens to suppress the replication of HIV within the host body. Nevertheless, they have to be administered in combination with other antiretroviral medications of the NRTIs. Classes of drugs include either Tenofovir with Emtricitabine or Abacavir with Lamivudine; with NNRTIs prospective function to inhibit viral replication in the central nervous system (Bean, 2005; Volberding and Deeks, 2010).

1.2.3.3. Protease Inhibitors (PIs)

The understanding of the HI Virus life cycle was crucial to develop such therapeutic regimen to inhibit its replication. Knowledge that HIV replication in the host cells requires the intervention of enzymes and the synthesis of proteins for the constitution of a new virion, the HIV proteases role were to cleave the polyprotein precursors that enable the production of essential proteins and enzymes made for the reconstitution and in the formation of a new virion. Thus, the activity of this class of HAART is focused and acts directly on the HI virus protein components and prevents HIV reproduction in an infected patient. FDA approved protease inhibitors includes Saquinavir (SQV), Indinavir (IDV), Ritonavir (RTV), Nelfinavir (NFV), Amprenavir (AMP), Lopinavir (LPV) and Atazanavir (ATV). LPV plus RTV is a protease inhibitor made from the combination of molecule from this class of HAART (Beans, 2005).

HIV patients are placed under protease inhibitor regimens either Tenofovir with Emtricitabine or Abacavir with Lamivudine in addition to their initial administered drug. The combination of different classes of HAART is to have a multiple synergistic effect to fight the virus and to maximize the chance to stop its replication, hence potentially leaving the person free of HIV (Volberding and Deeks, 2010).

1.2.3.4. Integrase Inhibitors (INSTIs)

Similar to the HAART cited above, the Integrase inhibitors act directly on the life cycle involving the production of a mature virion. The main objective of using this particular molecule is to inhibit and to interfere with the binding of the viral DNA to that of the host DNA, therefore preventing the fusion and the transfer of the pathogen genetic material to that of the host genetic material. The action of integrase inhibitors is made possible by disarming the bound metallic ions that are effective at the active site of HIV integrase, by influencing their positions within their binding pocket (Hare *et al.*, 2010).

A well-known integrase inhibitor is Raltegravir (RAL) and this regimen is proven to be safe, well tolerated and is highly effective to manage and treat HIV. Since the integrase inhibitor is not given as a first regimen for HIV infection, a patient receives this treatment as a supplementary regimen once the NRTIs treatment is failing (Beans, 2005; Volberding and Deeks, 2010).

1.2.3.5. Entry Inhibitors (EIs) or Fusion Inhibitors (FIs)

Whilst the above-cited HAART regimens mostly prevent HI Virus replication by acting inside the infected host cell, the entry inhibitors on the other hand execute their activity by acting on the infected or uninfected host cells. The conception of these treatment regimens was made to prevent the entrance of HIV to the host cells, by intervening at various stages of the virus entrance process. Implementing this approach, three major groups of entry inhibitors have been designed and have shown to be potential regimens to stop HIV replication. These entry inhibitors are comprised of (*i*) drugs blocking the gp120-CD4 interaction, (*ii*) drugs blocking the gp120-co-receptor interaction and finally (*iii*) drugs blocking gp41-mediated membrane fusion (Tilton and Doms, 2010).

1.2.3.5.1. Drugs blocking the gp120-CD4 interaction

It is well documented that binding of HIV gp120 to CD4+ molecule of monocytes, macrophages and T lymphocytes favour the attachment of the virus to these cells and the replication of the pathogen within these cells. Thus the development of drugs blocking the gp120-CD4 interaction was reasonable and these drugs could not come at a better time to prevent this replication to take place. The idea to prevent HIV replication enables the production soluble CD4 (sCD4) as oppose to cell-associated CD4. This molecule could mimic the CD4 function hence could favour the binding of the HI Virus and stop further infection of healthy human cells.

Though the clinical administration of sCD4 to HIV patient failed to inhibit replication of the virus (Daar *et al*., 1990), it nonetheless establish the baseline for the manufacturing of a class of sCD4 derivatives which include PRO-542 CD-IgG2 tetramer fusion protein, and the NBD-556 and NBD-557 compounds (Arthos *et al*., 2002; Martin *et al*., 2003; Schon *et al*., 2006). Other compounds from this group are the BMS-378806 and BMS-488043, which are small-molecule inhibitors. These small molecules could either compete with sCD4 for binding to gp120 (Ho *et al*., 2006) or they execute their activity by preventing gp120 to undergo conformational changes once CD4 is bound to gp120 (Si *et al*., 2004). The last approach was to utilise antibodies to target the binding between gp120 and the CD4 molecule. This antibody was the humanized antibody ibalizumab (TNX-355), which binds to the D2 domain of CD4 and disables the conformational change of gp120 (Moore *et al*., 1992).

Though most of the molecules in these groups look promising to inhibit the replication of HIV within the patient, they have not been granted approval by the FDA, thus more research have to be conducted, to optimise the treatment regimens so as to produce a non-toxic and safe product.

1.2.3.5.2. Drugs blocking the gp120-coreceptor interaction

In addition to the method mentioned above, another strategy to design an entry inhibitor was to block the next step in HIV infection, which is to prevent the viral gp120 from binding to the co-receptor, either CCR5 or CXCR4. Thus, the drugs blocking the gp120-coreceptor interaction were developed.

A number of compounds have been produced, which can stop HIV infection. Such compounds include CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) (Cocchi *et al.*, 1995). The main function of these compounds was to avoid the binding of the viral gp120 protein to the CCR5 co-receptor, by stimulating the internalisation of CCR5 from the cell surface (Alkhatib *et al.*, 1997). However, the activities of these molecules have developed an undesirable agonistic effect against CCR5.

Aplaviroc (GW873140), Vicriviroc (SCH-D, SCH-417690), cenicriviroc (SCH-C) and Maraviroc (UK-427857) are groups of small molecules developed to have antagonist function against HIV replication. Whilst the first compound was discontinued in phase IIb clinical trials in 2005, because it was reported to induce idiosyncratic hepatotoxicity in treated patients (Nichols *et al.*, 2008), the second compound is still in phase III clinical trials and was derived from the third compound, since this compound was also found to have serious side effects on the treated patients during the clinical trials and was terminated (Strizki *et al.*, 2005). The last compound, on the other hand, has received FDA approval in 2007 and is used as a treatment regimen for HIV-infected patients who have become drug resistant to the primary HIV regimens (Dorr *et al.*, 2005).

Another class of compounds used to prevent gp120 binding to CCR5 co-receptor are made from antibodies rather than the chemically synthesised molecules. PR-140 is an example of a compound developed from the humanised mouse anti-CCR5 antibody and which role is to counteract the ability of gp120 to interact with CCR5; however, this molecule does not block CCR5 to exercise its binding activity to gp120 (Jacobson *et al.*, 2008).

Whilst other drugs were directed towards CCR5 co-receptor, compounds such as T-22, T-134, T-140, and ALX40-4C were also developed from polypeptides, to prevent gp120 binding to CXCR4 co-receptor. These compounds act as an antagonist by mimicking the natural ligand of CXCR4 co-receptor, CXCL12 (SDF-1) and therefore these compounds bind the co-receptor thus preventing gp120 binding to CXCR4 (Arakaki *et al.*, 1999; Doranz *et al.*, 2001). Besides the polypeptides activities, other small-molecule antagonists are in the developmental stages and they include AMD3100 and ADM070. Both molecules were discontinued because of the side effects such as cardiac abnormalities and liver histological changes in preclinical

studies and the first compound was unable to considerably reduce HIV replication (Hendrix *et al.*, 2004; Stone *et al.*, 2007).

1.2.3.5.3. Drugs blocking gp41-mediated membrane fusion

The last group of entry inhibitor is composed of compounds that can block gp41-mediated membrane fusion. This group of entry inhibitors attempt to block gp41 to get into contact with the host membrane hence this group of compounds is mostly called fusion inhibitors. The FDA fusion inhibitor Enfuvirtide (T-20) was approved in 2003 for the treatment of HIV-infected patients. It is made up of a 32 amino acid peptide, whose sequence is similar to that of the HR2 region of gp41, hence preventing HR2 region by competitive binding with the HR1 region (Wild *et al.*, 1993). Though this compound has shown potential to inhibit HIV replication in clinical trials (Kilby *et al.*, 2002; Lalezari *et al.*, 2003), other derivatives of the molecules are under development so as to improve the pharmacodynamics and efficacy of the parental compound, enfuvirtide (Dwyer *et al.*, 2007). As opposed to other treatment regimens that are given orally to patients, Enfuvirtide, on the other hand, is given as an intravenous injection to the patients twice per day (Volberding and Deeks, 2010).

1.2.4. Entry Inhibitor based peptide: a sub-class of HAART from Antimicrobial Peptides and a way forward to treat HIV

Despite the progress made over 30 years of scientific research in finding potential and non-toxic HIV treatment regimens, two main directives have been implemented to design HAART medications. Medications that attempt to inhibit HIV replication within the host cells which includes NRTIs, NNRTIs, PIs and INSTIs; and the medications that inhibit HIV replication and prevent its entrance into the host cells, which are composed of entry inhibitors. While the medications that inhibit HIV replication within the host cells is of great importance to tackle the disease progression, they, however, exhibit a high level of side effects towards the human body (Volberding and Deeks, 2010; Dimock *et al.*, 2011). Another problem is that it is difficult to kill the infected cells, as other healthy human cells are also affected; and the human defense mechanism responsible for neutralising infectious pathogens will destroy the HIV-infected cells thus reducing the number of CD4+ T cells.

The entry inhibitors on the other side have shown to be a favourite route for the design of alternative treatment regimens for HIV infection. This is primarily due to their low toxicity as compared to the other treatment regimens cited above and also, because their condition of administration is well established and understood (Volberding and Deeks, 2010). Secondly, their methods of preventing HIV infection do not allow for the entry of the virus into the host cell but rather tackle the virus before it gets into the human cells. This concept is ideal as fewer cells will be infected and the host immune system will be able to defend itself against the pathology. Also, because the internalization of HIV or any pathological organism into the host cells makes it difficult for the host cell to establish a good clearance mechanism, hence the persistent presence of HIV reservoirs in the body (Cossart and Sansonetti, 2004; Pizzaro-Cerda and Cossart, 2006a). Thirdly, the minor side effects (irritation of the skin) encountered by some entry inhibitors-based peptides are of little concern, which is due to the intravenous administration of the medication and the unavailability of an oral dosage. Nonetheless, researchers still believe that the development of peptide inhibitors to prevent the fusion of HIV with the host cells is the gold standard for the design and development of a drug to treat HIV infection. Prospective solutions are already underway to design oral bio-available peptide-based drugs to prevent HIV entrance into the host cells (Welch *et al.*, 2007). Though other peptide-based drugs are either FDA approved (Kilby *et al.*, 2002; Lalezari *et al.*, 2003) or under clinical trials (Dwyer *et al.*, 2007), more peptides ought to be screened to develop a potent anti-HIV entry inhibitors treatment regimen.

1.3. Antimicrobial Peptides

The interest of using antimicrobial peptides (AMPs) to develop peptide-based drugs to inhibit HIV infection is that AMPs are sourced and/or similar in sequence from natural occurring defense peptide molecules. In addition, they have less cytotoxicity effects and are not proned to immunogenicity (Eckert *et al.*, 2006). Most of these peptide molecules are found in the human body and are used either as a primary human defense mechanism against pathogen invasion or are used as a secondary defense system (Ganz, 2003). Also, some of these peptides have proven to have versatile antimicrobial activity against gram-positive and gram-negative bacteria, protozoa, fungi, virus and particularly HIV (Andreu and Rivas, 1998; Brodgen, 2005;

Wang *et al.*, 2010). Thus these molecules stand a good chance to advance into clinical trials hence the search for putative anti-HIV AMPs.

1.3.1. AMPs, primary defence line of many organisms

The majority of molecules in charge of the organism's immune defence systems are made up of proteins and peptides. Though huge proteins such as immunoglobulin are the principal antibodies to defend the human body, there are other defence systems such as the human neutrophils, the human defensins, the gamma-defensins retrocyclins, etc., which play an important role in the protection of the human body against microbial invasion (Fritig *et al.*, 1998; Soderhall and Cerenius, 1998; Du Pasquier and Flajnik, 1999; Ganz, 2003).

From this statement, it can, therefore, be said that antimicrobial peptides and antibodies play a major role in the first line of defence of human and many organisms, such as the innate immunity of many plants, invertebrates and vertebrates species (Wong and Ng, 2003; Wang and Ng, 2005a). Experimental research on some antimicrobial peptides extracted from these organisms showed that they have activity against gram-positive and gram-negative bacteria, fungi, eukaryotic parasites as well as viruses (Shai, 2002). Furthermore, these antimicrobial peptides have proven to inhibit HIV replication using various mechanisms of suppressing the virus (Wang *et al.*, 1998; Munk *et al.*, 2003; Wang *et al.*, 2004).

Antimicrobial Peptides are a particular class of protein aptamers of relatively small size of approximately 6 to 100 amino acid residues. They are generally positively charged with charges varying from +2 to +9. An additional element that makes these biomolecules special is their high hydrophobic amino acid content, hence a hydrophobicity percentage of more than 30 % (Giuliani *et al.*, 2007). Furthermore, the positive charge, the high hydrophobicity are some of the elements that enable all antimicrobial peptides to fold properly into their three-dimensional structure and bind selectively to the negatively charged membrane of the microbe (Andreu and Rivas, 1998).

Despite their characteristic elements, one may think that antimicrobial peptides are originated from a common ancestry, they are however of diverse origin. The fact that these molecules constitute the defence system of various organisms may constitute the

reason for them to exhibit the same characteristics to fulfil their functions, which is to impart the host with adequate immunity to counter foreign attacks.

1.3.2. Physicochemical properties of AMPs

The computation of the physiochemical properties of a putative antimicrobial peptide shed light on the expectation of a biomolecule being a true antimicrobial peptide and exhibiting potent activity against an infectious microorganism with low toxicity. These physicochemical properties may include the charge, the hydrophobicity, their boman index, instability index, and the structural conformations just to name a few.

1.3.2.1. Charge

Though most antimicrobial peptides are of small size ranging between 6 to 100 amino acids and are expressed naturally from diverse origins, these biomolecules are positively charged with +2 to +9. These charges are favoured by the presence of a high number of positively charged amino acid residues such as lysine, arginine and histidine that are included in these AMP sequences (Hancock and Chapple, 1999). Also, negative charged AMPs exist and they are majorly constituted of residues such as aspartic acid and glutamic acid. The inhibitory activity of these AMPs is stabilized with the incorporation of zinc or calcium ions, which act as cofactor (Brogden *et al.*, 1997).

The reason behind the positive charge carried by AMPs is due to the fact that most microbial membranes are composed of phospholipid biomolecules including cardiolipins, lipopolysaccharides and phosphatidylglycerols. Therefore, the microbe membranes will be harbouring a negatively charged surface. The difference in charge will allow for an electrostatic attraction to take place between the pathogens negatively charged membrane and a potential AMP, which has activity against it (Matsuzaki *et al.*, 1995; Matsuzaki, 2009).

Although the positive charge transported by AMPs is an advantage to defeat the microbes, the excess number of positive charges could be detrimental to the activity exerted by the AMP, thus may reduce its activity or increase the peptide toxicity towards the host cell. Example to illustrate this is the AMP Magainin, for which a charge of +5 was found to intensify the peptide activity, however an increase in the

peptide charge to +7 changes the microbial activity of the peptide by increasing the haemolytic activity of the peptide (Powers and Hancock, 2003).

1.3.2.2. Hydrophobicity

Hydrophobicity is also an important physicochemical property that characterizes potency of a peptide to possess antimicrobial activity against pathogenic organisms. The computation of this property using an algorithm can give an idea on the potential activity of the AMP (Thomas *et al.*, 2010). A peptide predicted to have a hydrophobicity of 30 % or more are expected to have neutralizing activity (Giuliani *et al.*, 2007). This property is crucial for an AMP since it imparts to the biomolecule the ability to effectively penetrate the microbial lipid bilayer and lyse the microbe by membrane disruption (Brogden, 2005).

1.3.2.3. Structure

The folding of proteins and peptides into their secondary and three-dimensional conformation is essential for these biomolecules to be able to bind to the microbe receptor and exercise their biological function. As such, the antimicrobial peptides will have to enter into their final conformation and display their expected activity, in addition to their positive charged and high hydrophobicity. Such conformations may be the α-helix, the β-sheet, irregular or extended and loop conformations.

1.3.2.3.1. The α-helical class of antimicrobial peptide

This structure is mostly common with the AMPs and is made of helical conformations because either they lack or they have a little number of cysteine amino acid residues to form bridges. The structure is favoured by the presence of amino acids such as alanine, lysine, leucine, phenylalanine, tyrosine, tryptophan, cysteine, methionine, asparagine and valine. However, this structure is prohibited by the presence of serine, isoleucine, threonine, glutamic acid, aspartic acid, glycine, proline and hydroproline amino acid residues. AMPs with α-helical conformations are Magainin, Cecropin A, Andropin, Moricin, Ceratotoxin, Melittin, Cecropin P1 just to name a few examples. Although they present the same conformation, these peptides are originated from different organisms hence the diversity of AMPs (Zhang *et al.*, 1999; Anderson *et al.*, 2003; Brogden, 2005).

1.3.2.3.2. The β-sheet class of antimicrobial peptides

The β-sheet conformation of the peptide is illustrated by the presence of anti-parallel and/or parallel β-sheet structure, held together by 2-6 disulphide bridges. These bridges are mainly formed due to the presence of cysteine amino acid residue within the peptide sequence. The relevance of having a β-sheet structure may be an advantage for proper folding of the peptide and to hold structural integrity of the biomolecule. It also enhances the binding of the peptides to its receptor on the microbe, in order to inactivate the pathologenic microbe. Also, it enables the peptides to resist proteolysis attack as compared to their counterparts from the α-helical peptides (Scott *et al.*, 2008). The importance of this structure has been well illustrated with the AMP Tachyplesin, where a considerable reduction of antibacterial and antiviral activity was noticed when the peptide was linear (Tamamura *et al.*, 1993), and the peptide was less effective to penetrate a membrane model (Matsuzaki *et al.*, 1993). Research have shown that Defensins and other potent AMPs playing a role in the innate immunity in various organisms are rich in cysteine amino acids and, mostly formed β-sheet structures (Charlet *et al.*, 1996, Derua *et al.*, 1996, Ganz, 2003). The β-sheet structure may sometimes be alternated with α-helical structure, forming a mix conformation.

1.3.2.3.3. The extended structure

The extended structure is also called the irregular structure, as it is composed of neither the alpha nor the beta structure. It is usually depicted by the presence of one or more amino acids of the same residue. These amino acid residues could be histidine, tryptophan, proline and glycine; and well know AMPs having the extended structure are comprised of Histatin, Indolicidin, PR-09, Prophenin and Tritrpticin (Gallo *et al.*, 1994; Gao *et al.*, 2001; Selsted *et al.*, 1992). These AMPs have displayed potent activity and are able to cross the lipid membrane of the microbe (Rozek *et al.*, 2000; Zhang *et al.*, 2001).

1.3.2.3.4. The loops structure

The loops structure of antimicrobial peptides are predominantly illustrated with the presence of macrocyclic cysteine knots, thus the peptides of this structure will contain cysteine amino acid residues and will form either one or multiple disulphide bonds. The single disulphide bond is the most important feature as it enhances the formation

of a cysteine knotted motif in the peptide and the cyclic backbone (Cornut *et al.*, 1994; Powers and Hancock, 2003). AMPs known to have this structure are Kalata, Circulin A and B, Cyclopsychitride, Thanatin (Tam *et al.*, 1999; Fehlbaum *et al.*, 1996); and these peptides have proven to inactivate the replication of viruses and most especially HIV (Gustafson *et al.*, 1994).
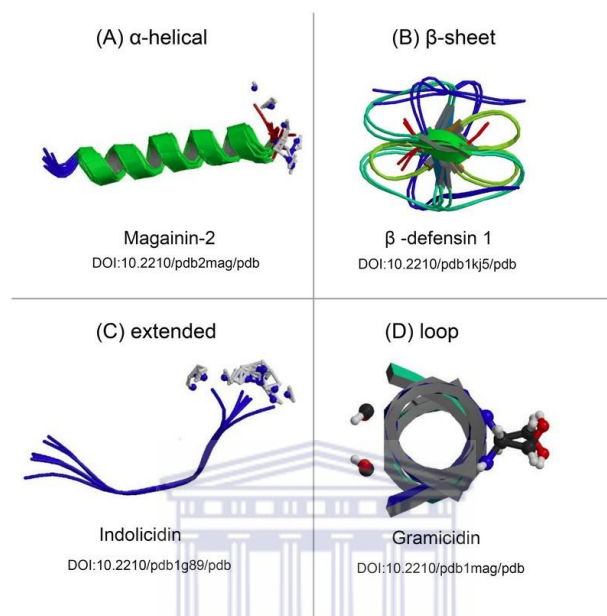
Figure 1.8: Antimicrobial Peptides represented by various structural conformations The figure was taken from Peters *et al.*, 2010.

1.3.2.4. Boman index

The boman index is a computational algorithm designed by late Prof. Hans G. Boman, to predict the potential of an antimicrobial peptide to interact or bind to different receptors of a pathogen. This prediction could be realized in an experimental laboratory environment and inactivate the intended pathogen. The index is defined as the sum of the free energies of the amino acid residue side chains divided by the total number of amino acid residues (Boman, 2003). Based on experimental data, it can be extrapolated that an antimicrobial peptide with a Boman index value lower or equal to 1 kcal/mol signified that the peptide will likely exhibit high antimicrobial activity with no side effects. However, peptides with Boman index value of 2.50 to 3.00 indicate that they have a multifunctional activity with hormone-like activities (Boman, 2003).

1.3.2.5. Instability index

Same as the Boman index, the introduction of computational biology and programming language have aided in the fast analysis of biological data through the utilization of mathematical predictions. As such, physicochemical of proteins and peptides could be computed. The instability index of AMP could predict if the biomolecule will be stable or unstable in an *in vivo* environment since it is known that protein-protein or protein-peptide interaction requires the biomolecules to be in their three-dimensional conformation, hence they have to be in a stable form. Using this index, it was proven that unstable proteins and peptides have an instability index of greater than 40, whereas proteins with an instability index smaller than 40 indicates the molecule is stable (Guruprasad *et al.*, 1990; Wang and Wang, 2004).

1.3.3. Therapeutic ability of AMPs

There is no doubt about the potential role that antimicrobial peptides play in the innate immunity of many organisms and in some cases in their adaptive immunity (Du Pasquier and Flajnik, 1999; Goldsby *et al.*, 2003). The majority of these peptides have been shown to have activity against many pathogenic microorganisms, ranging from bacteria, fungi, protozoa, cancer, spermicide activity and viruses (Zhang *et al.*, 1997; Stolzenberg *et al.*, 1997; Edgerton *et al.*, 1993; Soballe *et al.*, 1995; De Waal *et al.*, 1991; Qu *et al.*, 1996; Aboudy *et al.*, 1994; Wachinger *et al.*, 1992; Wang *et al.*, 2010).

The need for new therapeutic molecules due to drugs resistance experience in the health sector has encouraged the translation of these potent molecules into clinically available drugs. This new class of therapeutic agents has made a major contribution to the pharmaceutics research since the molecules are originated from natural sources, are components of the organism's defence system and are not easily predispose to proteolysis. In this regard, many antimicrobial peptides have made their way through clinical trials and some are daily used to treat certain disease conditions (Fjell *et al.*, 2012).

The progress made in clinical research of therapeutics antimicrobial peptides can be presented as follow. Pexiganan, is a peptide derive from the frog *Xenopus*, which activity indicates that this peptide could prevent or cure impetigo and diabetic foot

ulcers. Others peptides that have reached phase III clinical trials are the Omiganan variants, originated from cattle Indilicidin, where CD-226 or MX-226/MBI-226 is used as a topical antiseptic, catheter infections and; CLS001 or MX-594AN which are used in severe acne and rosacea (Hancock and Sahl, 2006; Fjell *et al.*, 2012). Another known therapeutic peptide that has passed clinical trial and is FDA approved is Enfuvirtide. This drug is used as a treatment regimen for HIV infection (Kilby *et al.*, 2002; Lalezari *et al.*, 2003). However, many AMPs are still at phase I and/or II clinical trials, but it is a matter of time before they progress into the later stage of testing (Silva *et al.*, 2011; Fjell *et al.*, 2012).

1.3.4. AMPs, potential anti-HIV molecule

The hunt for additional therapeutic agents for diseases due to drug resistance for diseases was not only limited to bacteria, protozoa, and fungi; it was also expanded to simple viruses such as HIV.

Members of the β-defensins, HBD2 and HBD3 showed dual anti-HIV activities similar to HNP 1, i.e. through direct interactions with the virus and indirectly by altering the target cell (Quinones-Mateu *et al.*, 2003, Sun *et al.*, 2005). However, the condition under which it was investigated was different from those for HNP1. One condition used mimics the oral mucosal environment, with low salt concentrations and the absence of serum (Quinones-Mateu *et al.*, 2003) and another condition used had high salt concentrations and the presence of serum (Sun *et al.*, 2005). Using electron microscopy, Quinones-Mateu *et al.*, 2003, showed the interaction between HBD2 and HBD3 with cellular membranes as well as HIV virions, although membrane disruption was not apparent. HBD2 does not affect cell-cell fusion but instead inhibits the formation of early reverse-transcribed HIV DNA products (Sun *et al.*, 2005).

Studies from Sun *et al.*, 2005 and Quinones-Mateu *et al.*, 2003 showed conflicting results on the down-regulation of HIV co-receptors by β-defensins. Sun *et al.*, 2005, reported HBD1 and HBD2 not to modulate cell-surface HIV co-receptor expression by primary CD4+ T cells whereas Quinones-Mateu *et al.*, 2003, in contrast, showed HBD2- and HBD3-mediated down-regulation of surface CXCR4 but not CCR5 expression by peripheral- blood mononuclear cells (PBMCs) at high salt conditions and in the absence of serum. The contrasting evidence might be a result of the

differences in the source of the defensins used and/or the experimental conditions under which the experiments were carried out (that is, the presence or absence of serum). Interestingly, HBD2 expression is diminished in HIV-infected individuals but is constitutively expressed in the healthy adult oral mucosa (Sun *et al.*, 2005).

The θ-defensins Retrocyclins, and RTD1, RTD2 and RTD3, function as lectins and can inhibit HIV entry (Munk *et al.*, 2003, Wang *et al.*, 2004). In addition, they inhibit several HIV-1 X4 and R5 viruses, including primary isolates (Munk *et al.*, 2003, Wang *et al.*, 2004). Retrocyclin does not seem to inactivate the HIV virion directly unlike α- and β-defensins, however, it binds to HIV gp120 as well as CD4 with high affinity. This observation is consistent with inhibition of viral entry (Munk *et al.*, 2003). Retrocyclin's high-affinity binding for glycosylated gp120 and CD4+ is mediated through interactions with their O-linked and N-linked sugars (Wang *et al.*, 1998). Serum strongly reduces the binding of retrocyclin to gp120 (Wang *et al.*, 2004). It remains to be determined whether the interactions with HIV glycoproteins are similar to those reported with influenza virus glycoproteins (Leikina *et al.*, 2005). Nevertheless, studies on retrocyclin-1 analogues indicate that modification of this peptide can enhance its potency against HIV *in-vitro* (Owen *et al.*, 2004), indicating the therapeutic potential of such analogues.

Besides the defensins family of AMPs, other AMPs have shown similar actions against HIV. Antimicrobial peptides such as Cecropin A from insects, Aurein 1.2 from frog, Mellitin from insect, Dermaseptin S1 from leaf frog, Circulin A from plant, Cycloviolin A-D from plant are just few peptides that have shown to have potential inhibitory activity against HIV replication *in-vitro* (Van Compernolle *et al.*, 2005; Wang *et al.*, 2010; Wachinger *et al.*, 1998; Daly *et al.*, 1999; Ireland *et al.*, 2007).

1.3.5. AMPs as Entry Inhibitors of HIV

The need for additional therapeutic regimens for HIV infection has allowed some peptide-based drugs to see the light. Even though fewer drugs made from this class of molecule have received FDA approval, there is hope that screening for new molecules and to optimize the existing anti-HIV AMPs might enable the output of additional drugs.

1.3.5.1. Examples of EIs or FIs derived from AMPs

The screenings of anti-HIV AMPs due to their physicochemical advantages and stability have yield potent peptide-based drugs, which are used as HAART regimen currently. Enfuvirtide (T-20) is a good example of a peptide-based drug that proved to have potent antiviral activity in the early 1990's (Wild *et al.*, 1992; Jiang *et al.*, 1993). The molecule later received FDA endorsement to treat HIV infection in clinical trials (Kilby *et al.*, 2002; Lalezari *et al.*, 2003). Though the drug is not recommended as a primary treatment regimen, it is however implemented at the later stage of the treatment when other treatment regimens have failed, thus this regimen might prevent further HIV invasion of the human host cells. The developments of other variants of Enfuvirtide are underway because the original parental molecule could not be administered orally. The new family of improved Enfuvirtide show better pharmacodynamics and efficacy to tackle HIV infection (Dwyer *et al.*, 2007; Lalezari *et al.*, 2005b).

Other major groups peptide-based drugs have been experimented to tackle HIV at a different stage of its evolution. Peptides including T-22, T-134 and T-140 and ALX40-4C were designed to mimic CXCR-4 receptor, CXCL-12. Though the initial activity proved to be non-effective and high side effects, subsequent testing revealed that these peptides have activity for patients who have CCR5-tropic strains of HIV (Doranz *et al.*, 2001). Optimization of these molecules to interact with the desired receptor will aid in the production of additional drugs to inhibit and/or prevent HIV infection.

1.3.5.2. Mechanism of action of EIs or FIs and the way forward for future peptide-based drugs

The mechanism of action of antimicrobial peptides was designed as entry inhibitors because the molecular mechanisms of HIV entry and fusion with host cells are well established and understood. Depending on the various stages of HIV infection, crucial steps have been highlighted and they include: gp120-CD4+ interaction, gp120-CCR5/CXCR4 interaction, and gp41 conformational change and membrane fusion. Also, the mechanism of action of some compounds to prevent HIV entry and fusion with the host cells has been well explained in sections 1.2.3.5.1, 1.2.3.5.2 and

1.2.3.5.3. Following the same principle, potent peptide-based compounds could be screened to tackle a single step or multiple steps of HIV infection.

To develop a more potent and non-cytotoxic peptide-based drugs, good pharmaceutics practice for drug discovery and development ought to be implemented so as to avoid the mistakes observed in the design of T-22, T-134 and T-140 (Doranz *et al.*, 2001). Such preliminary practice would include understanding the disease and steps of infection; choose a molecule to be targeted with the peptide, test the molecule activity on the target to confirm its role in the disease progression, find a promising molecule that could become a drug, early safety tests and lead optimization (Innovation, 2007; PhaRMA, 2015). Only after these first two steps have been achieved can a lead compound be accepted and the research would proceed to early safety testing.

The baseline set with the understanding of HIV infectious steps and the possible target points have encouraged researchers to believe that a good pharmacological practice of antimicrobial peptides would make AMPs potential lead compound for novel HIV regimens. Also, current anti-HIV AMPs could be optimized for oral intake and improved their pharmacodynamics and efficacy (Welch *et al.*, 2007).

1.4. Rationale of the research

The gaps in the literature regarding the treatment of HIV-1 and HIV-2 are the major consequence of raising the questions of additional therapeutic agents to slow the viral progress and its eradication, taking into account the importance of AMPs in many organisms defence system, the current published data on anti-HIV activity, and the prediction of putative AMPs binding to gp120 protein (Tincho *et al.*, 2016), thus the rationale for this study. These unfold as follows:

> ➢ AIDS is a disease that attacks the human immune system and is caused by the Human Immunodeficiency Virus (HIV). HIV can be transmitted to a healthy individual through body fluids or via mucosal surfaces. Viral entry is made possible by the interaction of the viral envelope glycoprotein 120 (gp120) to the cell surface on the CD4+ T lymphocytes and a chemokine receptor (either CXCR4 or CCR5) on the host cell surface (Chinen and Shearer, 2002). The disease interferes with the human immune system, exposing the patient to opportunistic infections and tumours.

➢ The disease is a major health problem in many parts of the world and is considered a pandemic (Kallings. 2008). The UNAIDS reported in 2009 that since the discovery of the disease in 1981, some 60 million people have been infected, with 30 million deaths, 34 million people living with HIV/AIDS, and 14 million orphaned children in Southern Africa alone as reported in 2009 (Wang *et al.*, 2010). This figure has now risen to 40 million people living with the disease, with 60 % of infected people in Sub-Saharan Africa alone. Swaziland has shown the worlds largest prevalence rate of 25.9 % whereas South Africa has the world largest HIV-infected population estimated at 5.6 million (Kendall, 2012).

➢ Though many advances have been made in HIV research to date, neither a cure, nor an HIV vaccine have been found and the disease can only be managed by using High Active Antiretroviral Therapy (HAART), which can only slow the course of the disease and reduce both deaths and new infections (Dybul *et al*. 2002). Besides reducing the mortality and disease progression, the antiretroviral therapy have several side effects such as microalbuminuria, dyslipidaemia, insulin resistance, Hepatotoxicity, Hyperglycemia, impaired glucose tolerance and increase risk of cardiovascular disease (Volberding and Deeks, 2010; Dimock *et al.,* 2011); and the patients have to adhere to the treatment for the rest of their lives.

➢ The need for a non-toxic therapeutic treatment has brought about the necessity for the discovery of additional HIV treatment regimens to lower mortality rates and avoid lifelong adherents to HIV treatment regimens. Antimicrobial Peptides (AMPs) are components of the first line of defense for prokaryotes and eukaryotes and have a wide range of activities against gram-negative and gram-positive bacteria, fungi, cancer cells, protozoa as well as viruses (Andreu and Rivas, 1998); and have been proven to be promising therapeutic agents against HIV (Wang *et al.*, 2010). The use of AMPs as promising anti-HIV molecules for therapeutic intervention of HIV are due to the fact that most AMPs have a net positive charge and are mostly hydrophobic molecules. This permits targeting of the net negatively charged pathogen membrane targets (protein or DNA). The unique mechanism of action and the diversity of these peptides may also be used as the basis to design peptide-based compounds or

backbone compounds to block the attachment and binding of HIV protein gp120 to the CD4+ molecules of T cells; and could form a novel class of HAART drugs, termed Entry Inhibitors (EIs).

➢ Previous *in-silico* docking and predictions of several putative AMPs have shown that some of these Antimicrobial Peptides bind to the gp120 HIV protein, at the area where the CD4+ T cells interact with the gp120 protein (Tincho *et al*., 2016). Using these anti-HIV AMPs might prevent infection of the T cells by HIV, bearing in mind that the contact between the gp120 and the CD4+ T cells favour the entry and infection of new human cells by HIV (Wilkinson, 1996). Blocking the interaction between the gp120 and CD4+ T cells can be a preventive measure to stop HIV contamination, thus paving an excellent opportunity for a promising therapeutic approach. This method could enable the eradication of the virus within the body since adhesion of pathogens to host cells is a key step during the establishment of an infection or contamination (Pelkmans and Helenius, 2003). Hence, the molecular validation of the *in-silico* prediction will be the focus of this research, to confirm earlier findings. This might be a way forward for an accurate drug design to eradicate HIV. These experimental validated anti-HIV AMPs might act as entry inhibitors and form a new class of HAART to block gp120 attachment and binding to CD4+ molecules. Therefore, the aims deriving from the rationale are as follow:

i. Optimize and increase the ability of the peptides to bind gp120 protein,

ii. *In-vitro* validation of the activity of the *in-silico* predicted anti-HIV AMPs as therapeutic agents,

iii. Determination of the range biological activities exhibited by the putative AMPs.

The objectives enumerated below will help improved some gaps encountered in the literature and they are as follow:

1) Perform mutation (s) on the initial putative anti-HIV AMPs, to increase the binding capacity between the peptides and gp120 protein, thus improve the HIV activity.

2) Perform the binding study of the putative anti-HIV AMPs and the recombinant protein gp120,

3) Study the effective concentration of the anti-HIV AMPs on HIV-1 pseudotypes (HIV-1 PV),

4) Perform binding activity between the putative AMPs and gp120 protein, using a molecular method,

5) Study the toxicity of these anti-HIV AMPs and cell viability of the host cells,

6) Study the activity of these putative antimicrobial peptides on various microorganisms (Gram positive and gram negative bacteria),

7) Study the activity of these putative antimicrobial peptides on various cancer cell lines.

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CHAPTER TWO: *IN-SILICO* OPTIMIZATION OF PUTATIVE ANTI-HIV PEPTIDES VIA SIDE-DIRECTED MUTAGENESIS

2.1. Introduction

The rising problem of drugs resistance, the lack of available and/or novel antibiotics to combat the current diseases have encouraged the exploration of new opportunities to search for future molecules that can serve as a lead compound for the development of potent antibiotics. Since the discovery of the first Antimicrobial Peptide (AMP) more than three decades ago (Rinaldi, 2002), the amount of discovered Antimicrobial Peptides has grown exponentially, due to their high potentiality against many pathogens. Furthermore, experimental data have proven that isolated AMPs could inhibit various pathogens from gram-negative, gram-positive bacteria, fungi, protozoa, cancer cells as well as viruses (Powers and Hancock, 2003). In addition, these AMPs have shown low toxicity, with minimal resistance been developed by the pathogenic organisms toward the AMPs, whilst these AMPs killed the target microbes rapidly (Jenssen *et al.*, 2006). AMPs have also been shown to have activity against the HI Virus and could stop viral replication, using different mechanisms (Chang *et al.*, 2003; Quinones-Mateu *et al.*, 2003; Wang *et al.*, 2004; Sun *et al.*, 2005).

Besides their potential being shown in experimental assays, the need for novel AMPs pushed for further research toward identifying these molecules since most AMPs occur naturally in many organisms, either in eukaryotes or in prokaryotes. Also, AMPs have been found to play a crucial role in the defence mechanisms of many organisms (Wong and Ng, 2003; Wong and Ng, 2005a; Wong and Ng, 2005b; Wang and Ng, 2002; Wang and Ng, 2005). The ability of these AMPs to carry out their activities mainly resides in the fact that most of these molecules are positively charged and are generally hydrophobic (Pushpanathan, *et al.*, 2013); consequently, the AMPs could bind to negatively charged receptors on the target microbe by electro-static interaction (Wang *et al.*, 2010).

The current molecular techniques utilized for the discovery, identification and characterization of these molecules have proven to be costly and time-consuming and make the availability of the drugs rare thus delaying the treatment and/or the eradication of some diseases. To satisfy the high demand for more potent therapeutic, less toxic and non-resistant molecules, additional cost-effective techniques ought to be put in place to satisfy these needs. Fortunately, the developments in Computational Biology and Bioinformatics have enabled rapid discovery of compounds of biology and therapeutic importance with the help of predictive tools such Quantitative Structure Activity Relationship (QSAR) (Torrent *et al.*, 2012), Linear Discriminant Analysis (LD), Support Vector Machine (SVM) and Random Forest (RF) (Thomas *et al.*, 2010), Sliding Window (SW) (Juretić *et al.*, 2011), profile Hidden Markov Models (HMMER) (Fjell *et al.*, 2007; Brahmachary *et al.*, 2004), Gap Local Alignment of Motifs 2 (GLAM2) (Fjell *et al.*, 2009). Furthermore, sophisticated algorithms have been designed to predict the physical and chemical properties of compounds, the 3-D structures of these molecules, their possible receptors and, their capacity to interact and bind to various receptors.

2.1.1. Tools utilised to predicted *in-silico* 3-D structures

In the past decades, conventional biophysical techniques such as Circular Dichroism, Nuclear Magnetic Resonance spectroscopy, X-ray crystallography, dual polarization interferometry and cryo-electron microscopy were utilized to determine the Three-Dimensional (3-D) structures of most proteins, peptides, DNA and RNA. However, the development of computational biology has also been marked by the design of sophisticated algorithms, which predict, with high accuracy the structures of these molecules. These computational tools implemented to predict these *in-silico* structures utilized methods that include: (1) the "fold recognition and threading" methods, (2) the "integrative" or "hybrid" methods, (3) the "comparative" or "homology" modelling approach and (4) the "*de novo*" or "*ab initio*" methods (Schwede *et al.*, 2008).

To describe an *in-silico* method for proteins and peptides 3-D structures prediction, the "*de novo*" or "*ab initio*" method will best suit this purpose as the molecules used in this work do not have defined structures. The Iterative Threading ASSembly Refinement (I-TASSER) server is a predictive server, which utilised the principle

underlined by the *de novo* method, to predict the 3-D structures of proteins and peptides based on their simple amino acids sequences (Wu *et al.*, 2007; Zhang, 2008; Roy *et al.*, 2010). The *de novo* method uses principles of physics that governs protein folding and/or using information derived from known structures but without relying on any evolutionary relationship to known folds. This tool completes its task by implementing a prediction of multiple alignments of the target sequence to look for a suitable and available template, followed by iterative structural assembly simulations, to generate a final structure (Schwede *et al.*, 2008).

The resultant 3-D structures predicted by the server generate various parameters, which aid in the results analysis and evaluation. These include the C-score, the TM-score and the Root Means Square Deviation (RMSD). The C-score given by the I-TASSER software is a confidence score, which estimates the quality of predicted models and ranges from -5 to 2. A C-score cut-off superior to -1.5 indicates that the model has a correct fold. The TM-score is a scale to measure the structural similarity between the predicted 3-D structure and the template structure used for this prediction. A TM-score greater than 0.5 indicates a model has a correct topology, however, a TM-score lower than 0.17 means a random similarity (Roy *et al.*, 2010). The Root Means Square Deviation (RMSD) measures the distance between atoms of superimposed proteins hence there are a strong correlation between the TM-score and the RMSD. An RMSD inferior to 1 Å is considered ideal as it indicates an identical structure. Nonetheless, an RMSD value around 2Å or above show that there was less distance between atoms of the proteins/peptides and the templates that were used for their 3-D structure prediction (Wei *et al.*, 1999; Carugo and Pongor, 2001). The number of Decoys represents the number of structural decoys that are used in generating each predicted 3-D model. Conversely, the cluster density is defined as the number of structure decoys at a unit of space in the SPICKER cluster (Zhang, 2008; Roy *et al.*, 2010).

2.1.2. Computational tools used for *in-silico* protein-protein interaction

Similar to the 3-D predictive tools, a swift shift has been applied for the screening of potential compounds of biological interest by performing an interaction to their various receptors using computational tools, to save time and money, and other factors that are demanding in molecular techniques. *In-silico* methods such as

PatchDock and SymmDock (Schneidman-Duhovny *et al.*, 2005), GRAMM-X (Tovchigrechko and Vakser, 2006), RosettaDock (Lyskov and Gray, 2008), PepSite (Petsalaki *et al.*, 2009), HexServer (Macindoe *et al.*, 2010), Haddock (Dominguez *et al.*, 2003), ClusPro (Comeau *et al.*, 2004) and ZDOCK (Chen *et al.*, 2003) have been highly utilised in this exercise. Though these tools aim to achieve the same objective, various principles are implemented in these servers, such as the FFT-based docking in GRAMM-X (Tovchigrechko and Vakser, 2006), ClusPro (Comeau *et al.*, 2004), ZDOCK (Chen *et al.*, 2003) and HexServer (Macindoe *et al.*, 2010); and the geometric hashing in PatchDock (Schneidman-Duhovny *et al.*, 2005), RosettaDock (Lyskov and Gray, 2008) and Haddock (Dominguez *et al.*, 2003) servers. These differences raise the questions of the effectiveness of each of tool and the advantages one may get from using one over the other. However, the protein's interaction result would be valuable if additional information is provided to explain how good the binding affinity is during the virtual screening of the receptors/targets and their ligands in drug discovery, diagnostics, or enzymatic reactions. As such, a scoring system ought to be added to each tool to make the prediction valuable. Yet, this characteristic is still posing a problem in the interpretation of various protein-protein interaction predictions (Kitchen *et al.*, 2004).

Taking the scoring system as an essential element for a good docking predictive tool, Patchdock server will be a good example as an *in-silico* predictive tool to accomplish protein-protein or protein-peptide interaction. Besides using a geometric hashing algorithmic system, the server is based on a rigid-body, which works on the principle of molecular shape complementarities between the 3-D structures of the two proteins or the protein-peptide involved in the complex formed. The tool performs a fast transformation, to search the six-dimensional transformational spaces created by the formation of the complex (Schneidman-Duhovny *et al.*, 2005).

2.1.3. Computational tools utilised for *in-silico* site-directed mutation

The interaction of two proteins or a protein-peptide interaction is made possible by the individual amino acids that contribute towards the binding affinity within the interaction formed by the complex. Such binding affinity and/or binding capacity might be impossible or disrupted if a single amino acids contributing to the complex formation is substituted or altered. These phenomena could enable the increase in

binding affinity in a complex formation by changing key amino acids in the interaction area (Schubert *et al.*, 2002).

To facilitate the possible effect of an amino acid residue on a given protein-protein or protein-peptide complex, computational tools in structural bioinformatics have also enabled a fast prediction of the consequences the substituted residue(s) might have in the binding affinity or in the orientation of the ligand on the receptor. Such *in-silico* tools include the Knowledge-based FADE and contacts (KFC) server (https://kfc.mitchell-lab.biochem.wisc.edu/KFC_Server/upload.php) (Darnell, *et al.*, 2007; Zhu and Mitchell, 2011). This server serves the purpose of site-directed mutagenesis, as the primary step of the tool is to determine residues that are essential for the complex formation and that keep the ligand at the right orientation with its receptor. However, amino acids residues called "hotspots" found at the interface of the complex formation of the protein or peptide are sensitive to mutation and as such cannot be mutated during the site-directed mutagenesis process because their substitution might alter the orientation of the ligand. Thus, only amino acids residues with no influence on the ligand orientation should be changed so as to increase the binding affinity of the ligand within the complex with its receptor.

2.1.4. Previous study

The putative anti-HIV AMPs were identified using an *in-silico* mathematical algorithm called Profile Hidden Markov Models (HMMER). During this process, experimentally validated anti-HIV AMPs were retrieved from various publicly available AMP databases, Antimicrobial Peptide Database (APD) (Wang and Wang, 2004; Wang *et al.*, 2009), Collection of Anti-Microbial Peptides (CAMP) (Thomas *et al.*, 2010), Bactibase (Hammami *et al.*, 2007), Uniprot Knowledgebase (UniprotKB) (Uniprot, 2009) and Dragon Antimicrobial Peptide Database (DAMPD) (Sundararajan *et al.*, 2011), and the peptides sequences classified according to super-families. Hidden Markov Model (HMMER) profiles were built for each super-family of anti-HIV AMPs. Following the scanning of proteome sequence databases using the trained profiles, 30 putative anti-HIV AMPs were identified.

The identified putative anti-HIV AMPs were ranked based on their E-values with only the 10 best AMPs with the lowest E-values used for the rest of the study. The putative anti-HIV AMPs physicochemical properties including: (*i*) the number of

basic residues, (*ii*) acidic residues, (*iii*) net charge, (*iv*) the Isoelectric point, (*v*) the Boman Index (or protein binding potential), (*vi*) Hydrophobic residues, (*vii*) the instability index of the proteins (Hammami *et al.*, 2007; Hammami *et al.*, 2010), (*viii*) the number of arginine (Arg) or lysine (Lys) residues, (*ix*) the presence of cysteine (Cys) residue of the putative anti-HIV AMPs and HIV protein gp120 were calculated using the prediction interface of Bactibase and APD. Following the prediction of the peptides physicochemical properties, their 3-D structures were predicted using I-TASSER, and the modelled AMPs were docked against the HIV protein gp120 using the PatchDock online server and the binding complexes visualized using PyMOL software, version 1.3.

Docking analysis of putative AMPs against HIV protein gp120 showed that only Molecules 1, 3, 7, 8 and 10 firmly binds the HIV protein gp120 at the point where this viral protein interacts with CD4+ of T cells, macrophages/monocytes and dendritic cells. Since the interaction of the viral protein gp120 to CD4+ of the host cells is vital for HIV to gain entry into the human cells and to replicate and proliferate (Kwong *et al.*, 1998; Zhou *et al.*, 2007; McLellan *et al.*, 2001), the implementation of the identified molecules to prevent this interaction could prevent the attachment of the virus to the host cells via their CD4+ surface molecules. Additionally, the binding affinity of the putative anti-HIV AMPs could be strengthened if the positive charged and/or hydrophobicity percentage of these peptides are increased by an amino acid substitition. Thus, this chapter aims to derive additional putative anti-HIV AMPs from the five parental anti-HIV AMPs, through an *in-silico* substitution of specific amino acids to increase the binding affinity of these AMPs to gp120. The specific objectives arising from these aims will be to:

➢ Identify mutation sensitive amino acids or "hotspot" amino acid residues,

➢ Complete an *in-silico* site-directed mutagenesis of the putative anti-HIV AMPs,

➢ Generate 3-D predictive structure of the derived AMPs, and complete an *in-silico* protein-peptide interaction of the derived putative anti-HIV AMPs and the HIV protein gp120,

➢ Analyse and visualize the *in-silico* binding studies using PyMOL, to confirm

that the binding of the complex is unchanged and the peptide still binds at the expected area.

2.2. Methods

2.2.1. Preparation of biological samples

To develop mutated anti-HIV AMPs, parental AMPs predicted to have anti-HIV activity in our previous work (Tincho et al., 2016) was utilized. Additionally, the HIV protein gp120 was used as receptor, to which the mutated AMPs will be docked to, to confirm their presumed activity.

2.2.1.1. Selection of the peptides

The AMPs selected for the site-directed mutagenesis were those peptides which were predicted to have anti-HIV AMPs due to the fact that these AMPs bind the HIV protein gp120, at the point where gp120 interacts with the surface protein CD4+ found at the surface of T cells, macrophages/monocytes and dendritic cells. These AMPs include Molecule 1, Molecule 3, Molecule 7, Molecule 8 and Molecule 10 (Supplementary material, Table A.1; Tincho *et al.*, 2016). These peptides were considered most relevant since they could prevent the binding of the viral gp120 protein to the host CD4 surface protein, by blocking this interaction. Thus these putative anti-HIV AMPs were good candidates for development as future entry inhibitor compounds

2.2.1.2. Selection of the HIV protein and extraction of gp120 sequence

The HIV protein gp120 was chosen as potential receptor during the conceptualization and development of entry inhibitors based peptides since this protein, which is a viral surface protein mediates the attachment and entrance of HIV into the host cells, and favours its replication and proliferation during the infection process (Kwong *et al.*, 1998).

The HIV protein gp120 utilized in this study was retrieved from the Protein Data Bank (PDB), previously solved with the X-ray crystallography technique (Zhou *et al.*, 2007). The gp120 protein 3-D structure was solved in complex with CD4 and 17b antibody, with PBD ID 2NXZ with the complex deposited into the Protein Data Bank

(PBD: http://www.rcsb.org/pdb/explore.do?structureId=2NXZ). The full sequence of the HIV protein gp120 sequence was downloaded from this database and this protein represents the chain A of the 2NXZ PBD file.

2.2.2. Optimization of the putative anti-HIV AMPs

2.2.2.1. Search for "Hotspot" residues on the parental putative anti-HIV AMPs

The site-directed mutagenesis performed on peptide molecules, to increase their binding affinity for a specific receptor, can only be possible if the substitution of an amino acid on the ligand does not alter its position and the orientation of its receptor. The substitution should also strengthen the electrostatic attraction between the two molecules in their complex formation so as to increase the binding affinity as compared to that of the parental peptide. Implementing this concept, crucial amino acids intervening in this complex affinity ought to be identified so the ligand does not shift after the mutation. These amino acids are termed "hotspot" residues, and are sensitive to substitution of amino acid residues due to the fact that they generate the forces and free energy responsible for maintaining the interaction between the ligand and the receptor in their complex formation (Darnell, *et al*., 2007; Zhu and Mitchell, 2011). To identify the potential "hotspot" amino acids in the complex formed between the parental putative anti-HIV AMPs and gp120, the task was achieved by uploading the complex formed between the individual parental putative anti-HIV AMPs and HIV protein gp120 from the previous study (Tincho *et al*., 2016) into the Knowledge-based FADE and contacts (KFC) online server (https://kfc.mitchell-lab.biochem.wisc.edu/KFC_Server/ upload.php) (Darnell, *et al*., 2007; Zhu and Mitchell, 2011). The outputs of this task generated the list of amino acid residues that contributes mostly to the interaction of gp120 and the putative anti-HIV AMPs, with the "hotspot" residues amongst these amino acid residues being highlighted in yellow colour (Table 2.1).

2.2.2.2. *In-silico* prediction of site-directed mutagenesis

Following the identification of "hotspot" residues within the interface of the interaction of the parental anti-HIV AMPs and gp120, *in-silico* site-directed mutagenesis could be carried out on the amino acid residues, which were not

considered as being crucial "hotspot" residues for maintaining the ligand in its right orientation when interacting with the gp120 protein.

The substitutions of amino acids introduced into the parental anti-HIV AMP sequences were done in consideration of the physicochemical properties of the individual amino acid changed. We also had to make sure that the introduced amino acid(s) still conserve their position and their role on the peptide, and increase their binding affinity when bound to gp120. Characteristics such as longer R-group amino acid residues of the same amino acid class, positive charged, hydrophobic amino acids were used in the mutation experiment. The sequences of the mutated AMPs, after *in-silico* site-directed mutagenesis are represented in the supplementary material (**Table A.2**).

2.2.2.3. Physicochemical characterization of the mutated AMPs

Several parameters such as: (*i*) the number of basic residues, (*ii*) acidic residues, (*iii*) net charge, (iv) the Isoelectric point, (v) the Boman Index (or protein binding potential), (*vi*) Hydrophobic residues, (*vii*) the instability index of the proteins (Wang and Wang, 2004); (*viii*) the number of arginine (Arg) or lysine (Lys) residues, (*ix*) the presence of cysteine (Cys) residue (Wang *et al*., 2010; Wang *et al*., 2011) were utilized to predict the physicochemical properties of the mutated putative anti-HIV AMPs and the HIV protein go120. Predictive online tools such as: Bactibase (http://bactibase.pfba-lab-tun.org/physicochem) (Hammami *et al*., 2007 and Hammami *et al*., 2010) and Antimicrobial Peptides Database (http://aps.unmc.edu/AP/design/design_improve.php) (Wang and Wang, 2004) were used to determine these parameters for the mutated AMPs.

2.2.2.4. *De novo* prediction of the mutated AMPs and gp120 3-D structure

The mutated anti-HIV AMPs and the HIV protein gp120 3-D structures were predicted using I-TASSER (Iterative Threading ASSembly Refinement) server, which is an example of a *de novo* method to predict the structure of unsolved peptides and/or proteins molecules, using *in-silico* predictive methods. I-TASSER server is a free online tool, (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and it is held at the University of Michigan, USA (Schwede *et al*., 2008, Zhang, 2008).

The 3-D structures of the derived (mutated) anti-HIV AMPs and that of HIV protein gp120 were predicted by uploading each sequence onto the I-TASSER website. The user enters their email address to which the results link is sent. After naming the uploaded sequence, the menu "Run I-TASSER" was selected.

2.2.2.5. Prediction of protein-peptide interaction of the AMPs and gp120

The docking of the individual derived anti-HIV AMP to the HIV protein gp120 was performed using PatchDock Beta, version 1.3. PatchDock is a free online web-server that allows for protein-protein and protein-small ligand molecule docking and is available at http://bioinfo3d.cs.tau.ac.il/PatchDock/.

Docking was done by uploading the PDB files of the respective putative anti-HIV AMPs and that of the HIV protein gp120 onto the PatchDock server website, after which the user enters an email address. The cluster RMSD was set to 4.0 Å and the complex type was selected as "protein-small ligand". The task was submitted by selecting "Submit Form". The docking results were sent via an email notification, containing the web link to the docking results. The result provided the highest scoring complexes between the HIV protein gp120 and the respective anti-HIV AMP as a PDB output file (Schneidman-Duhovny *et al.*, 2005). Interaction analysis of the complex formation between the HIV protein and the putative anti-HIV AMP was done using PyMOL software, version 1.3.

2.3. Results

2.3.1. "Hotspot" identification

The submission of the previous docking studies performed by the interaction of putative anti-HIV AMPs and gp120 protein into the Knowledge-based FADE and contacts (KFC) online server has enabled us to determine the important amino acid residues, which are indispensable to maintain the binding interaction of these putative anti-HIV AMPs) to the gp120 receptor. By doing so, we could effectively increase the binding affinity of the AMPs to gp120, and still keep these mutated AMPs at their right orientation with gp120 protein. The "hotspot" residues selected after uploading the initial complexes formed by the parental anti-HIV AMPs bound to gp120 into KFC online server are represented in Table 2.1, and are highlighted with a yellow

colour. However, the other amino acid residues not selected as "hotspot" residues are those that are not highlighted with the yellow colour (Table 2.1), on which mutation can be performed without affecting the shifting and/or change is the orientation of the mutated peptide or decreasing the binding affinity of the peptide.

Table 2.1: Hotspot residues at the interface of the interaction between the parental anti-HIV AMPs and gp120 protein. The amino acid residues highlighted in yellow colour represent the "hotspot" residues, whilst the amino acids residues not highlighted in yellow are the residues not selected as "hotspots" residues.

Molecule 1									
	Cys1	Leu2	Arg3	Tyr4	Lys5	Lys6	Pro7	Glu8	Cys9
	Ser11	Asp12	Gln14	Cys15	Pro16	Gly17	Lys18	Lys19	Arg20
	Cys30	Leu31	Asp32	Pro33	Val34	Asp35	Thr36	Pro37	Asn38
	Pro39	Arg41	Arg42	Lys43	Pro44	Gly45	Lys46	Cys47	Pro48
	Arg71	Asp72	Lys74						
Molecule 3									
	Arg1	Trp2	Lys3	Leu4	Phe5	Lys7	Ile8	Lys10	Val11
	Asn14	Val15	Arg16	Gly18	Leu19	Ala22	Pro24	Ala25	Ile26
	Ala27	Val28	Ile29	Gly30	Gln31	Ala32	Lys33	Ser34	Leu35
	Lys37								
Molecule 7									
	Arg1	Trp2	Ile4	Phe6	Ile8	Glu9	Met11	Gly12	Ile15
	Arg16	Ile19	Val20	Gly23	Pro24	Ala36	Ile26	Val28	Leu29
	Gly30	Ser31	Ala32	Lys33	Ala34	Ile35	Gly36	Lys37	
Molecule 8									
	Cys1	Lys3	Ser4	Gly5	Val11	Phe12	Cys13	Pro14	Arg15
	Arg16	Tyr17	Lys18	Gln19	Ile20	GLy21	Thr22	Cys23	Gly24
	Leu25	Pro26	Lys29	Cys30	Lys32	Lys33	Pro34		
Molecule 10									
	Trp1	Asn2	Pro3	Lys5	Glu6	Leu7	Lys9	Ala10	Gly11
	Gln12	Arg13	Val14	Arg15	Ala17	Pro23	Ala24	Val25	Asp26

	Val27	Val28	Gly29	Gln30	Ala31	Thr32	Ala33	Ile34	Ile35
	Lys36								

2.3.2. Site-directed mutagenesis prediction

After determining the amino acid residues that should not be substituted as they formed the binding affinity "hotspot" residues, mutation(s) of specific amino acid residues of the parental anti-HIV AMPs sequences were ready to be carried out. The criterion to mutate an amino acid was based on the group similarity of the substituted amino acid with that of the parental molecule because these amino acids have the same physicochemical property as the expectation was for the AMPs to remain at the same binding area of gp120, following site-directed mutagenesis.

Following the criteria mentioned above, amino acids with a high hydrophobic property could be used if the predictive percentage hydrophobicity of the AMP was less than 37 % as it has been established that the hydrophobicity will impact on the ability of a peptide to interact with its receptor (Biro, 2006). Besides the hydrophobicity percentage, positively charged amino acid was used because it is well known that most AMPs are positive charged and the positive charge is one of the elementary principles of selecting a good AMP. This property enables AMPs to have selective activity toward the pathogen rather than the host (Lee *et al*., 2011). In addition, amino acids with longer R-group could be used to reduce the distance and the area between the anti-HIV AMPs and gp120 protein; as it is said to strengthen the interaction between the ligand and the receptor, in this case, the interaction between the anti-HIV AMPs and gp120 protein (Biro, 2006).

From the concept enumerated above about the R-group length, shorter R-groups within the parental AMPs were substituted with longer R-groups for Molecule 3 ($V_{28}L$) and Molecule 10 ($V_{25}L$) (Table 2.2). Molecule 1.1 ($F_{62}W$) mutation was made by substituting the phenylalanine residue of the parental peptide with tryptophan amino acid. The introduced amino acid has the advantage of increasing the hydrophobicity of the mutated AMP. Molecule 7 was mutated by replacing the hydrophobic amino acid tryptophan with a positively charged and more hydrophobic amino acid residue namely histidine (Table 2.2). The last mutation was carried out on Molecule 8 where phenylalanine was replaced with histidine, at position 12 of the parental anti-HIV AMP ($F_{12}H$) (Table 2.2).

Table 2.2: Table displaying the position of each amino acid on the parental Molecule and the amino acid substitution on that same AMP.

Putative anti-HIV AMPs	Mutation
Molecule 1.1	$F_{62}W$
Molecule 3.1	$V_{28}L$
Molecule 7.1	W_2H
Molecule 8.1	$F_{12}W$
Molecule 10.1	$V_{25}L$

2.3.3. Physicochemical properties of the derived putative anti-HIV AMPs

The physicochemical properties of the derived anti-HIV AMPs were predicted so as to ascertain that these AMPs retained the same physicochemical properties as the parental AMPs and would still bind selectively to gp120 protein. The parameters used to determine the physicochemical properties of the mutated AMPs indicated that these findings correlated well with previous AMPs showing potent anti-HIV activity, as suggested by previous studies (Wang and Wang, 2004; Wang *et al.*, 2010; Wang *et al.*, 2011). It could be observed from Table 2.4 that the parameters predicted for the derived anti-HIV AMPs are not different from that of the parental anti-HIV AMPs (Table 2.3).

Table 2.3: Characterisation of the different parameters of the five putative anti-HIV AMPs

	Mass	Most common amino acids and %	Lys %	Arg %	Cys %	Isoelectric point	Net charge	Total hydrophobic ratio	Instability Index	Protein-binding Potential (Boman Index)	Half-Life in Mammals	Similarity with other molecules and percentage
Molecule 1	8903.716 Da	Cys: 16	11.39	6.33	16	8.37	+6	34 %	43.71	2.17 kcal/mol	1.2 hour	SLPI: 68.22 %
Molecule 3	4040.889 Da	Lys: 18.92	18.92	8.11	0.00	11.86	+8	43 %	27.80	1.37 kcal/mol	1 hour	Hyphancin IIIF: 81.08 %
Molecule 7	4073.94 Da	Lys: 18.92	18.92	8.11	0.00	11.46	+7	43 %	61.88	1.45 kcal/mol	1 hour	Cecropin B: 94.59 %
Molecule 8	3670.552 Da	Cys: 17.65	14.71	5.88	17.65	9.60	+8	38 %	48.28	1.07 kcal/mol	1.2 hour	hBD2: 82.92 %
Molecule 10	3908.564 Da	Ala: 16.67	11.11	5.56	0.00	10.33	+2	47 %	7.89	1.33 kcal/mol	2.8 hour	Cecropin D: 80.55 %

Table 2.4: Characterisation of the different parameters of the five mutated putative anti-HIV AMPs

	Mass	Most common amino acids and %	Lys %	Arg %	Cys %	Isoelectric point	Net charge	Total hydrophobic ratio	Instability Index	Protein-binding Potential (Boman Index)	Half-Life in Mammals	Similarity with other molecules and percentage
Molecule 1.1	8942.752 Da	Cys: 16	11.39	6.33	16	8.37	+6	34 %	44.30	2.18 kcal/mol	1.2 hour	SLPI: 67.28 %
Molecule 3.1	4054.916 Da	Lys: 18.92	18.92	8.11	0.00	11.86	+8	43 %	27.80	1.35 kcal/mol	1 hour	Hyphancin IIIF: 78.37 %
Molecule 7.1	4024.868 Da	Lys: 18.92	18.92	8.11	0.00	11.46	+8	40 %	58.00	1.64 kcal/mol	1 hour	Cecropin XJ: 97.29 %
Molecule 8.1	3660.516 Da	Cys: 17.65	14.71	5.88	17.65	9.60	+9	35 %	48.28	1.29 kcal/mol	1.2 hour	hBD 2: 80.48 %
Molecule 10.1	3922.591 Da	Ala: 16.67	11.11	5.56	0.00	10.33	+2	47 %	12.06	1.30 kcal/mol	2.8 hour	Cecropin D: 77.77 %

2.3.4. Three-dimensional structure prediction of the derived AMPs and gp120

The output from the I-TASSER server contains statistical parameters used to interpret the docking results. These statistical parameters are important indicators, which provide an estimate of accuracy scoring of the predicted derived AMPs and gp120 proteins 3-D structure. These statistical parameters are based on the C-score, TM-score, and RMSD (Roy *et al.*, 2010). The results of the predicted 3-D structures of the five mutated anti-HIV AMPs showed that these peptides have C-score values, which ranged from -1.96 to 0.68, with Molecule 1.1 having the lowest C-score and Molecule 8.1 having the highest C-score. It could be observed from Table 2.5 that all the peptides had C-score values higher than -1.5, except for Molecule 1.1, which had a C-score of -1.96. Whilst the TM-score of the five mutated AMPs oscillated between 0.48 and 0.81 all their TM-score values were above 0.5 except again for Molecule 1.1, which had a TM-score of 0.48. It should be noted that only mutated AMP Molecule 8.1 had a RMSD score that was less than 1Å. Whilst Molecule 3.1, Molecule 7.1 predicted 3-D structures had RMSD scores higher than 1.4 to 2.0 Å; Molecule 1.1 RMSD value was above 4 Å and was reported to be 7.6 Å (Table 2.5).

Although the 3-D structure of HIV protein gp120 was already solved, an *in-silico* prediction of this protein structure was performed to confirm that the *in-silico* tool used for the prediction of the AMPs 3-D structures was indeed accurate and those predicted structures satisfied the basis profiling of structure determination (Schwede *et al.*, 2008). The C-score of gp120 protein was reported to be 2, which is the highest value that can be obtained for C-scoring. Additionally, the TM-score was observed to be 0.99 and the RMSD of gp120 was less than 2Å and reported to be 1.7 Å.

Table 2.5: The mutated anti-HIV AMPs structure prediction and scoring

Putative AMPs	C-score	Exp. TM-score	Exp. RMSD (Å)
Molecule 1.1	-1.96	0.48 ± 0.15	7.6 ± 4.3
Molecule 3.1	0.43	0.77 ± 0.10	1.4 ± 1.3
Molecule 7 .1	0.15	0.73 ± 0.11	1.8 ± 1.5
Molecule 8.1	0.68	0.81 ± 0.09	0.8 ± 0.8
Molecule 10 .1	0.01	0.71 ± 0.11	2.0 ± 1.6
gp120 protein	2.00	0.99 ± 0.03	1.7 ± 1.5

Besides the statistical results from the structures prediction, a full-length secondary, as well as the tertiary structure prediction of each AMP, was generated. The

visualizations of these 3-D structures PDB files were done using the PyMOL software, version. 1.3. The mutated AMPs exhibited various secondary structures, represented by extended or loop structure with α-helical secondary structure (Molecule 1.1), anti-parallel and parallel β-sheeted secondary structure mix with a loop structure (Molecule 8.1) and α-helical structure (Molecule 3.1, Molecule 7.1 and Molecule 10.1). Besides the perfect C-score recorded for gp120 protein, the predicted secondary structure of gp120 protein was also noted to be the same as the solved structure because it is composed of the same number of α-helical, anti-parallel and parallel β-sheeted, and extended or loop structures (Figure 2.4).

Molecule 1   **Molecule 1.1**

Figure 2.1: Displays the predicted 3-D structure of the parental Molecule 1 and the derived Molecule 1.1. It was observed that the derived Molecule 1.1 is now displaying an extended partial α-helical structure, which was not present in the parental Molecule 1.

Molecule 8   **Molecule 8.1**
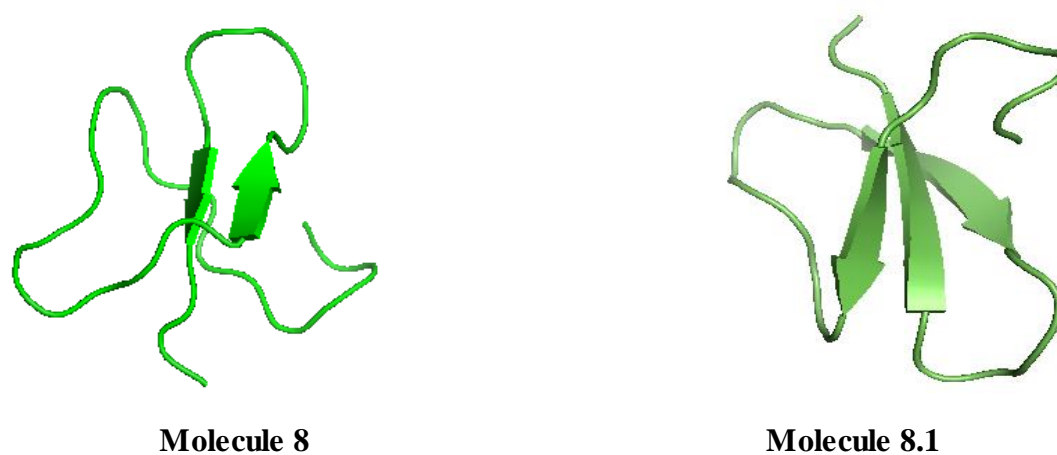
Figure 2.2: Displays the predicted 3-D structure of the parental Molecule 8 and the derived Molecule 8.1. It was observed that the derived Molecule 8.1 is now displaying an extended partial antiparallel β sheet structure, which was not present in the parental Molecule 8.

Molecule 3.1   **Molecule 7.1**   **Molecule 10.1**
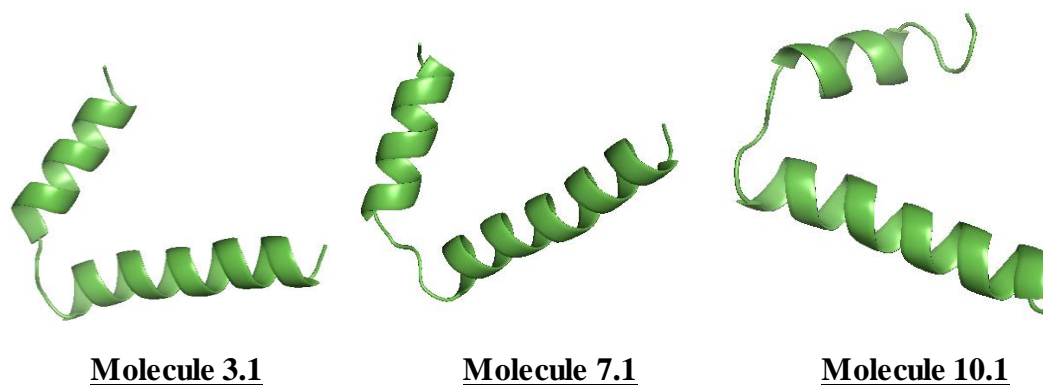
Figure 2.3: Displays the predicted 3-D structure of the derived of Molecule 3.1, Molecule 7.1 and Molecule 10.1. The derived AMPs have the same α-helical secondary structure as their parental counterparts AMPs, which are Molecule 3, Molecule 7 and Molecule 10 (Supplementary material **Figure A.1**).
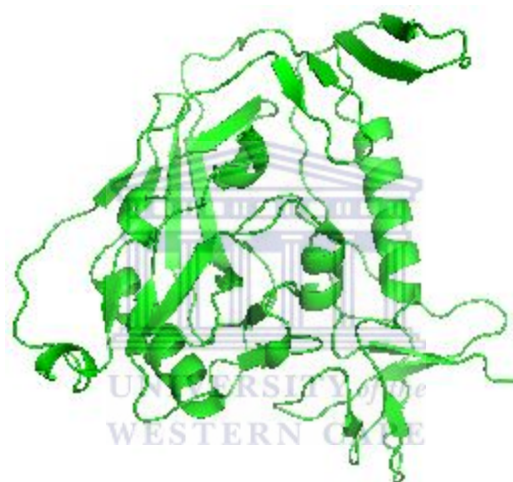
Figure 2.4: Cartoon representations of the 3-D structures of HIV protein gp120, predicted using I-TASSER server. The protein consists of α-helices, antiparallel and parallel β-sheets, and loop structures.

2.3.5. Protein-peptide interaction studies of gp120 and putative anti-HIV AMPs

Predicting the capacity of the derived anti-HIV AMPs to bind HIV protein gp120 using PatchDock server could provide insight into the potential roles of these AMPs to inhibit HIV replication using *in-vitro* studies. The results generated geometric scores of the binding affinity of the individual mutated anti-HIV AMP with gp120 protein, and these scores ranged from 14236 to 12140. From the results it was observed that the binding of the derived anti-HIV AMPs to gp120 had very high binding scores (Table 2.6). At closer examination of the binding predictions, it was observed that the binding of certain parental AMPs was higher than their mutated

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counterparts. The binding affinity of Molecule 1.1 and that of Molecule 3.1 was reduced as compared to the binding affinity of the parental AMPs (Molecule 1 and Molecule 3), bound to gp120 (Table 2.6). Conversely, the binding affinity of the parental AMPs of Molecule 7, Molecule 8 and Molecule 10 compared to that of their mutated AMPs were positive, with Molecule 8.1 having the highest binding difference of all, thus Molecule 8.1 could be expected to have the best anti-HIV activity.

Table 2.6: Binding affinities, position of parent AMPs and derivative AMPs on HIV protein gp120 and percentage increase or decrease

AMPs	Mutation	Parental AMPs binding score	Derived AMPs binding score	Binding difference	% Increase
Molecule 1.1	$F_{62}W$	14926	14236	- 690	-4.6 %
Molecule 3.1	$V_{28}L$	13686	12548	-1138	-8.3 %
Molecule 7.1	W_2H	13648	13338	310	2.3 %
Molecule 8.1	$F_{12}H$	11086	12140	1054	9.5 %
Molecule 10.1	$V_{25}L$	12208	12276	68	0.6 %

Besides the geometric scoring generated by PatchDock server, we had to study the position of the derived anti-HIV AMPs bound to HIV protein gp120 and their spatial arrangement in the three-dimensional coordinates.

The spatial arrangement of the docked molecules represents the position of the interaction space, in three-dimensions when they are placed at the coordinates X, Y and Z. The transformation gives the position of the complex in the (X), (Y) and (Z) axes (Table 2.7). The area reported in Table 2.7 represent the surface covered during the interaction of the mutated anti-HIV AMPs and gp120; and the force that contributed to the interaction of these two molecules are calculated from the various individual atoms that forms Hydrogen bonds, van der Waal's Forces, hydrophobic interactions and ionic interactions (Salt Bridges). The total force of this interaction is calculated to generate the Atomic Contact Energy (ACE), and Table 2.7 depicts the ACE of each interaction, of the mutated anti-HIV AMP and gp120.

Table 2.7: The area cover and the ACE's from the docking the gp120-Mutated-anti-HIV AMP using the PatchDock docking server.

	gp120		
	Area (Å^2)	ACE	Transformation coordinates
Molecule 1.1	1948.40	332.53	-0.19 -0.36 2.18 86.57 -2.78 -38.62
Molecule 3.1	1760.20	-157.17	-2.72 0.05 -1.02 -31.93 62.17 40.00
Molecule 7.1	1741.40	38.07	0.44 -0.49 -2.46 49.50 47.26 -50.38
Molecule 8.1	1563.10	2017.21	-1.82 0.81 1.57 36.38 -61.29 -9.24
Molecule 10.1	2216.90	-283.32	-1.70 0.58 -032 -62.81 -22.42 33.32

This visual check of the interacting structure was to confirm that these AMPs in addition to their high binding score truly binds to gp120, at the point where gp120 binds CD4+ surface molecule to invade and attack the human T cells, macrophages/monocytes, and dendritic cells.

The PDB files from the docking study of the mutated AMPs and gp120 using PatchDock server were visualized using PyMOL software, version 1.3. The analysis of the cartoon representation of gp120 interaction with the derived AMPs proves that Molecule 1.1, Molecule 3.1, Molecule 7.1, Molecule 8.1 and Molecule 10.1 bind gp120 at various areas of the protein (**Figure 2.5**, **Figure 2.6**, **Figure 2.7**, **Figure 2.8** and **Figure 2.9**). The binding of these mutated anti-HIV AMPs to various positions of gp120 and these various interactions to gp120 protein might have different implications on the ability of HIV to replicate and might regress through interacting with either of these AMPs.
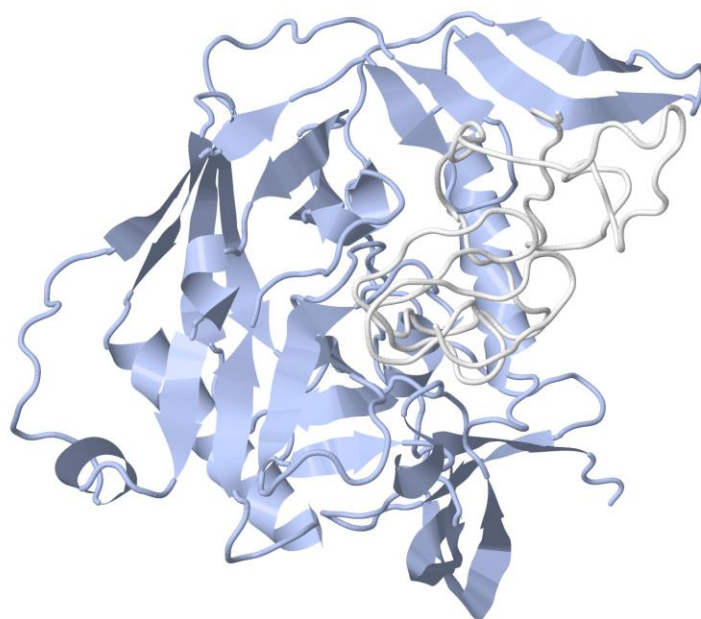
Figure 2.5: gp120-Molecule 1.1 complex formation during the anti-HIV-gp120 interaction. The cartoon representation in purple colour is the HIV protein gp120 and the derived anti-HIV AMP (Molecule 1.1) is represented in light grey colour.
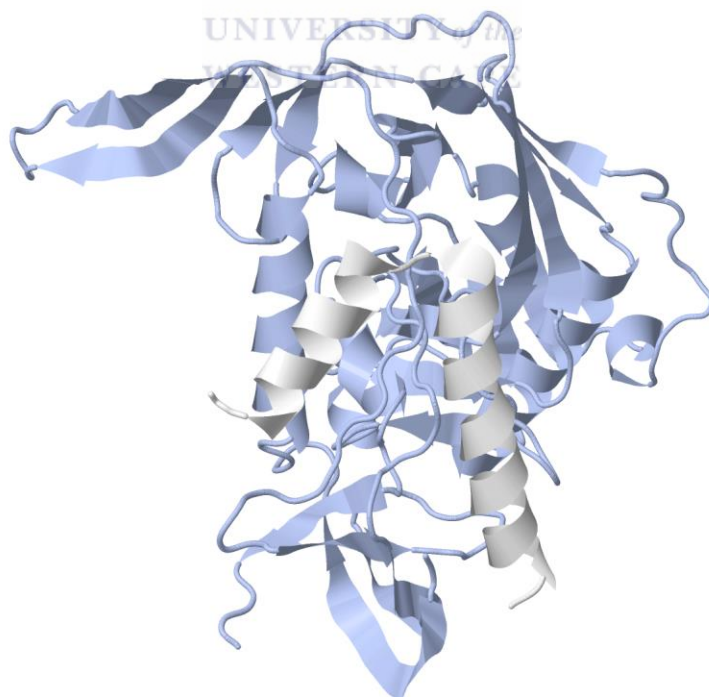
Figure 2.6: gp120-Molecule 3.1 complex formation during the anti-HIV-gp120 interaction. The cartoon representation in purple colour is the HIV protein gp120 and the derived anti-HIV AMP (Molecule 3.1) is represented in light grey colour.
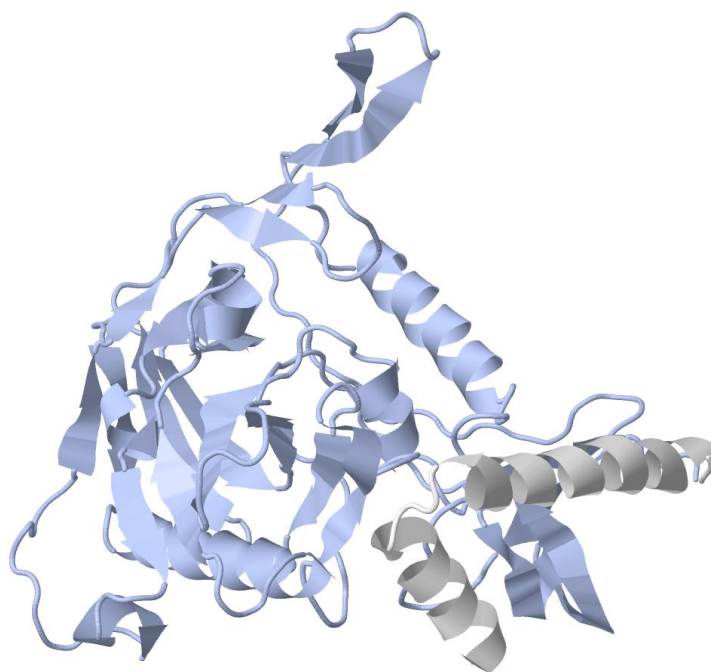
Figure 2.7: gp120-Molecule 7.1 complex formation during the anti-HIV-gp120 interaction. The cartoon representation in purple colour is the HIV protein gp120 and the derived anti-HIV AMP (Molecule 7.1) is represented in light grey colour.
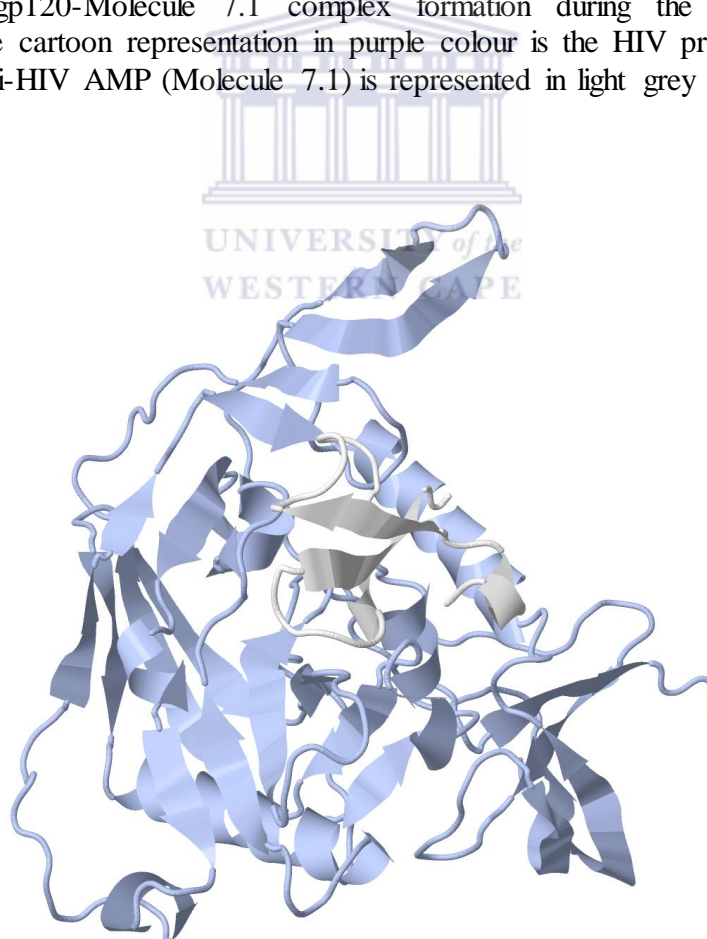
Figure 2.8: gp120-Molecule 8.1 complex formation during the anti-HIV-gp120 interaction. The cartoon representation in purple colour is the HIV protein gp120 and the derived anti-HIV AMP (Molecule 8.1) is represented in light grey colour.
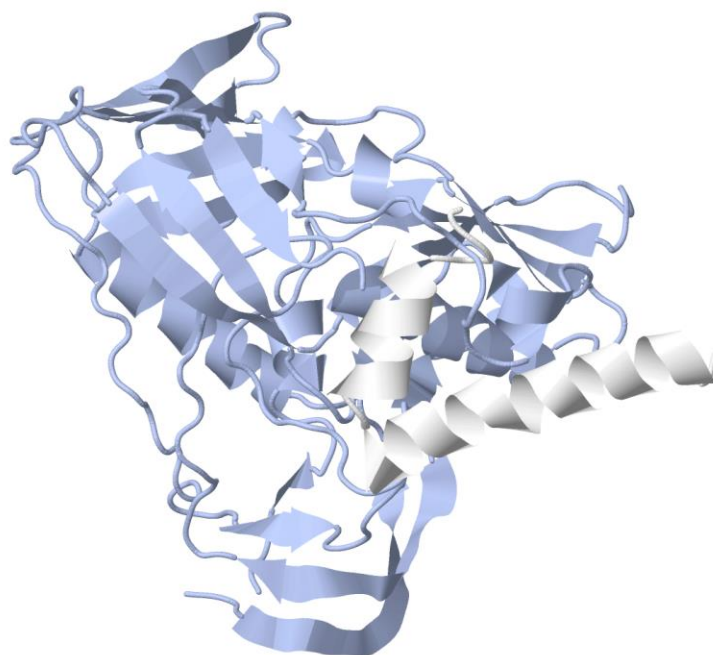
Figure 2.9: gp120-Molecule 10.1 complex formation during the anti-HIV-gp120 interaction. The cartoon representation in purple colour is the HIV protein gp120 and the derived anti-HIV AMP (Molecule 10.1) is represented in light grey colour.

2.4. Discussion

The world has still not recovered from the havoc caused by HIV since the discovery of the virus. This was mostly due to the poor information about the virus routes of infection and the predispositions to get infected with this deathly pathogen. Though tremendous efforts have been made to understand the pathogenesis and the mechanisms of HIV infection, many strategies have been used to design therapeutic regimens to inhibit the virus replication at any stage of its life cycle. However, these therapeutic regimens are still unable to eradicate the virus from the human body. Entry inhibitors of HIV ought to be used to stop the virus to get into the host's cells through more specific avenues. Recent research has identified Antimicrobial Peptides (AMPs) that could help prevent or stop HIV entrance into T cells, macrophages/monocytes, and dendritic cells, by binding gp120 at the area where gp120 interacts with CD4+ of these cells (Tincho *et al.*, 2016). These putative anti-HIV AMPs could now also permit the development of derivative anti-HIV AMPs from these initial AMPs with a higher and more potent anti HIV activity.

Mutating the parental anti-HIV AMPs could increase their binding affinity to the HIV protein gp120. In this regard, the Knowledge-based FADE and Contacts (KFC) online

server were used to select possible amino acid residues, which could be subjected to mutation. From the results generated from the KFC server, "hotspot" amino acid residues were identified which are the main residues responsible for the binding affinity between the AMPs and gp120, at a specific area of interaction. Thus these residues ought not to be changed during the site-directed mutagenesis exercise, which aims to increase the binding affinity of these AMPs to gp120. The non-"hotspot" residues can thus be subjected to mutation, to increase the binding affinity between the derived anti-HIV AMP and gp120, without expecting any major change of the anti-HIV AMPs initial binding position (Darnell *et al.*, 2007; Zhu and Mitchell, 2011).

Following the determination of the parental anti-HIV AMPs "hotspot", site-directed mutation of these peptides were performed using specific amino acid residues, and each amino acid used in the substitution exercise can be justified from a physicochemical property point of view i.e. the derived AMPs have similar properties as their mutated counterparts. To this end, mutations of the parental anti-HIV AMPs to generate mutated peptides: Molecule 1.1, Molecule 3.1 and Molecule 10.1 were performed using amino acids of longer R-group but of the same amino acid class. This decision was based on the fact that longer R-group amino acids could be used to reduce the distance and the area between the anti-HIV AMPs and gp120 protein, thus strengthening the interaction between the peptide and gp120 protein (Biro, 2006). Nonetheless, the geometric binding score of Molecule 1.1 interacting with gp120 protein, and Molecule 3.1 bound to gp120 seem to deviate from the aim to increase the binding affinities as the binding scores for the mutated AMPs showed a decrease, when compared to the binding affinities of their parental molecules to gp120.

Binding affinity of Molecule 10 bound to gp120 compared to Molecule 10.1 bound to gp120 gave a net positive difference (increased binding affinity) (Table 2.2). The unexpected result that Molecule 1.1 binding affinity to gp120 did not show an increase as compared to his parental AMP since it is well known that the tryptophan amino acid residue has been proven to be relevant in increasing the binding affinity of AMPs (Chan *et al.*, 2006). The inconsistency in results could be due to the fact that that the use of a hydrophobic amino acid residue to replace the previous amino acid found in the parental sequence for Molecule 1 did not have the desired effect. Thus, additional mutations could be employed making use of a positively charged and

hydrophobic residue such as the amino acid histidine so as to increase the charge of this AMP, thus increase the binding affinity of Molecule 1 to gp120 protein (Lee *et al.*, 2011). The same emphasis will be placed when performing additional mutations to Molecule 3 and Molecule 10, where more hydrophobic and positively charged amino acid will be used as substitutes.

Besides the substitutions performed on the Molecule 1, Molecule 3 and Molecule 10, mutations done on Molecule 7 and Molecule 8 were achieved by introducing amino acids which have a greater hydrophobicity and positively charged such as histidine. For Molecule 8.1, the change of phenylalanine to histidine was to strengthen the electrostatic interaction of this mutated peptide and gp120, without shifting the peptide from its original point of interaction, with a net positive percentage of 9.5 % obtained when calculating the binding difference between the parental peptide and the derived one (Table 2.6). Furthermore, the effect of the positively charged amino acid histidine introduced into Molecule 7 to generate Molecule 7.1 resulted in a noticeable increase in its interaction with gp120. The substitution of a tryptophan residue to a histidine residue also showed an increase in the binding affinity of the derived AMP to gp120, when compared to the binding affinity of the parental AMP, bound to gp120 for the same molecule (Table 2.6). The significance of introducing a histidine amino acid finds its usefulness in increasing the binding affinity of the peptide to its target (Yeaman and Yount, 2003).

After the substitution of amino acids performed on the five parental AMPs, the next step was to determine that the mutated versions were not similar to any existing AMP and that these AMPs had no altered activity following mutagenesis, by characterising their physicochemical properties. It was confirmed following alignment of the mutated peptides sequences that none of the mutated AMPs sequences were similar to known AMPs sequences in any of the antimicrobial peptide databases (Table 2.4), thus these AMPs have not yet described as AMP and/or implicated in the inhibition of HIV.

The AMPs properties could only be considered meaningful if certain characteristics are taken into consideration during the determination of their physiochemical properties. As such, the computation of the mutated AMPs net charged showed that all the mutated anti-HIV AMPs had the same net positive charge as observed for the

parental anti-HIV AMPs except for Molecule 7.1 and Molecule 8.1 which have an additional + 1 charge as compared to the parental peptides, Molecule 7 and Molecule 8 (Table 2.3 and Table 2.4). This additional charge to both AMP compounds was due to the additional positive amino acid histidine residue introduced during the substituting exercise. This increase in the positive charged has repercussions on the ability of Molecule 7.1 and Molecule 8.1 to bind gp120 with high binding affinity, as compared to the parental peptides, Molecule 7 and Molecule 8 that binds to gp120 with a lower binding affinity (Table 2.6). It could thus be deduced that positive charged amino acids are crucial for AMPs binding to their targets and could exhibit their activity with high performance. Another very important element considered during thus characterisation was the hydrophobic percentage of each mutated AMP.

Despite the mutations performed on the parental AMPs, it was observed that the hydrophobicity percentage, another major contributor of an AMP binding ability to its target and its mechanism of action has not majorly increased in all the mutated AMPs. On the contrary, the introduction of the hydrophobic and positively charged histidine amino acid residue did not seem to increase the hydrophobic percentage of Molecule 7.1 and Molecule 8.1, the introduction of this residue rather contributed to the decrease of the hydrophobic percentage of these two AMPs (Table 2.3 and Table 2.4). However, all the mutated AMPs had a hydrophobic percentage higher than 30 %, the minimum hydrophobicity value required for a peptide to be a good AMP, to carry out its biological activity (Hancock and Diamond, 2000; Hancock and Sahl, 2006).

Another characteristic to take in consideration for a good AMP is the Boman Index of the mutated peptides. The Boman Index is the assessment of a peptide to firmly bind their receptor (proteins, DNA or RNA), and it is calculated as the sum of free energies of all amino acid residue side chains divided by the total number of amino acid residues (Boman, 2003). To put this parameter into perspective, it has been demonstrated that AMP with a Boman Index value lower or equal to one (≤ 1) could suggest that this particular AMP is likely to have higher antimicrobial activity without many side effects. Whilst, AMPs having Boman Indices less than zero could only have antibacterial activity, AMPs with higher Boman Index value (2.50-3.00) could signify that these peptides have multifunctional roles, with hormone-like activities (Boman 2003). There were no major changes in the Boman Indices of the mutated peptides as compared to the parental peptides despite the mutations carried out on the

parental peptides. Nevertheless, Molecule 7.1 and Molecule 8.1 have some considerable shifts in their Boman Indices as compared to their parental peptides, Molecule 7 and Molecule 8 (Table 2.3 and Table 2.4). The Boman Indices of the mutated anti-HIV AMPs were appreciated since their values were less than 2.5 kcal/mol, demonstrating that these AMPs have the likelihood to individually bind gp120 protein and prevent the binding of gp120 protein to CD4 and thus inhibiting the virus replication.

The other parameter that is essential for an AMP to harbour anti-HIV activity is the presence of cysteine amino acid residue in their sequence. The results depicted in Table 2.4 showed that only Molecule 1.1 and Molecule 8.1 have cysteine residue in their sequence. Thought all of the AMPs have been predicted to bind HIV gp120, at the area where gp120 interacts with CD4 surface protein of the hosts, not all of them had the presence of cysteine amino acid residues (Tincho *et al.*, 2016). This residue has however been proven to be essential for the folding of the peptide sequence and enhances the ability of the AMP to exhibit proper anti-HIV activity *in-vitro* (Wang *et al.*, 2011).

The binding of the mutated AMPs to HIV protein gp120 could only be possible if the AMPs and gp120 protein have taken on their 3-D structure conformations. As such, their structures were predicted using the I-TASSER server. The statistical results proved that the predicted structures of all the mutated AMPs except for Molecule 1.1 had C-scores higher than -1.5, implying that the predicted structures have the correct fold. However, the C-score of Molecule 1.1 which was lower than the expected C-score could signify that its structure was randomly predicted and that a proper template was not available to gather the necessary details for an accurate 3-D structure prediction (Roy *et al.*, 2010). A C-score of 2 was obtained when gp120 3-D structure was predicted using I-TASSER lending credibility in using this tool for 3D structure prediction (Table 2.5). This score could be justified by the fact that the structure of gp120 has been solved already (Kwong *et al.*, 1998; Zhou *et al.*, 2007) and this solved structure was utilised as a template for the *in-silico* prediction of gp120 structure, thus the perfect C-score obtained.

The other statistical parameter to evaluate the relevance of the predicted structures was to evaluate the TM-score of the mutated AMPs. All the five mutated AMPs had

TM-score above 0.5, except for Molecule 1.1, with a TM-score of 0.48. The TM-scores being superior to 0.5 signify that the predicted 3-D structures of the mutated AMPs were similar to the templates utilized for the prediction of their conformation. The low TM-score obtained for Molecule 1.1 meant that the template used for its structure prediction was not similar to the peptide which structure was to be predicted (Zhang, 2008; Roy *et al.*, 2010).

The Root Mean Square Deviation (RMSD) of the mutated AMPs generating scores of 2 Å or below signifying that there were fewer distance differences between atoms of the putative peptides and atoms of the templates which were used for their 3-D structure prediction (Wei *et al.*, 1999; Carugo and Pongor, 2001). This parameter was however very high for Molecule 1.1, meaning that there was huge atomic deviation of this peptide as compared to its template. Whilst predictive results could be obtained either for the structures well predicted with good templates or those structures predicted with less accuracy due to the lack of a proper template, observation would be made that there is a strong correlation between the C-score, the TM-score and the RMSD of the predicted AMP structures (Roy *et al.*, 2010). The same observations were made by Roy and co-workers who showed that their predicted structures showed a strong correlation between these three algebraic parameters (Roy *et al.*, 2010). This strong correlation between the TM-score and the RMSD is a key element that permits the generation of good topologies observed in the predicted 3-D structures of the mutated AMPs and the HIV protein gp120.

The secondary structures generated during 3-D prediction of the five mutated AMPs displayed different conformation ranging from the α-helical, antiparallel and parallel β-sheeted, the extended and the loop structures proving that AMPs despite being diverse in origin and conformation could exhibit the same biological activity. The presence of α-helical conformation in Molecule 1.1 was observed which could be justified by the introduction of tryptophan amino acid residue in the parental AMP. The presence of this amino acid has been proven to favour the presence of α-helical conformation, thus the presence of a α-helical conformation in the structure of Molecule 1 following its mutation (Lee *et al.*, 2011). An additional β-sheeted conformation was also present in Molecule 8.1 with the introduction of the amino acid histidine into the parental AMP, Molecule 8 thus having a combination of parallel and antiparallel β-sheeted in its mutated version Molecule 8.1. However, the

same β-sheeted was not visible in Molecule 7.1 despite the introduction of the same histidine amino acid residue in the parental AMP, after the site-directed mutagenesis.

The confirmation that the mutated AMPs would still prevent the interaction of gp120 of HIV to the CD4+ of T cells, macrophages/monocytes, and dendritic cells could be shown if predictive experiments are performed to demonstrate their binding to this particular area. As such, the geometric score obtained from docking of HIV protein gp120 to the five mutated AMPs yield good prediction despite the fact that the score was decreased for Molecule 1.1 and Molecule 3.1 as compared to the parental AMPs Molecule 1 and Molecule 3, bound to gp120 (Table 2.6).

Despite the substitution of amino acid residues in Molecule 1, Molecule 3 and Molecule 10 with amino acid residues with longer R-group, the decrease in binding score could be explained by the fact that the substituted amino acids were not positively charged, even though these three AMPs carried the recommended hydrophobicity percentage (Table 2.4). On the contrary, an increase in binding affinity was obtained for Molecule 7.1 and Molecule 8.1 when these AMPs were bound to gp120, signifying that the histidine amino acid plays a huge role in the AMP's ability to bind the protein gp120. Furthermore, the role of this particular amino acid residue has been proven to increase the interaction of AMPs to their receptors during binding (Chan *et al*., 2006). The recommendation should then be made that further site-directed mutagenesis has to be performed using positively charged amino acid residues so as to increased the net charge of the AMP.

Although the geometric score of the binding of the mutated AMPs and HIV protein gp120 looks promising, the probability of the mutated AMPs to prevent the binding of HIV protein gp120 to CD4+ surface molecule of T cells, macrophages/monocytes and dendritic cells still require close examination. It was observed from the binding results that Molecule 3.1, Molecule 7.1 and Molecule 10.1 did not bind HIV protein gp120 at the point where this protein interaction with CD4+ of the host cells: T cells, macrophages/monocytes and dendritic cells (Figure 2.6, Figure 2.7 and Figure 2.9) as compared to the parental AMPs (Supplementary material, Figure A.3, Figure A.4, Figure A.6). However, Molecule 1.1 and Molecule 8.1 bind gp120, at this point (Figure 2.5 and Figure 2.8), as was observed for the parental compounds (Supplementary material, Figure A.2, Figure A.5).

The Atomic Contact Energy (ACE) generated during this interaction could also justify the results obtained in the visual representation of the complex formed after the docking of gp120 and the mutated AMPs. It was noticed that only Molecule 1.1 and Molecule 8.2 showed a rise in the ACE parameter as compared to the ACE generated for the interaction of the same protein and the parental AMPs, Molecule 1 and Molecule 8 (Supplementary material, Table A.2). Conversely, Molecule 3.1, Molecule 7.1 and Molecule 10.1 showed a decrease in ACE as compared to the interaction of their parental AMPs with gp120. The rise in ACE for Molecule 1.1 and Molecule 8.1 is as a consequence of introducing more hydrophobic and positively charged residues into the parental AMPs (Table 2.2). Nonetheless, the same rise was not observed for Molecule 7.1, which was obtained by introduction of a histidine residue. Another explanation of the appropriate binding of the mutated AMPs to gp120 could be justified by the presence of cysteine amino residue in Molecule 1.1 and Molecule 8.1, whose role in inhibiting HIV has been proven (Wang *et al.*, 2011).

Since it is well demonstrated that the interaction of HIV protein gp120 to CD4+ surface of T cells, macrophages/monocytes and dendritic cells are an advantage for HIV to penetrate the host cells (Kwong *et al.*, 1998; Zhou *et al.*, 2007; McLellan *et al.*, 2001), the results obtained during docking of Molecule 1.1 and Molecule 8.1 with gp120 protein is unique following the concept that HIV inhibition could be stopped by preventing the interaction of gp120 to CD4+ of the host cells. Thus, only Molecule 1.1 and Molecule 8.1 should be considered as potential candidates to be utilized as future anti-HIV compounds, to prevent the binding of HIV protein gp120 to their preferential host receptors, T cells, macrophages/monocytes and dendritic cells. Implementing this concept with the usage of AMPs is more advantageous since these peptides are able to selectively bind to the pathogens molecules than the host cells or components.

2.5. Conclusion

The discovery of these novel compounds has not only paved the route for the design of peptide-based drugs that could act as entry inhibitors, to prevent the replication and spread of the HI Virus, the identification of the AMPs highlight the possibility of these molecules to be mutated by introducing amino acid residues that can favour the increase in the AMPs binding capacity to gp120. The work conducted previously

identified putative AMPs, which could block the binding of HIV protein gp120 to host cells CD4+ surface protein (Tincho *et al.*, 2016).

Although site-directed mutagenesis of the parental AMPs was done by performing single amino acid substitution, with amino acids with longer R-groups, hydrophobic residues and positively charged residues, more mutational studies ought to be conducted so as to obtain more AMPs that can bind gp120 protein, at the site where it interacts with CD4 surface protein. Nonetheless, mutations performed showed that the physicochemical characterization of these mutated compounds could already indicate the possibility of the AMPs having good binding affinity towards gp120. Furthermore, after predicting the 3-D structure of the mutated AMPs and gp120 and performing docking study, the results indicated that the AMPs with a positive binding difference are the ones which had an increase in positively charged amino acids as shown in the *in-silico* characterization. Additional confirmation was provided through visual image inspection of the complexes formed between gp120 and the mutated AMPs during the docking study of this chapter. It could be observed that only Molecule 1.1 and Molecule 8.1 bind gp120 at his particular area of interaction with CD4+, prompting us to only select these two compounds as potential potent entry inhibitors of HIV in a molecular validation study. Further site-directed mutagenesis would help identify additional AMPs that can be applied as entry inhibitors compounds to block HIV binding to T cells and other host cells.

CHAPTER THREE: BINDING CAPACITY OF SELECTED PUTATIVE ANTI-HIV AMPS TO HIV PROTEIN GP120

3.1. Introduction

Since the onset of HIV in the late 1980's, one of the major devotions to reduce HIV progression has been the development of potential drugs, which can prevent the virus replication so as to stop its spread to other cells and/or infect other healthy individuals. These efforts has enabled the appearance of many drugs implemented for HIV treatment termed High Active Antiretroviral Therapy (HAART) and includes many classes as discussed in section 1.2.3 of Chapter 1 (Delaney, 2006). These molecules main objective is preventing viral progression by attacking HIV at various stages of the virus life cycle, and to reduce the mortality rate of the infected patients.

Whilst these molecules slow down virus replication and/or progression, the efficiency of HAART are still questionable due to the fact that NRTIs, NNRTIs, INSTIs and PIs are used to fight HIV progression when the virus has already penetrated into the human T cells, macrophages/monocytes and dendritic cells (Bean, 2005; Pang *et al.*, 2009; Hare *et al.*, 2010; Volberding and Deeks, 2010). Since the goal was to develop therapeutic molecules that would inhibit the virus before it gets into the human cells, the design of Fusion or Entry Inhibitors (FIs or EIs) were eminent.

Though the research on FIs or EIs is still in its developmental stages and that only one such molecule has received FDA approval (Kilby *et al.*, 2002; Lalezari *et al.*, 2003), efforts ought to be doubled so that more FIs are approved. Thus, the design of molecules/peptides that could prevent HIV gp120 protein interaction with the CD4+ of the T cells and macrophages/monocytes were developed, using a mathematical prediction algorithm. The binding of these peptides to this particular site was demonstrated using a structural bioinformatics method (Tincho *et al.*, 2016). The selection of these molecules could be relevant due to certain points: Firstly, these peptides bind to HIV gp120 protein, at the area where the protein interacts with CD4+

of T cells and macrophages/monocytes. Secondly, the only FIs or EIs that has received FDA approval are of peptide origin.

Although the function of these peptides was demonstrated with *in-silico* methods, the validation of their role(s) should be confirmed using molecular methods. For the purpose of showing specific binding of the peptides to gp120, examples of such techniques include Isothermal Titration Calorimetry (ITC), Circular Dichroism (CD), Surface Plasmon Resonance (SPR), Bio-Layer Interferometry (BLI), Dual Polarization Interferometry (DPI), Fluorescence Resonance Energy Transfer (FRET) and Microscale Thermophoresis (MST) (Berggård *et al.*, 2007). Whilst the techniques cited above are mostly used to demonstrate and confirm protein-protein interaction, predicted in an *in-silico* study, this current study has made use of another technique, which demonstrates protein-protein interaction in an on/off experimental setup, where the receptor and the ligand are allowed to bind in a Lateral Flow Devise (LFD) set-up. The technique has been implemented in a study published by Williams *et al.*, 2016 related to the interaction of HIV p24 protein with a number of selected AMPs. Though the function described here is not the same, the need for such technique was to show in a fast and simple experiment putative anti-HIV AMPs binding to HIV gp120 protein. This study could serve as a confirmatory method to justify the *in-silico* prediction made in Chapter Two on the interaction of HIV gp120 protein with various putative anti-HIV AMPs, and HIV gp120 protein as well as mutated versions of these AMPs. The results of a positive interaction are observed as the intensity displayed by bands represented in the window of the LFD and are interpreted as Dot-Blot intensity, using an in-house colour rating design by Medical Diagnostech (Pty) Ltd, with the scale ranging from G1 to G10. Whilst a negative result is the absence of a band in the LFD window thus signifying that there was not interaction between the receptor and the ligand.

The major goal of this part of the project was to express and purify a recombinant HIV gp120 and to use this protein in an "in house" LFD binding experimental disposition, with the putative AMPs and the mutated AMPs, predicted to bind gp120, with the work performed in collaboration with our industrial partner, Medical Diagnostech (Pty) Ltd.

The specific objectives to achieve in this piece of work were to:

➢ Express and produce a purified recombinant HIV gp120 protein,

➢ Bio-conjugate the putative and mutated AMPs with gold nanoparticles,

➢ Perform the interaction study of the HIV gp120 protein and the AMPs, to confirm the *in-silico* prediction.

3.2. Methods

3.2.1. Expression of recombinant HIV protein gp120

3.2.1.1. Designing of the gp120 expression insert

The HIV gp120 gene sequence was generated using a computational method, by translating the gp120 amino acid sequence utilized in the protein-AMP docking study (Chapter Two). The gp120 amino acid sequence was taken from the PDB ID: 2NXZ, representing the A-chain of this complex (Zhou *et al.*, 2007). The HIV gp120 amino acid sequence was reverse-translated into a nucleotide sequence using the online tool: Sequence manipulation Suite Reverse translate (http://www.bioinformatics.org/sms2/rev_trans.html) (Stothard, 2000). The DNA sequence was submitted to GenScript® Company (USA) for optimization so that the gene can be expressed in bacterial cells. GenScript® Company cloned the optimized insert into a pGEX-6P-2 vector, using the restriction sites *Bam H1* and *Not I* (**Figure 3.1**). These restriction sites were selected using the online tool called "WebCutter Server", version 2, which generates all the possible restriction sites that may be found within the gene to be expressed. The selected restriction sites were selected since they have no recognition sites within the gp120 gene.

pGEX-6P-2 (27-4598-01)

PreScission™ Protease

Leu Glu Val Leu Phe Gln↓Gly Pro Leu Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
CTG GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG

BamH I   EcoR I   Sma I   Sal I   Xho I   Not I
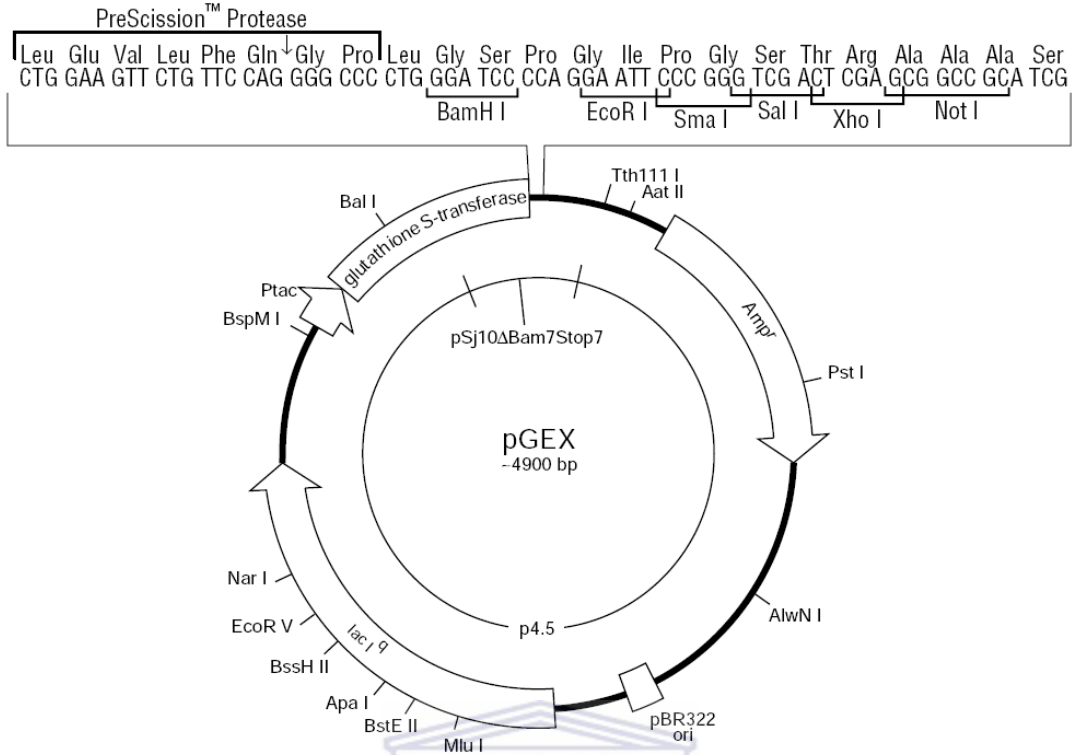
Figure 3.1: Map of the pGEX-6P-2 vector, utilized for protein expression in *E. coli* BL 21 Gold. The picture was taken from Amersham, 2000.

3.2.1.2. Preparation of pGex-6P-2-gp120

The purchased vector was prepared as per the manufacturer instructions. In brief, before the vial was opened, the lyophilised plasmid DNA was centrifuged at 6000 x *g* for one minute at 4 °C. after the vial was opened the plasmid DNA was diluted with 20 µl of sterilized water, then vortexed for an additional minute to reconstitute the DNA, and was ready for further use.

3.2.1.3. Transformation and Expression screening for pGex-6P-2-gp120

To perform the transformation reaction, the competent *E. coli* BL 21 Gold cells were thawed on ice. After, 100 µl of the competent BL 21 Gold cells were placed into a tube with 1 µg of the vector DNA. These components was gently mixed by tapping and the tube incubated on ice for 30 minutes. Next, the transformation mixture was heat shocked at 42 °C for 45 seconds and incubated immediately on ice for 5 minutes. Following incubation, the transformation mix was added to 900 µl pre-warmed Luria Broth (LB) containing no antibiotics and was further incubated at 37 °C for one and a half hours. To evaluate the transformation reaction, four LB agar plates were prepared

in the following disposition: one LB agar only plate and three LB agar + Ampicillin plates with a concentration of 100 µg/ml. The transformation mixture (50 µl) was then plated onto the LB agar plate and, LB + Ampicillin plates respectively. Furthermore, the rest of the transformation mixture (900 µl) was plated on another LB agar + Ampicillin plate. The reason to plate such as high volume was to compensate for a low transformation efficiency that may arise. The plating of 100 µl of the BL 21 competent cells on a LB + Ampicillin plate was utilized as a control, to confirm that the competent cells did not contain any plasmid DNA before the transformation reaction. These plates were incubated overnight at 37 °C using a 211DS Shaking Incubator (Labnet).

Following overnight incubation, three single colonies were picked from the LB + Ampicillin plates and were inoculated into 10 ml pre-warmed LB containing 100 µg/ml Ampicillin. The tubes were incubated overnight at 37 °C shaking at 225 revolutions per minute (RPM). Thereafter, glycerol stocks of each colony were prepared from the overnight culture and were stored at – 80 °C.

From the overnight culture, 0.5 ml was taken from it and was added to 4.5 ml LB containing Ampicillin (100 µg/ml), and the mixture was further incubated for an additional hour at 37 °C, in a shaker at 225 RPM. After the one-hour incubation, 1 ml was removed from the initial solution mixture and represents the un-induced sample. The un-induced sample was centrifuged at 12000 RPM for 10 minutes using a 5415D Benchtop Microcentrifuge from Eppendorf. The pellet was subsequently stored at – 80 °C for further analysis by SDS-PAGE. The remaining 4 ml was subjected to protein expression, in which the sample was induced with 1 mM IPTG for 3 hours at 37 °C, in an incubator shaker at 225 RPM. The induced sample was centrifuged at 12000 RPM for 10 minutes using a 5415D Benchtop Microcentrifuge from Eppendorf, and only the pellet was stored at – 80 °C for further analysis by SDS-PAGE.

3.2.1.4. Large-scale expression of gp120

Once the colony that shows the highest protein expression was selected in the screening process, a 1 ml glycerol stock representing this sample was added into 100 ml LB supplemented with 100 µg/ml ampicillin and the mixture was incubated

overnight at 37 °C at 225 RPM. The overnight culture was then added to 900 ml LB supplemented with 100 µg/ml ampicillin in a 2 liter Erlenmeyer flask to ensure sufficient aeration. The sample was incubated at 37 °C at 225 RPM until an optical density (O.D) of 0.5 to 0.6 was reached. Once this O.D range was reached, 10 ml of the sample was removed which represented the un-induced sample. This sample was then centrifuged and the pellet was stored at - 80 °C for SDS-PAGE analysis. The remaining 990 ml was subjected to induction of protein expression with 1 mM IPTG and was incubated for three hours at 37 °C, shaking at 225 RPM. The induced culture was divided into five equal volumes of 198 ml and placed into 350 ml centrifuge bottles, and subjected to centrifugation using a Beckman Coulter centrifuge at 5000 x *g* for 10 minutes. Each pellet was re-suspended in 10 ml Sodium Chloride-Tris-EDTA (STE)/lysozyme and the sample mixtures were incubated on ice for 15 minutes. All re-suspended samples were combined into a 50 ml tube and one 1 X Complete™, EDTA-free protease inhibitor cocktail tablet (Roche) was added to the combined sample and the tablet was left to dissolve. The sample was later sonicated on ice for three cycles (one minute sonication and two minutes rest on ice). This process was repeated until the lysate became clear and, it was centrifuged at 5000 x *g* for 15 minutes at 4 °C, thereafter. The pellet and supernatant were stored at – 80 °C for further evaluation either for protein purification or for SDS-PAGE analysis.

3.2.2. Purification of recombinant GST-gp120

3.2.2.1. Column preparation

To effectively isolate the gp120 protein, a column of 5 ml glutathione agarose was prepared by allowing 350 mg of Glutathione agarose to swell in 70 ml distilled H_2O overnight at 4 °C. After the swelled beads were poured into a 50 ml centrifuge tube and the column purification method was used to purify the target protein. The column was washed with dH_2O and was equilibrated with 10 resin volumes of equilibration buffer (PBS-T) and the buffer was discarded.

3.2.2.2. Purification of gp120 protein

After the preparation of the column, the protein lysate (10 ml) was added to the equilibrated beads (5 ml) and mixed onto the Tube roller (Stuart SRT9dD) for 60

minutes at 4 °C. After, the mixture within the tube was allowed to settle, the sample was collected as flow through and stored for SDS-PAGE analysis. The beads were washed with 5 resin bed volumes of PBS-T and the supernatant was collected, stored and denoted as Wash 1. This step was repeated thrice, that is Wash 2 to Wash 4 were collected and also stored for downstream analysis. The GST-gp120 protein was eluted by addition of one resin bed volume of elution buffer. The elution buffer and GST-gp120 suspension were mixed at room temperature for 30 minutes to dissociate the GST-gp120 protein from the beads. This step was repeated twice to generate Elution 2 and Elution 3, and these solutions were also collected and stored on ice for analysis. The beads were washed with cleansing buffers to remove any excess protein within the column and thereafter stored in storage buffer.

3.2.3. Analysis of extracted samples

To analyse all samples collected during the purification process, a 12 % SDS-PAGE were used as recommended by Laemmli, 1970. The SDS-PAGE solutions were prepared as shown in Table 3.4 and cast using a Mini-PROTEAN Tetra Cell, with TEMED being added right before the gel was cast. For the sample analysis of the expression screening, 10 µl of protein sample was added to 10 µl of 2X SDS sample buffer and subjected to 3 cycles of heating at 95 °C for 3 minutes and vortexing for 3 minutes, respectively. These samples were then centrifuged to sediment debris and 15 µl of sample was loaded onto the gel. For the samples from the purification, 10 µl of sample and 10 µl of 2X SDS sample buffer were mixed and 15 µl of sample were loaded onto the gel. The gel was electrophoresed at 150 Volts for 70 minutes. Thereafter the gel was stained with Coomassie Brilliant Blue R-250 stain for 15 minutes and destained overnight. Gel images were viewed and captured by a UVP BioSpectrum Imaging System and Canon CanoScan LiDE 120 electronic scanner.

Table 3.4: Reagents used for the preparation of 12 % SDS-PAGE.

Reagents	12 % separation buffer	5 % stacking buffer
dH$_2$O	4.3 ml	3.65 ml
1.5 M Tris-Cl pH 8.8	2.5 ml	-
0.5 M Tris-Cl pH 6.8	-	630.0 µl
10 % SDS	100.0 µl	50.0 µl
Acry/Bis (40 %)	3.0 ml	400.0 µl
10 % APS	200.0 µl	50.0 µl
TEMED	20.0 µl	20.0 µl

3.2.4. Acquiring of putative anti-HIV AMPs and gp120 recombinant protein

3.2.4.1. Peptide synthesis

The seven AMPs utilised for the binding study were composed of the five initial putative anti-HIV AMPs taken from previous work (Tincho MSc thesis, 2013) and the two mutated AMPs which were found to bind gp120 as demonstrated in Chapter two. The chosen AMPs were chemically synthesised by GL Biochem Ltd. (Shanghai 200241, China) using the solid-phase method and they were purified to > 98 % by reverse-phase High-Pressure Liquid Chromatography and the AMPs were shipped in a lyophilised form.

3.2.4.2. Acquiring of recombinant proteins gp120 and CD4

Due to the fact that the recombinant HIV gp120 protein could not be purified after its expression, the binding experiment would be carried out using a commercially available protein. Thus, the HIV-1 gp120 protein (group M, subtype CRF07_BC) was purchased from Sino Biological Inc. (Biological Solution Specialist), with the catalog number 11233-V08H, and the protein was shipped in lyophilised form. In addition, the CD4 protein (Human CD4/LEU Protein (HisTag)) was purchased from the same company with the product catalog number 10400-H08H, and the product was also shipped in lyophilised form.

3.2.5. Interaction of gp120 and the putative AMPs

The reason for purchasing gp120 and CD4 proteins was justified by the fact that the scope was to determine if the AMPs could compete with gp120 binding to the CD4 protein.

3.2.5.1. Preparation of gp120 protein

The commercial HIV gp120 and CD4 proteins samples were prepared by dissolving the 100 µg of the lyophilized protein in 1 ml PBS and were stored for further application. The same was done with the various AMPs, where 1 mg of each peptide was dissolved in 1 ml PBS.

3.2.5.2. Preparation of AuNPs-gp120 and AuNPs-CD4 conjugates

Stable colloidal gp120 and CD4 conjugates were prepared according to an "in house" protocol designed by Medical Diagnostech (Pty) Ltd. CD4 was used in the conjugate preparation whereas gp120 was used on the membrane of the LFD. A biotinylation kit from Thermo Scientific (EZ-Link NHS-PEG4-Biotinylation kit) was used to biotinylated gp120 and CD4 used in conjugates. Biotinylated gp120 or CD4 and streptavidin stocks were made up to 0.05 mg/ml. The Biotinylated gp120 or CD4 were added to 10 µg of streptavidin in the ratio 1:1, 1:2, 1:3, and 1:4. This was allowed to complex for 30 minutes. This mixture was conjugated to 2 µg gold nanoparticles and allowed to incubate for 15 minutes. Thereafter 500 µl of 10 % BSA was added and incubated for a further 15 minutes. The conjugated mixture was then centrifuged for 30 minutes at 3300 RPM. The supernatant was discarded and the pellet re-suspended in 50 µl of borate suspension buffer. To each of the re-suspended conjugates, 1 % casein, 8 % sucrose, 0.75 % Tween 20, and 0.01 % glycerol were added. The conjugate was then blotted at 2 µl per stick and run as per test instructions.

3.2.5.3. Preparation of membranes attachment with gp120 or CD4 proteins

It is known that gp120 is a surface protein found on the HIV virus particle, and bind to the CD4 receptor found on T cell, macrophages and monocytes. In theory, purified CD4 should bind to gp120 in an electrostatic manner. Therefore, it was postulated that one of the AMPs might bind to the CD4 receptor and therefore compete with gp120

for the binding to CD4. Thus, conjugates were made with both CD4 and gp120 with both CD4 and gp120 placed on the membrane side of lateral flow strips to determine whether gp120 does in fact bind to CD4, and thereafter, adding the peptides into the running buffer in order to determine if the peptides can compete with gp120 for binding to CD4.

3.2.5.4. Assembling the strips

Strips were assembled in accordance with the current "in-house" HIV system created by Medical Diagnostech (Pty) Ltd. CD4 or gp120 were blotted onto the nitrocellulose membranes in triplicate at a concentration of 100 µg/ml. The successfully produced CD4 conjugate and gp120 conjugate were formulated and blotted onto the conjugate pads of the strips. Different combinations of the CD4 and gp120 were experimented upon to determine the optimal setting pair that would enable the scope of this chapter. The strips were then placed into cassettes and the test was run.

3.2.5.5. Interaction of the recombinant AMPs and AuNPs-gp120 or AuNPs-CD4 on the LFD

Before the interaction of the proteins with the peptides could be performed, the right combinations of the proteins had to be determined as described in section 3.2.6.4. The control standards were established where CD4 and gp120 are used on the membrane and conjugate respectively, with the peptides used as positive control. If the peptides are used on the conjugate, then either CD4 or gp120 are used as positive controls.

To carry out the interaction study, recombinant AMPs samples were serially diluted, and 10 µg/ml was applied to the sample well of the LFD with 120 µl of buffer spiked with CD4 or gp120 protein added to the sample well. Negative controls were tested with buffer only. The results were read after 15 minutes and results were not considered after 30 min.

3.2.5.6. Result interpretation

After performing the testing, a difference in dose response between a negative and positive sample could be distinguished as shown in Figure 3.2 below. The visual interpretation was done using the in-house G1-G10 colour coded chart to determine whether any dose response to the controls was observed.
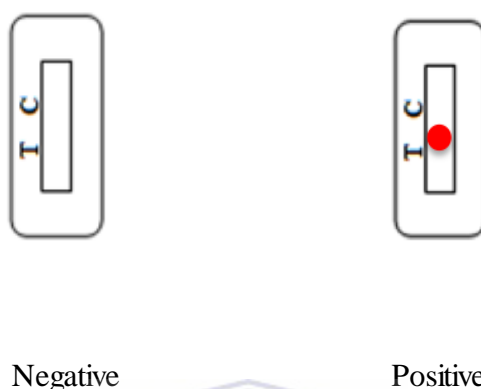
Negative   Positive

Figure 3.2: Diagram showing how the results are interpreted on an LFD made by Medical Diagnostech (Pty) Ltd. Negative is indicated by the absence of a dot. Positive is indicated by the presence of a red dot in the window. NOTE: If there is a significant difference between the intensities of the dots between negative and positive, this indicates a different in dose response between the negative and positive samples and can be viewed as a confirmation result irrespective of whether a faint dot appears or not when a negative sample is used. Signal intensities were measured using the MD G1-G10 Gold Colour Chart.

3.3. Results and Discussion

3.3.1. *In-silico* reverse translation

To obtain the protein expression of the recombinant protein, the HIV gp120 protein amino acids sequence was reverse translated to a nucleotide sequence using the online server http://www.bioinformatics.org/sms2/rev_trans.html, and the result is as depicted below.

```
ATGGAAGTGGTGCTGGTGAACGTGACCGAAAACTTTAACATGTGGAAAA
ACGATATGGTGGAACAGATGCATGAAGATATTATTAGCCTGTGGGATCA
GAGCCTGAAACCGTGCGTGAAACTGACCCCGCTGTGCGTGGGCGCGGGC
AGCTGCAACACCAGCGTGATTACCCAGGCGTGCCCGAAAGTGAGCTTTG
AACCGATTCCGATTCATTATTGCGCGCCGGCGGGCTTTGCGATTCTGAAA
TGCAACAACAAACCTTTAACGGCACCGGCCCGTGCACCAACGTGAGCA
CCGTGCAGTGCACCCATGGCATTCGCCCGGTGGTGAGCAGCCAGCTGCT
GCTGAACGGCAGCCTGGCGGAAGAAGAAGTGGTGATTCGCAGCGTGAAC
TTTACCGATAACGCGAAAACCATTATTGTGCAGCTGAACACCAGCGTGG
AAATTAACTGCACCGGCGCGGGCCATTGCAACATTGCGCGCGCGAAATG
GAACAACACCCTGAAACAGATTGCGAGCAAACTGCGCGAACAGTTTGGC
AACAACAAAACCATTATTTTTAAACAGAGCAGCGGCGGCGATCCGGAAA
TTGTGACCCATTGGTTTAACTGCGGCGGCGAATTTTTTTATTGCAACAGC
ACCCAGCTGTTTAACAGCACCTGGTTTAACAGCACCTGGAGCACCGAAG
GCAGCAACAACACCGAAGGCAGCGATACCATTACCCTGCCGTGCCGCAT
TAAACAGATTATTAACATGTGGCAGAAAGTGGGCAAAGCGATGTATGCG
CCGCCGATTAGCGGCCAGATTCGCTGCAGCAGCAACATTACCGGCCTGCT
GCTGACCCGCGATGGCGGCAACAGCAACAACGAAAGCGAAATTTTTCGC
CCGGGCGGCGGCGATATGCGCGATAACTGGCGCAGCGAACTGTATAAAT
ATAAAGTGGTGAAAATTGAATAA
```

Figure 3.3: The sequence of HIV gp120 gene with start codon (ATG) and stop codon (TAA), after the reverse-translation predicted from its protein sequence, using an online *in-silico* server.

3.3.2. Expression of recombinant GST-gp120 protein

Due to the fact that the protein expression was supposed to be expressed in a bacterium cell, HIV gp120 gene sequence from the reverse translation was optimized for this purpose by GenScript® Company (USA). The cloning of the optimized HIV gp120 gene into the pGEX-6P-2 vector was carried out by the GenScript® Company (USA), and the lyophilized construct used for the protein expression. The result of the optimization, the restriction sites predicted by the "WebCutter Server" are also included and are as shown in Figure 3.4.

Item >1 Gene Synthesis:

Gene name: GP120_opt, Length: 971 bp, Additional 5' sequence: ggatcc, Additional 3' sequence: gcggccgc, Start with: GGATCC,

Sequence:

ATGGAAGTGGTCCTGGTCAACGTCACGGAAAACTTTAACATGTGGAAAA
ACGACATGGTGGAACAAATGCACGAAGATATTATTAGCCTGTGGGATCA
GAGTCTGAAACCGTGCGTTAAACTGACCCCGCTGTGCGTCGGTGCAGGC
AGTTGTAACACCAGCGTGATTACGCAAGCTTGTCCGAAAGTTTCTTTTGA
ACCGATTCCGATCCATTATTGCGCGCCGGCCGGCTTTGCGATCCTGAAAT
GTAACAATAAAACCTTCAACGGTACGGGCCCGTGCACCAATGTGAGCAC
GGTTCAGTGTACCCACGGCATTCGTCCGGTGGTTAGCTCTCAACTGCTGC
TGAACGGTTCACTGGCGGAAGAAGAAGTGGTGATCCGCTCGGTGAACTT
CACGGACAATGCCAAAACCATTATCGTCCAGCTGAACACGAGCGTGGAA
ATTAATTGCACCGGTGCAGGCCATTGTAATATCGCACGTGCTAAATGGAA
CAATACCCTGAAACAGATTGCGTCTAAACTGCGCGAACAATTCGGTAAC
AACAAAACGATCATCTTCAAACAGAGTTCCGGCGGTGATCCGGAAATCG
TCACCCACTGGTTTAACTGCGGCGGTGAATTTTTCTATTGTAATAGCACG
CAACTGTTTAACTCTACCTGGTTCAATAGCACCTGGTCTACGGAAGGTAG
TAACAATACCGAAGGCTCCGACACCATTACGCTGCCGTGCCGTATCAAA
CAGATTATCAACATGTGGCAAAAAGTTGGTAAAGCGATGTACGCACCGC
CGATTAGCGGCCAGATCCGTTGTTCATCGAACATTACGGGTCTGCTGCTG
ACCCGCGATGGCGGTAATTCAAACAATGAATCGGAAATCTTCCGCCCGG
GCGGTGGCGATATGCGTGACAATTGGCGCTCCGAACTGTATAAATACAA
AGTTGTCAAAATTGAATAA,

End with: GCGGCCGC,

Item >2 Custom Cloning: Direct Cloning GP120_opt_pGEX-6P-2:

Vector name: pGEX-6P-2,

Vector size (kb): -,Resistance: -, Copy number: High, Cloning site: BamHI-Notl,

Figure 3.4: The optimization of HIV gp120 gene sequence by GenScript® Company (USA), for the expression of recombinant protein in *E. coli* BL21 Gold since amino acids code for different codons in the bacteria, viruses and mammalians.

3.3.2.1. Expression screening of the transformed cells

The expression screening of three selected transformed colonies showed that the cell was able to take up the plasmid and express the protein. The expression screening of the isolate are shown in Figure 3.5.
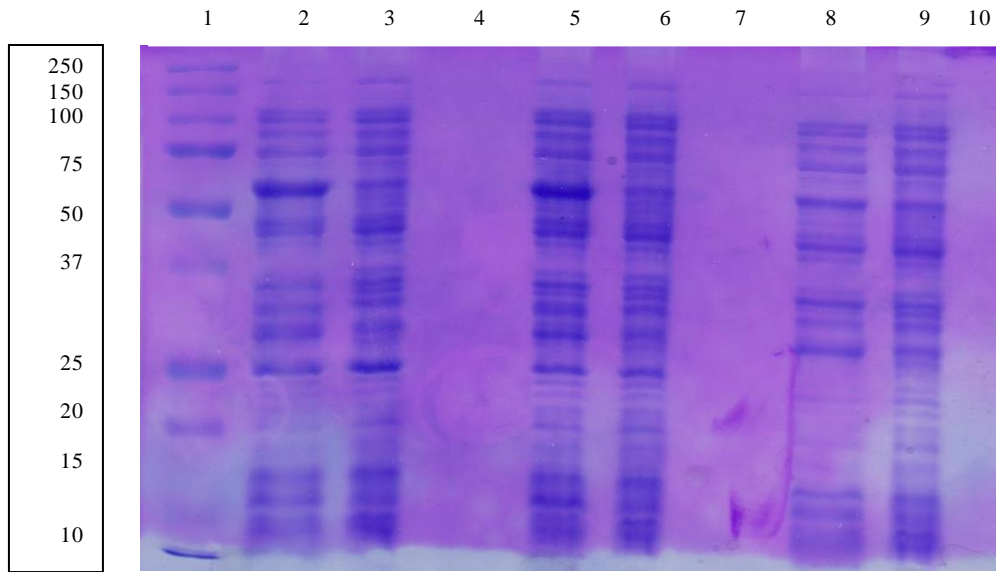
Figure 3.5: SDS-PAGE analysis displays the expression screening of 3 isolated colonies. Lane 1: 250 kDa Precision plus marker; lane 2: colony 1 induced; lane 3: colony 1 un-induced; lane 4: colony 1 supernatant; lane 5: colony 2 induced; lane 6: colony 2 un-induced; lane 7: colony 2 supernatant; lane 8: colony 3 induced; lane 9: colony 3 un-induced and lane 10: colony 3 supernatant.

From the SDS-PAGE results as displayed in **Figure 3.5**, we noticed that the size of the protein band on the gel of the sample induced with IPTG represents protein size of either colony 1, colony 2 or colony 3, which are of expected size (GST + gp120). This is due to the fact that the gp120 gene was tagged with a GST-linker gene, thus the expression of this fusion protein (GST + gp120), would have a protein band with the expected size around 61 kDa (26 kDa GST + 35 kDa gp120) on the gel. The un-induced samples for these colonies did not express the protein of interest (Figure 3.4). Furthermore, the protein was not shed into the supernatant. After confirming the protein expression in either of the colonies, the colony with the highest expression was utilized for large-scale protein expression.

3.3.2.2. Large-scale expression of the recombinant protein GST-gp120

After confirming the protein expression, the expression had to be done on a larger scale. Thus, isolate from colony 2 was grown on an agar plate containing Ampicillin, with one colony taken to start the large-scale expression of the protein. No protein expression was noticed in the un-induced *E. coli* BL 21 Gold cells and produced the same amount of the protein (Lane 2, Figure 3.5). However, It was noticed that the protein was expressed when the *E. coli* BL 21 Gold cells were induced at 37 °C with

IPTG (Lane 3, Figure 3.5), confirming the present of the protein in this fraction, after the induction. The same observation was made that the protein was not released into the supernatant (LB broth media), after its expression (Lane 4, Figure 3.6).
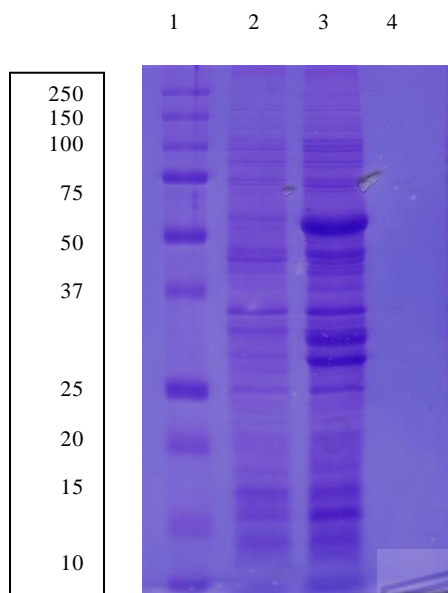
Figure 3.6: SDS-PAGE analysis depicting large-scale expression of the GST-gp120 fusion protein, for colony 2. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced; lane 3: colony 2 induced; lane 4: supernatant colony.

3.3.2.3. Purification of GST-gp120
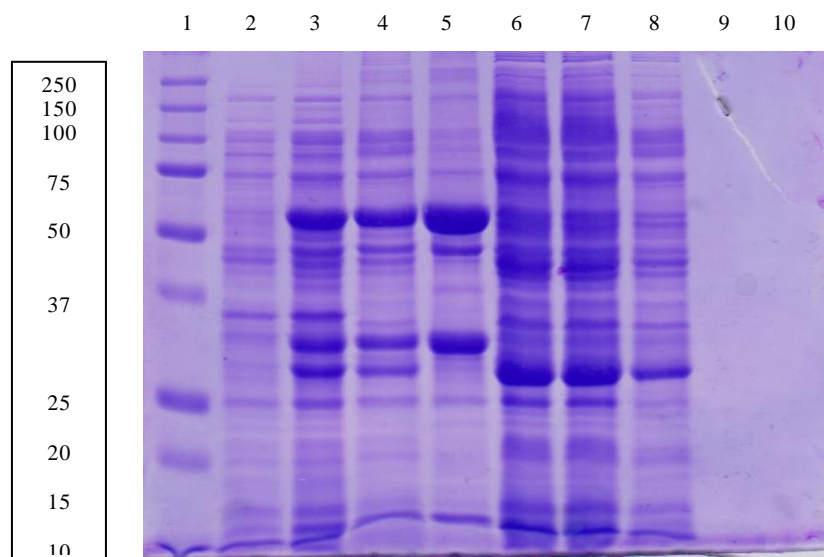
Figure 3.7: SDS-PAGE analysis depicting large-scale expression and purification of the GST-gp120 fusion protein, for colony 2. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced; lane 3: colony 2 induced; lane 4: total protein or lysate; lane 5: lysate pellet fraction; lane 6: lysate supernatant fraction; lane 7: flow through; lane 8: wash 1; lane 9: elution 1; lane 10: elution 2.

After performing the purification step of the fusion protein (GST + gp120) on a large scale, the SDS-PAGE results revealed that the fusion protein was not well purified (Figure 3.6). Even though the induced protein (Lane 3, Figure 3.7) showed that the fusion protein was expressed as compared to the un-induced protein (Lane 2, Figure 3.7), it was later realized that large amount of the fusion protein was mostly found in the lysate pellet fraction (Lane 5, Figure 3.7) and some of the protein was found in the lysate supernatant fraction (Lane 6, Figure 3.7). The bands on the gel represented by lane 3, lane 4 and lane 5 corresponded to that of the fusion protein GST-gp120, signifying that the fusion protein (GST + gp120) was well expressed but the protein was not soluble when the sample was spun down to give the various fractions of the sample. Furthermore, this notion was confirmed when it was noticed that the bands displayed by lane 6, lane 7 and lane 8 (Figure 3.7), emanating from the lysate supernatant fraction, did not contain the expected size of the fusion protein. This could be explained by the fact that this protein might have been degraded once it became soluble thus an alternative method was sought for protein expression that would allow it to be soluble and allow for its purification.

To resolve the protein solubility problem, a detergent named N-lauroylsarcosine or sodium lauroyl sarcosinate (INCI) was added to the STE/lysozyme during the protein induction and extraction protocol as described in section 3.2.2.4, to solubilize the fusion protein found within the lysate. After lysing of the cells and fractionation, the lysate supernatant fraction was subjected to purification with the same protein purification protocol as described in section 3.2.3.2. From the image of the protein analysis displayed below, the result indicated that the protein became soluble with the addition of this detergent (Figure 3.8). However, the purification was still not successful due to the fact that the expected fusion protein GST+gp120 did not appear in lane 7, lane 8 and lane 9. The lack of bands in these lanes (Figure 3.8) could indicate that the GST-tagged protein did not bind to the beads, thus not allowing for the protein to be purified. This could be explained by the fact that the fusion protein was not in its native conformation and did not fold properly to allow the GST-tag to bind to the glutathione beads.
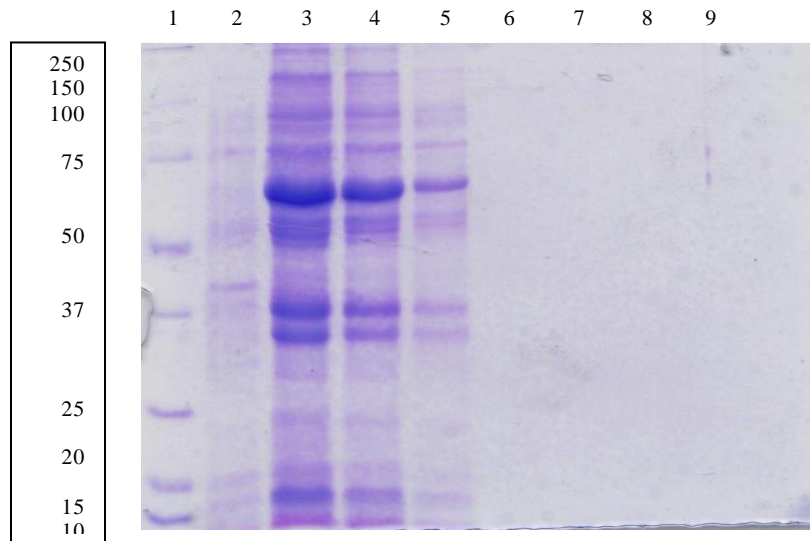
Figure 3.8: SDS-PAGE analysis depicting large-scale expression and purification of a GST-gp120 fusion protein with INCI treatment. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced; lane 3: total protein or lysate; lane 4: lysate supernatant fraction (flow through); lane 5: wash 1; lane 6: wash 4; lane 7: elution 1; lane 8: elution 2; lane 9: elution 3.

Despite the fact that the protein was made soluble, the inability to purify this protein was corrected by supplementing the extraction solution with 5 mM DTT so that the denatured protein could be folded back to its native conformation and allow for the protein to be purified. As such, the same expression and extraction protocol were followed as described in section 3.2.2.4 and the purification was performed as described as in section 3.2.3.2 above.

The purification result showed that the protein could not be purified once again since no bands representing the protein were found in the elution fraction (Lane E7, Lane E8 and Lane E9, Figure 3.9). Furthermore, the SDS-PAGE image depicts that the size of the protein (lane 4 and lane 5, Figure 3.9) on the gel do not correspond to that of the expected size for the fusion protein of 61 kDa, implying the protein may have been degraded once it became soluble in solution. The solubility of the GST + gp120 fusion protein was confirmed by the presence of a band corresponding to the fusion protein within lane 3 of Figure 3.8. However, concern was mostly raised that no protein bands were observed in the elution fractions (lane 7, lane 8 and lane 9, Figure 3.9). This concern comes with the knowledge that if the protein were folded back into its native conformation with DTT treatment, after denaturation with INCI, the protein would bind to the glutathione beads, which would allow its purification. Furthermore,

if the protein were being degraded after becoming soluble, the gel image would show the presence of the protein in lane 7, lane 8 and lane 9 corresponding to the size of GST i.e. 26 kDa.
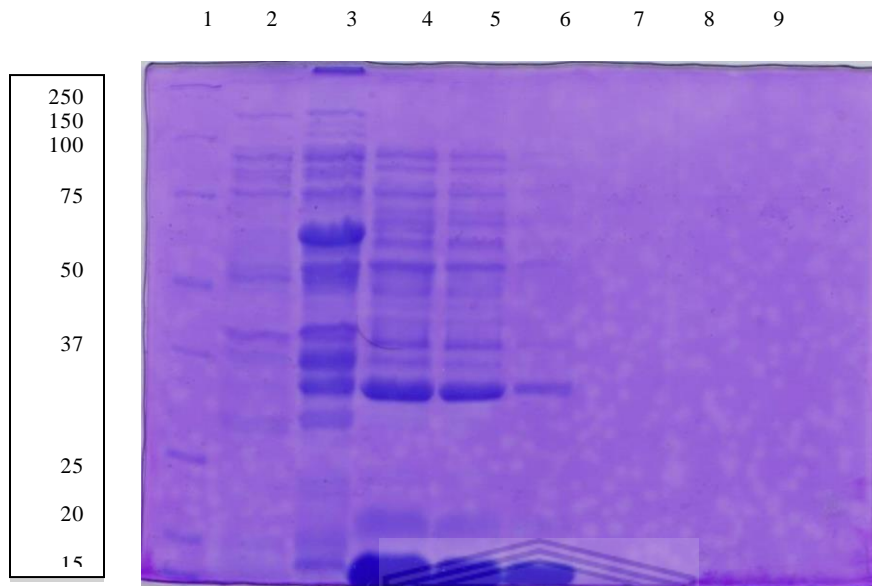
Figure 3.9. SDS-PAGE analysis depicting large-scale expression and purification of a GST-gp120 fusion protein with INCI and DTT treatment, with purification performed at 4 °C, for 1 hour. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced protein; lane 3: total protein or lysate; lane 4: lysate supernatant fraction (flow through); lane 5: wash 1; lane 6: wash 4; lane 7: elution 1; lane 8: elution 2; lane 9: elution 3.

The non-binding of the protein to the beads motivated the change of the purification protocol, where the incubation of the lysate supernatant with the beads was changed from an 1 hour incubation at 4 °C, to incubation at room temperature, for an hour. After the incubation and purification, the SDS-PAGE result still exhibits the same outcome as previously demonstrated in Figure 3.8, meaning that the fusion protein was still not able to bind to the agarose beads and the soluble protein was degraded (Figure 3.10).
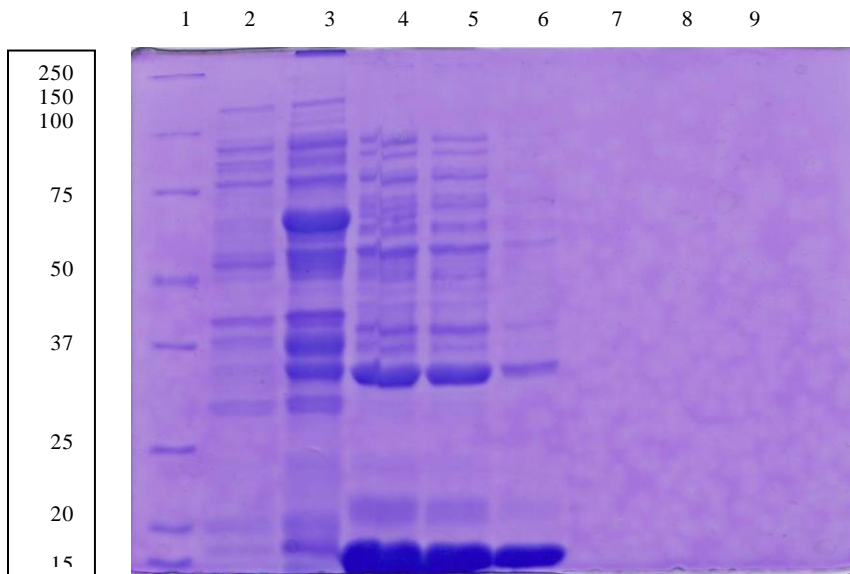
Figure 3.10: SDS-PAGE analysis depicting large-scale expression and purification of a GST-gp120 fusion protein with INCI and DTT treatment, with purification performed at room temperature for 1 hour. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced protein; lane 3: total protein or lysate; lane 4: lysate supernatant fraction (flow through); lane 5: wash 1; lane 6: wash 4; lane 7: elution 1; lane 8: elution 2; lane 9: elution 3.

Due to the fact that it was difficult to obtain a soluble protein under the previous conditions and that it was possible to solubilize the protein using N-lauroylsarcosine as explained above, it was however difficult to purify the fusion protein since the GST-tag which is fused to the protein did not bind to the agarose beads to allow the purification of the fusion protein as shown on the SDS-PAGE images of Figure 3.7; Figure 3.8; Figure 3.9; Figure 3.10).

Based on the limitation encountered in the above protocol, a new protocol was implemented to optimize the expression of a soluble protein. Thus, N-lauroylsarcosine detergent was replaced by Urea, to denature the fusion protein and to allow its solubility so as to facilitate its purification. To achieve this, the same steps as described in section 3.2.2.4 were followed and the lysate from these steps was separated from the supernatant by centrifugation. The cell lysate was re-suspended with Wash buffer (8 M urea, 50 mM Tris, 5 mM EDTA and cOmplete EDTA-free protein inhibitor tablet) and was centrifuged for 20 minutes at 5000 x g. The pellet from this washing step was dissolved into U-buffer (50 mM Tris, 5 mM EDTA, 5 mM DTT and cOmplete EDTA-free protein inhibitor tablet) and the mixture was incubated on ice for 2 hours. Following incubation, the mixture was centrifuged for 20 minutes at 5000 x g, and the supernatant was harvested and 1 % Triton-X100

added to the solution. The supernatant was placed into a dialysis bag and was placed into PBS/glycerol buffer for 2 hours. Afterwhich, the dialysis bag was transferred into PBS/cOmplete EDTA-free Protease Inhibitor and the dialysis reaction was allowed to continue overnight. The following day, the contents of the dialysis bag was placed into a 15 ml tube and the tube was centrifuged at 5000 x g for 20 minutes, and the supernatant containing the fusion protein was recovered. The various samples were analysed with SDS-PAGE and the result is depicted below (Figure 3.11).
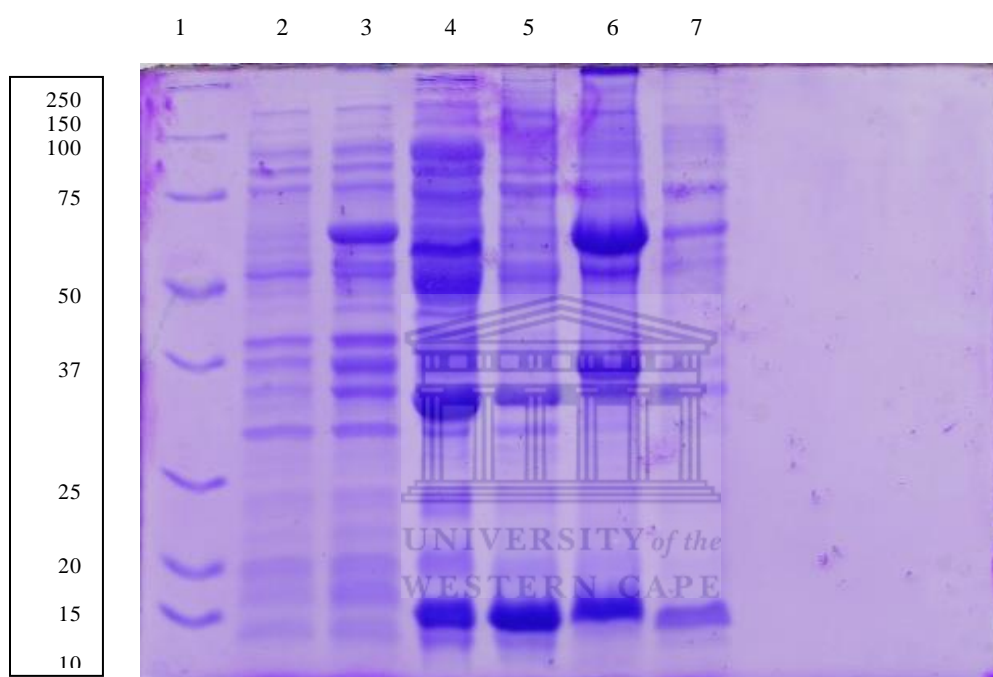
Figure 3.11: SDS-PAGE analysis depicting large-scale expression after treating pellet with U-buffer and dialysis. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced protein; lane 3: colony 2 induced; lane 4: lysate supernatant fraction after sonication; lane 5: washing with a wash buffer; lane 6: supernatant after incubation with U-buffer; lane 7: supernatant after dialysis.

The image above shows that the fusion protein is still not found in the supernatant following sonication as shown in lane 4, Figure 3.10. The protein becomes visible only once the U-buffer was added to the cell pellet, therefore it can be deduced that the U-buffer allows the solubility of the protein (Lane 6, Figure 3.11). However, the end result did not seem promising due to the fact that the amount of fusion protein is minimal as compared to the amount of the fusion protein in Lane 6. Despite the fact that the end result was not promising, purification was carried out on the sample prepared with the U-buffer before dialysis since the dialysis sample showed little to no protein. The column was prepared by cleaning the beads with 1-bed volume of 5

M Urea to denature all proteins in the column, followed by 1-bed volume of 10 mM of reduced glutathione, and then the beads were washed with distilled water. The column was equilibrated with 3-bed volumes of PBS buffer and the supernatant was added to the cleaned glutathione agarose column and was incubated at 4 °C on an end-to-end rotator for 1 hour, and the purification carried out as described in section 3.2.3.2 and the sample analysed with 12 % SDS-PAGE.
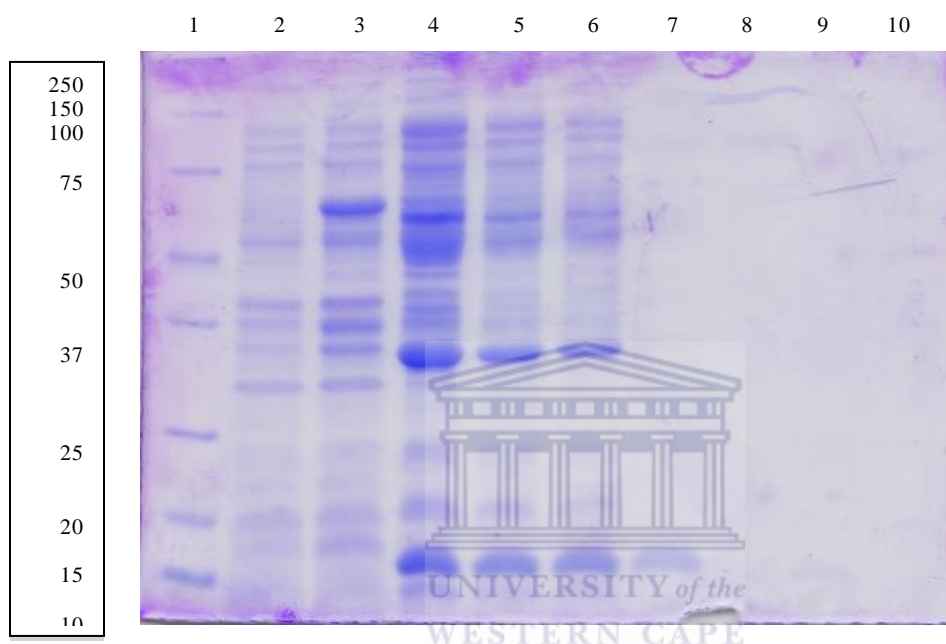
Figure 3.12: SDS-PAGE analysis depicting large-scale expression after treating pellet with U-buffer. Lane 1: 250 kDa Precision plus marker; lane 2: colony 2 un-induced protein; lane 3: total protein or lysate; lane 4: lysate supernatant fraction (flow through); lane 5: wash 1; lane 6: wash 2; lane 7: wash 3; lane 8: elution 1; lane 9: elution 2; lane 10: elution 3.

As observed in figure 3.12, no clear bands could be seen in the elution fraction represented by lanes 8, 9 and 10. However, distinct bands of ≈ 35 kDa were observed in the wash fractions (lane 4, lane 5 and lane 6, Figure 3.11), and the band size was neither that of the fusion protein (61 kDa) nor that of the GST-tag (26 kDa), implying the fusion protein was degraded or spliced once it becomes soluble. However, the portion of the fusion protein that was being degraded could not be ascertained. It could be speculated that the GST-tag underwent splicing rather than the gp120 protein since no band was shown in the elution lanes implying the protein could not be purified. Due to the inability to purify the fusion protein despite the various protocols utilised, and the fact that the fusion protein was spliced once becoming soluble, the construct utilised for the transformation was sequenced so as to confirm that the DNA

sequence was the correct one as to produce the fusion protein. The result was analysed with CLC Sequence Viewer, version 7.7 (QIAGEN Bioinformatics, Aarthus A/S).

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ATGGAAGTGGTCCTGGTCAACGTCACGGAAAAYTTTAACATGTGGAAAAA
CGACATGGTGGAACAAATGCACGAAGATATTATTAGCCTGTGGGATCAGA
GTCTGAAACCGTGCGTTAAACTGACCCCGCTGTGCGTCGGTGCAGGCAGT
TGTAACACCAGCGTGATTACGCAAGCTTGTCCGAAAGTTTCTTTTGAACCG
ATTCCGATCCATTATTGCGCGCCGGCCGGCTTTGCGATCCTGAAATGTAAC
AATAAAACCTTCAACGGTACGGGCCCGTGCACCAATGTGAGCACGGTTCA
GTGTACCCACGGCATTCGTCCGGTGGTTAGCTCTCAACTGCTGCTGAACGG
TTCACTGGCGGAAGAAGAAGTGGTGATCCGCTCGGTGAACTTCACGGACA
ATGCCAAAACCATTATCGTCCAGCTGAACACGAGCGTGGAAATTAATTGC
ACCGGTGCAGGCCATTGTAATATCGCACGTGCTAAATGGAACAATACCCT
GAAACAGATTGCGTCTAAACTGCGCGAACAATTCGGTAACAACAAAACG
ATCATCTTCAAACAGAGTTCCGGCGGTGATCCGGAAATCGTCACCCACTG
GTTTAACTGCGGCGGTGAATTTTTCTATTGTAATAGCACGCAACTGTTTAA
CTCTACCTGGTTCAATAGCACCTGGTCTACGGAAGGTAGTAACAATACCG
AAGGCTCCGACACCATTACGCTGCCGTGCCGTATCAAACAGATTATCAAC
ATGTGGCAAAAGTTGGTAAAGCGATGTACGCACCGCCGATTAGCGGCCA
GATCCGTTGTTCATCGAACATTACGGGTCTGCTGCTGACCCGCGATGGCG
GTAATTCAAACAATGAATCGGAAATCTTCCGCCCGGCSGTGGCGATATGC
GTGACAATTGCCGCTCCGAACTGTATAATACCAAGTTGTCAAAATTGAAT
AA
```

Figure 3.13: The sequencing result of the optimised HIV-gp120 construct from GenScript® Company (USA) for the expression of the recombinant protein in *E. coli* BL21 Gold

A close examination of the gene sequence produced proves that the sequenced gene is the same as that of the optimised HIV gp120 construct, therefore confirming the fact that the correct gene sequence was cloned into the vector. This is further confirmed by the presence of a protein of expected size from the SDS-PAGE results, which corresponded to the size of the fusion protein. With all the information collected during the expression and purification of the protein, the inability to obtain a pure protein could be explained by the chemical composition of the protein and the host utilised for the protein expression.

The HIV gp120 protein is in fact a glycoprotein, made of a protein backbone with associations of carbohydrate chains highly made up of N-linked glycosylation sites

and the N-linked glycans (Matthews *et al.*, 1987; Leonard *et al.*, 1990), where the glycans residues help in the stabilisation of the protein in its final conformation and structure. Using a bacterium such as *E. coli* to perform the expression and the purification of gp120 could be challenging since the bacterium does not undergo post-translational modification to produce the sugar moieties required to keep gp120 in its native conformation once it is released from the bacterium cell into the extraction buffer. Even though the original HIV gp120 gene was optimised to be expressed in a bacterium cell, since different organisms amino acids residues code differently, the protein could not purified since the sugar residues were absent on the gp120 protein backbone, to maintain its stability in solution. The fact that the right band size of the fusion protein was observed on the SDS-PAGE images (Lane 3 of Figure 3.5 to Figure 3.12) confirmed that the protein was expressed but purification failed because the protein was not stable due to the absence of sugar residues on its backbone, thus the spliced forms of the fusion protein observed on the SDS-PAGE images of Figure 3.7, Figure 3.9, Figure 3.10 and Figure 3.12.

Various research have generated opposite results on the role of these glycan residues for its interaction with CD4 surface molecules of T cells, macrophages and monocytes; however, molecular biologists and structural biologists are certain that the overall structure and conformation of HIV gp120 depends on these sugar moieties (Li *et al.*, 1993). The importance of these glycan moieties prove that it will be impossible to have a stable protein if expressed in a bacterium cell, thus the results obtained in the current work. Even though detergent and urea were used to solubilise the protein in solution, a pure protein could not be obtained because the expressed gp120 did not have sugars moieties in its backbone to maintain the protein in its native form hence splicing of the protein (Figure 3.7, Figure 3.9, Figure 3.10 and Figure 3.12). Moreover, it will thus prove difficult to unfold this protein with urea and attempt to fold it back into its native conformation using dialysis due to the reasons mentioned above. An alternative solution should be the optimisation of the extraction protocol since the sequence ought to express the correct GST-gp120 size, after purification. Additional technique such as Western bloting would be performed subsequently to track the protein expression and in downstream purification processing steps. A further modification would be to utilise different hosts such as mammalian cells for the protein expression.

3.3.2.4. Binding studies of HIV gp120 protein with putative anti-HIV AMPs

Due to the fact that the HIV gp120 protein could not be purified, the protein was purchased as mentioned in section 3.2.4.2 and the working stock was made up as stipulated in section 3.2.5.1.

The first experiment was to establish whether gp120 does in fact, bind to CD4 (Figure 3.14) where gp120 was blotted onto the membrane and a CD4 conjugate was used, and *vice versa*. It was discovered that when gp120 was on the conjugate side and CD4 was blotted onto the membrane, a dot becomes observable but the same not possible when the test was ran with gp120 on the membrane and CD4 on the conjugate side. However, It is important to note that it was a challenge to produce a CD4 conjugate.

Figure 3.14: A test run with gp120 on the membrane and CD4 as a conjugate (left of the two tests) and a test run with CD4 on the membrane and gp120 as a conjugate (right of the two tests).

Thereafter a test with CD4 on the membrane and gp120 on the conjugate was run with buffer only as a control and with gp120 spiked into the buffer at a final concentration of 8 µg/ml (Figure 3.15). This was done in triplicate and it was found that free-floating gp120 was able to compete with the gp120 conjugate. The free-floating gp120 was, therefore, able to bind to the blot sufficiently to block off the gp120 binding sites. Hence gp120 conjugates was therefore unable to bind to CD4.

Figure 3.15: Picture showing tests run with CD4 on the membrane and gp120 on the conjugate. The left test was run using buffer only and the right test was run with gp120 spiked into the buffer at a concentration of 8 µg/ml in triplicate.

In a similar fashion, a test was run with CD4 on the membrane and gp120 on the conjugate side and using CD4 as the positive control (Figure 3.16). Observation showed that the CD4 was able to bind to the gp120 conjugate, therefore making it unable to bind to CD4 on the membrane.

Figure 3.16: Picture showing tests with CD4 on the membrane and gp120 on the conjugate. The left test is run with buffer only and the right test was run with buffer spiked with CD4 to 8 µg/ml.

Using the same strip format as above with CD4 on the membrane and gp120 on the conjugate side, all the peptides (AMPs) were run after spiking the running buffer with each one respectively. The results shown in Figure 3.17 indicate that most of the peptides were able to dim the blot compared to the negative control. At this stage, the result was promising but still inconclusive since it was later established that the peptides contain a buffer that was not compatible with gold particles.

Figure 3.17: Test with CD4 on the membrane and gp120 on the conjugate run with each of the peptides spiked into the running buffer. From the pictures depicted here, (1): Molecule 1; (2) Molecule 3; (3): Molecule 7; (6): Molecule 8; (9): Molecule 1.1; (10): Molecule 8.1.

To proceed, the next step was to produce conjugates with the putative AMPs. Out of the AMPs used only Molecule 1 and Molecule 7 did not cause the conjugate to spontaneously collapse. After successful conjugation, tests were run with CD4 on the membrane side, and with Molecule 1 and Molecule 7 on the conjugate side respectively, using buffer only to simulate negative controls. For a positive control, gp120 and CD4 were spiked into the running buffer respectively, to determine whether gp120 and CD4 competed with the AMP for binding to CD4. When Molecule 1 was used on the conjugate side, there was very little response, but when Molecule 7 was used on the conjugate, there was a significant dose response when comparing buffer only and spiked buffer (with CD4 and gp120) (Figure 3.18 a and b).
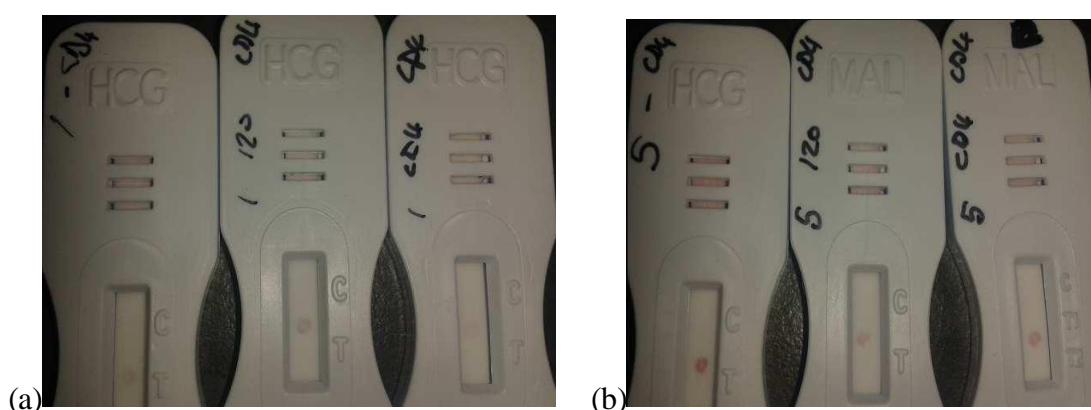
Figure 3.18: a) Figure showing test run with CD4 on the membrane and Molecule 1 on the conjugate and b) picture showing test run with CD4 on the conjugate and Molecule 7 on the conjugate. Both were run using gp120 (middle test of each picture) and CD4 (right test of each picture) as positive control standards.

The experiment using Molecule 7 on the conjugate above was repeated but using buffer spiked with a higher concentration of 18 µg/ml (Figure 3.19) It was shown that the test spiked with gp120 gave a weaker intensity blot, showing that gp120 may be competing with Molecule 7 for CD4 binding, and when the concentration was increased to 18 µg/ml, the blot intensity was still weaker.

Figure 3.19: Tests run with CD4 on the membrane and Molecule 7 on the conjugate. The left test was run with buffer only. The middle test was run with buffer spiked with gp120 to 8 µg/ml. The right test was run with buffer spiked with gp120 to 18 µg/ml.

The results from different test runs proved to be inconsistent. In one experiment tests were run with different concentrations of gp120 spiked into the buffer and compared to un-spiked buffer (Figure 3.20a). Even though there appears to be a difference between the test not spiked and the test spiked, the difference observed was small. On this occasion, there was no difference between the three different concentrations of the spiked buffer. In another experiment when the same conditions were run in triplicate completely different results were observed (Figure 3.20 b). Tests run with CD4 on the membrane and Molecule 7 on the conjugate and gp120 as control standard showed one set of tests giving reverse results (no blot on the negative with a visible blot on the positive), one set of tests with no delta, and one set of tests with the expected result. This occurred about 15 % of the time.
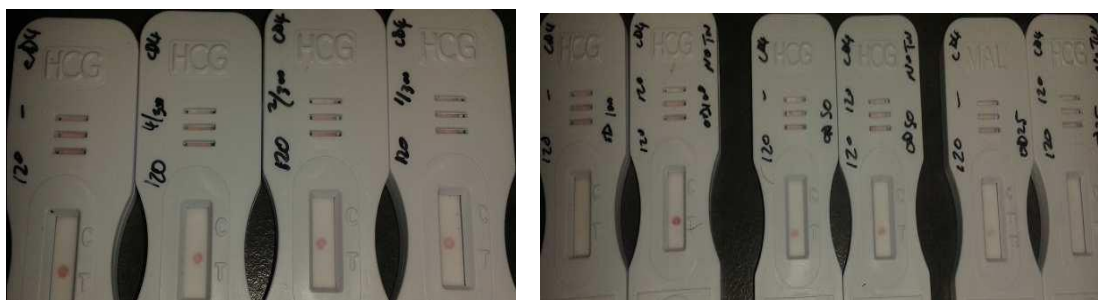
Figure 3.20: a) Tests run with CD4 on the membrane and Molecule 7 on the conjugate run with buffer only (left test) and different concentrations of gp120 spiked into running buffer. b) 3 sets of a test run in triplicate with completely different results.

3.4. Conclusion

The work performed in this chapter has shown that the optimised HIV gp120 gene, cloned into pGex-6P-2 vector was able to express the right fusion protein size proving that the reverse translation and optimisation of the amino acid sequence was done correctly. However, the purification of the protein was problematic either by the inability of the bacterium to undergo post-translational modification to produce a full gp120 glycoprotein, or instability of the protein to stay in its native form once in solution. Nevertheless, the protein was expressed as it was demonstrated with the SDS-PAGE results however, could not be purified.

Though the initial concept to bind gp120 with the various AMPs was not proved in this work, it can be concluded as shown from the binding results that when CD4 is on the membrane and gp120 is on the conjugate side, the conjugate is capable of binding to CD4 and this binding generally seems to be inhibited by the presence of free-floating gp120 and to a certain extent, the AMPs. Conversely, it was possible to show that when Molecule 7 was used on the conjugate and CD4 on the membrane, gp120 was able to dim the blot, indicating some sort of competition with the Molecule 7. It is also important to note that making a conjugate with gp120 was straight forward and there was nothing in the buffer of the gp120 sample that would interfere with the conjugate stability, therefore there was little chance that the dimmer blot was as a result of the constituents which make up the buffer and not as a result of gp120 itself.

It was however impossible to generate conjugates with the putative AMPs except with Molecule 1 and Molecule 7 as a constituent within the buffer interacted with the gold

particles and caused them to spontaneously collapse, making the bio-conjugation impossible. This inability could be attributed to the buffer used to produce these peptides thus the buffer constituents should be re-looked in future. In addition, incorrect or inconsistent results were obtained for about 15-20 % of the time of test performed prompting the need for a more optimised experimental protocol or the use of other techniques to perform binding studies such as SPR.

CHAPTER FOUR: ANTI-HIV ACTIVITY OF PUTATIVE ANTIMICROBIAL PEPTIDES

4.1. Introduction

More than three decades have passed since the first case of HIV was reported in the early 1990's in the USA. The path leading to the alleviation of the high infection rate of HIV spread, the rapid evolution of the disease pathogenesis, an accurate early diagnostic system and non-toxic therapeutic regimens have not been easy. However, scientific research have enabled the implementation of acceptable HIV therapeutic regimens, the first anti-HIV molecule, Zidovudine (AZT) received FDA approval as a HIV therapeutic regimen (Bean, 2005; Volberding and Deeks, 2010).

Although helpful, this drug was found to be very toxic to the patient and had unexpected side effects. Nevertheless, many anti-HIV drugs have been developed to satisfy the desired activity without causing more harm to the patients, and these compounds vary from nucleotide analogue reverse transcriptase inhibitors (NRTIs), non-nucleotides analogue reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and integrase inhibitors (INSTIs) (Pang *et al.*, 2009; Hare *et al.*, 2010; Volberding and Deeks, 2010). These medications were specifically designed based on the HIV life cycle and could act at a particular point in the virus life, to slow and/or prevent its replication into the patient body.

Despite the effort to formulate treatment regimens made of a combination of different classes of anti-HIV compounds, hence the name high active antiretroviral therapy (HAART), the virus was still not eradicated in the patient. Though major headway have been made to tackle this deathly pandemic through various therapeutic molecules, these treatment regimens have not yet achieved their intended success since these drugs cannot eradicate HIV but could only slow the virus progression. These shortcomings might be due to the fact that the virus is already within the human cells and HIV reservoir has been formed within the infected person with the virus hidden in some parts of the body (Pierson *et al.*, 2000; Pomerantz, 2002; Chun *et al.*, 2015).

Taking into account that the most crucial component of HIV eradication has been to develop and provide adequate medication(s) that ought to stop the progression as well as viral propagation and prevent its spread to healthy human cells. Further research has allowed a new class of anti-HIV drugs termed Entry Inhibitors (EIs) or Fusion Inhibitors (FIs) to emerge (Tilton and Doms, 2010). Whilst this class of HIV therapeutic regimen is also based on the virus life cycle, the remarkable element of the class of HIV drug is that it prevents viral entrance at different stages of HIV penetration into the T cells, Macrophages/monocytes, during the infective stage. Thus drugs blocking the gp120-CD4+ interaction, gp120-coreceptor interaction, and gp41-mediated membrane fusion have experimented with great success (Tilton and Doms, 2010). However, the Food and Drugs Administration (FDA) has approved only one drug belonging to this class of anti-HIV therapeutics, which is a peptide-based drug (Kilby *et al.*, 2002; Lalezari *et al.*, 2003),

This result although coming to fruition after many years of research has redirected the research for HIV treatments towards the use of peptides as lead compounds to develop therapeutic molecules. The implementation of AMPs has yielded substantial results to demonstrate their activity against gram-positive and gram-negative bacteria, protozoa, fungi, virus and specifically HIV (Andreu and Rivas, 1998; Munk *et al.*, 2003; Lalezari *et al.*, 2003; Wang *et al.*, 2004; Brodgen, 2005; Dwyer *et al.*, 2007; Wang *et al.*, 2010).

With the broad activity exhibited by AMPs, the search for novel peptides has enabled the development and discovery of putative anti-HIV AMPs as they prove to bind HIV gp120 protein at the side where this protein interacts with CD4+ surface protein of T cells, macrophages/monocytes as demonstrated in Chapter Two, the aim of this Chapter was thus to prove the ability of these putative AMPs, to inhibit HIV replication.

The objectives emanating from this aim could be elaborated as follow:

➢ Validate the activity of these AMPs as potential anti-HIV molecules,
➢ Demonstrate the cytotoxicity of these antimicrobial peptides,
➢ Show their broad activities again various HIV-1 pseudotypes,
➢ Demonstrate the mechanism of action of these antimicrobial peptides.

4.2. Methods

4.2.1. Cell lines utilized

The cell lines utilized for testing the anti-HIV activity of the putative antimicrobial peptides included the HIV-1 pseudotyped virus NL4-3 and the human T cells utilized to confirm the cytotoxicity of the same peptides on the host cells.

4.2.2. Antimicrobial peptides compounds

The peptides to be tested consisted of five putative anti-HIV AMPs taken from previous research (Tincho *et al.*, 2016) and the two mutated AMPs, which were shown to bind gp120 as demonstrated in Chapter two. In brief, these peptides were chemically synthesized by GL Biochem Ltd. (Shanghai 200241, China) using the solid-phase method and they were purified to > 98 % by reverse-phase High-Pressure Liquid Chromatography and shipped in a lyophilized form.

4.3. Biological assays

4.3.1. Anti-HIV assays of the putative AMPs.

The anti-HIV activity of the putative AMPs against a HIV-1 pseudotyped virus-based assays were performed by the Biomed-Advanced Materials Division, Mintek (Pretoria, South Africa), as described in (Montefiori, 2005). In brief, T cells were seeded the day before the antiviral testing at 3×10^5 cells/ml. The following day, the viability was checked via automated cell count and 2×10^5 cells/ml were placed into a 50 ml conical tube and HIV-1 NL4-3 stock added. The cells were incubated with the virus for 90 minutes. Cells were subsequently washed four times with 0.01 M DPBS to remove any unbound virus. A control set of cells were incubated without the virus and washed four times with 0.01 M DPBS to replicate the test cells. A total of 10 ml of 10 % RPMI media was then added to the cells and 100 µl of cells were added to each well of a Corning® Costar® 96-Well Cell Culture Plate (Sigma-Aldrich, USA). The plate was placed into a 37 °C, 5 % CO_2 incubator to equilibrate for one hour. During the incubation, compounds (AMPs) were made up in 10 % RPMI media containing 10 % heat-inactivated FCS (Merck, Germany). The compounds were made up to the desired concentrations ranging from 12,5 to 150 µg/ml. A total of 100 µl of compound solution was added to the wells containing cells and mixed to ensure they were homogeneous. The plate was placed into a 37 °C, 5 % CO_2 incubator for five

days. Following incubation, the microtiter plates were stained with XTT tetrazolium dye to evaluate the efficacy of the putative AMPs. The plates were then read on a multi-plate reader at 450 nm (xMARK^{TM}, Bio-Rad, USA) to determine the value of EC$_{50}$ (50 % inhibition of virus replication) of each AMP. A concentration of 50 μg/ml peptide was used in the screening process of the anti-HIV effect of the putative AMPs since the laboratory internal control achieves 50 % HIV inhibition at this concentration, and subsequent anti-HIV activity of the peptides for the dose-dependent effect experiment was done with serial dilutions from 12.5 μg/ml to 150 μg/ml.

4.3.2. Cell viability assay.

In-vitro cytotoxicity test of putative anti-HIV peptides were performed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H tetrazolium) (MTS) procedure (Cory *et al.*, 1991). Briefly, 100 μl of human T cell lines were seeded into 96-well plates at a density of 1 x 10^6 cells/ml the day of the experiment, and was incubated in 5 % CO$_2$ atmosphere at 37 °C, during which, test compounds were made up in 10 % RPMI media to the desired concentrations ranging from 12,5 to 150 μg/ml. A total of 100 μl compounds was added to the wells containing the cells and mixed to ensure the solution was homogeneous. The plate was placed in a humidified 37 °C incubator with 5 % CO$_2$ atmosphere for five days. Following 5 days incubation, 10 μl of MTS was added and mixed. The plate was further incubated for a further four hours at 37 °C in a humidified, 5 % CO$_2$ atmosphere, and the absorbance was recorded at 450 nm (xMARK^{TM}, Bio-Rad, USA).

4.3.3. Statistical analysis

Absorbance results were exported into an Excel file, where they were transformed into a percentage, in a process called Normalizing, using Microsoft Excel. The normalized results from the anti-HIV assays and cell viability assays were analysed using the statistic algorithm GraphPad Prism Software, version 7 (GraphPad software, San Diego, CA, USA). The data were expressed as mean ± SD (Standard deviation) of the normalized values from the three experiments.

4.4. Results

4.4.1. Anti-HIV activity of the putative AMPs

The putative antimicrobial peptides from the docking prediction, during gp120-AMP interaction, were subjected to the screening process with a fixed dose of 50 µg/ml, and only those AMPs with significant inhibition were carried to the dose response experimental reactions.

4.4.1.1. Experimental screening of the putative anti-HIV AMPs

The evidence that the predicted AMPs could exhibit anti-HIV activity was proven through well-elaborated methods. HIV type 1 based assays with T cells were utilized to demonstrate the anti-HIV effect of the putative AMPs and their mutated counterparts. The results revealed that Molecule 7, Molecule 8, Molecule 10 and Molecule 8.1 could prevent the replication of the NL4-3 virus in the preliminary experimental setup, as compared to all other putative anti-HIV AMPs tested (Figure 4.1).
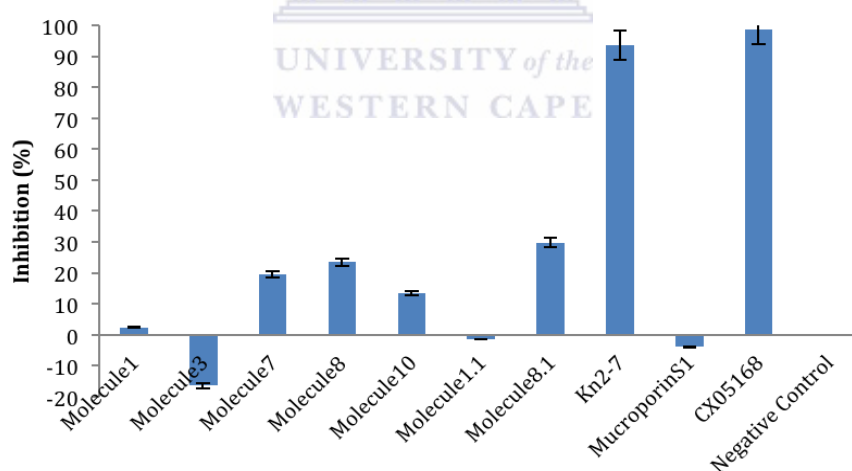
Figure 4.1: Preliminary screening of the putative AMPs against HIV-1 NL4-3 using a single dose. The inhibition of HIV-1 NL4-3 by the putative AMPs was measured by using 50 µg/ml. Only Molecule 7, Molecule 8, Molecule 10 and Molecule 8.1 showed some inhibition of the virus replication, by preventing their entry into the host cell to multiply. Thus Molecules 7, 8, 10 and 8.1 could block the binding of HIV gp120 to CD4+ of T cells to allow the virus replication. At this concentration, the Kn2-7 (positive control) showed approximately 93.63% inhibition whilst Mucroporin-S1 (negative control) showed no inhibition of the virus. All the data represent the mean values for three independent experiments and are reported as mean ± SD of the three replicated samples of each Molecule.

4.4.1.2. Dose-response to determine the effective concentration of the anti-HIV AMPs

Subsequent dose-dependent experiments revealed that only Molecule 7, Molecule 8 and Molecule 8.1 were able to significantly inhibit the HIV-1 NL4-3 replication as compared to Molecule 10, with Molecule 7, Molecule 8 and Molecule 8.1 showing the highest anti-HIV percentage inhibition against HIV-1 NL4-3 (Figure 4.2). It was possible to extrapolate from the dose-response curve represented in Figure 4.2 that Molecule 7 and Molecule 8 had effective concentrations (EC_{50}) of 37.5 µg/ml and 93.75 µg/ml respectively. Hence, these molecules potentially pave the way for the development of an entry inhibitor drug using these peptides as the lead compound. Consequently, Molecule 7 and Molecule 8 were selected to continue the peptides anti-HIV testing and to clarify their mechanisms and application. However, Molecule 8.1 effective concentration (EC_{50}) could not be determined and additional experiments are underway, where peptide with lowest concentrations will be used to determine the EC_{50} of Molecule 8.1. However the dose response results thus far show that this AMP may have an EC_{50} less than 12.5 µg/ml, thus a more promising result. Conversely, the inhibitory ability of Molecule 10 was not convincing since concentration up to 150 µg/ml was unable to inhibit more than 30 % of the virus (Figure 4.2).
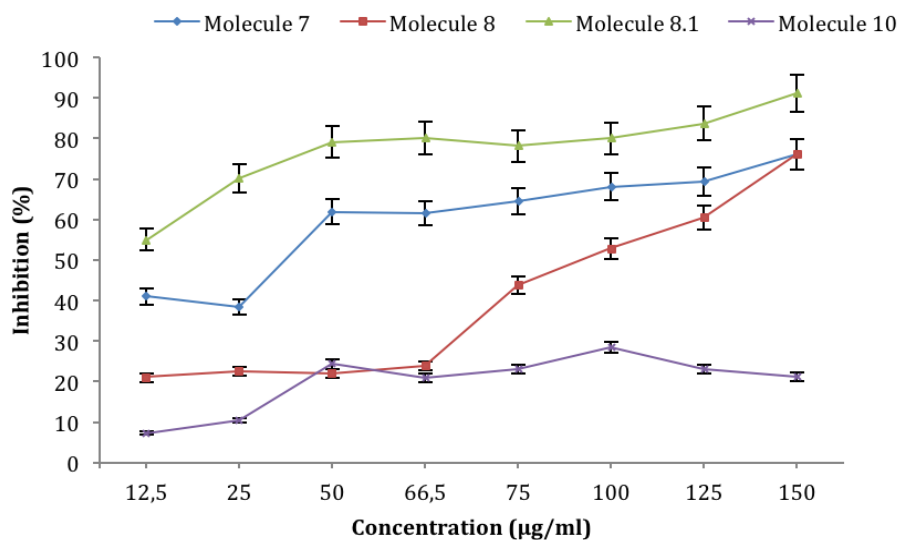
Figure 4.2: The dose-dependent effects of Molecule 7, Molecule 8, Molecule 10 and Molecule 8.1 against HIV-1 NL4-3. The EC_{50} of Molecule 7 was 37.5 µg/ml and that of Molecule 8 was 93.75 µg/ml. We could not calculate the EC_{50} of Molecule 10 due to the fact that even at a concentration of 150 µg/ml it did not inhibit 30 % of the virus. However, the EC_{50} of Molecule 8.1 could be lesser than 12.5 µg/ml since more

that 50 % of HIV cells were still viable at this concentration. The data represent the mean values for six independent experiments and are reported as mean $\pm$ SD of the six replicated samples of each Molecule.

4.4.2. Cell viability assay and the selective effect the anti-HIV AMPs

The ability of the putative AMPs to exhibit anti-HIV activity was confirmed by carrying out a non-selective cytotoxicity assay on the AMPs that showed strong anti-HIV activity, that is Molecule 7, Molecule 8, Molecule 10 and Molecule 8.1. This experiment was performed on T cell lines to establish the selective potential of these compounds, by treating the T cell lines with different concentrations of the AMPs for 5 days and measuring the viability of the cells by taking the absorbance at 450 nm, on an ELISA plate reader (xMARK^{TM}, Bio-Rad, USA). The "Statistical Package for the Social Sciences" (SPSS) was used to calculate the Cytotoxic Concentration (CC) values and Figure 4.3 showed the CC_{50} of the three molecules achieved with various doses of the peptides. Even thought the CC_{50} was not established, it should be noted that at 150 µg/ml 80 % of T cells was still not inhibited by any of the peptides. Therefore, increasing the peptides concentration in our next step of experiments would help determine the CC_{50} of each compound hence should enable the determining their individual Therapeutic Index (TI) or Selective Index (SI).
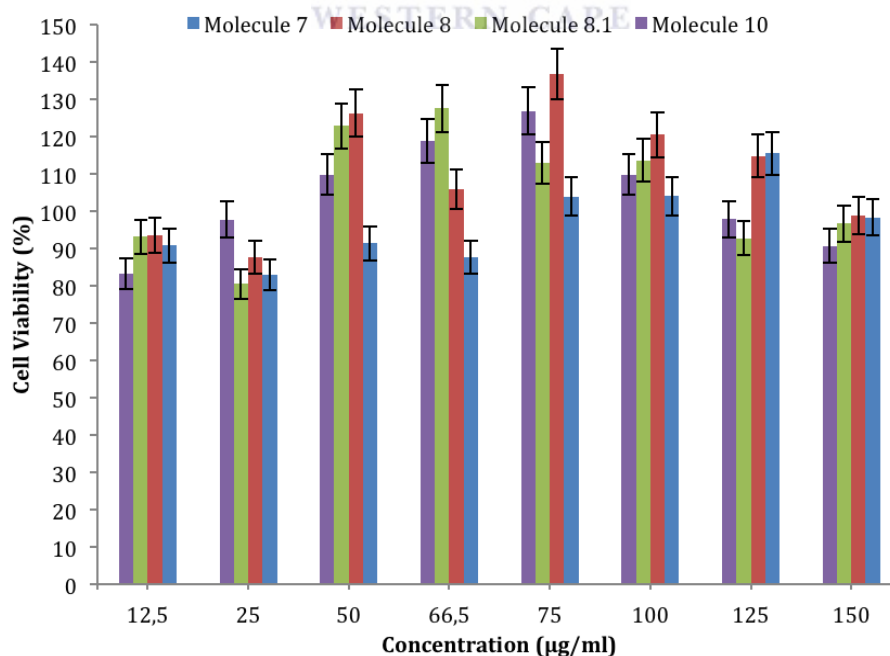
Figure 4.3: Cytotoxicity of Molecule 7, 8, 10 and 8.1 on T cell lines. All the data represent the mean values for six independent experiments and are reported as mean $\pm$ SD of the six replicated samples of each Molecule.

4.5. Discussion

The possibility to prove that the putative AMPs and the two mutated ones have anti-HIV properties is a crucial step in the design of AMPs that would serve as lead compounds for the development of potent drugs against HIV. The confirmation of the activity of the seven putative anti-HIV AMPs against HIV-1 NL4-3 virus during the screening process demonstrates that only Molecule 7, Molecule 8, Molecule 10 and Molecule 8.1 could inhibit HIV replication with the concentration of 50 µg/ml of each peptide (Figure 4.1). At a closer look an observation was made that at this concentration, the percentage of the HIV NL4-3 cell inhibited by Molecule 1 is more than its mutated counterpart (Figure 4.1). This result correlated well with that observed in the *in-silico* prediction made in Chapter Two which showed that there was a decrease in the binding score of Molecule 1.1 when it was docked to HIV gp120 (Table 2.6). The inverse observation was noticed for Molecule 8.1, which percentage inhibition of HIV NL4-3 cell was higher than the parental peptide Molecule 8, which showed an increase in its binding score during the docking of the mutated peptide to HIV gp120 protein (Table 2.6). This preliminary result could imply that the binding of the AMPs to gp120 protein would play an important role in preventing the interaction of HIV gp120 protein to the surface molecule CD4+ of T cells, macrophages/monocytes, and dendritic cells.

The subsequent reaction of the dose-dependent response assays was performed to determine the EC_{50} of the AMPs, which were found to have anti-HIV activity in the screening assays. During this process, it was demonstrated that Molecule 7 and Molecule 8 showed inhibitory capacity against HIV-1 NL4-3, with EC_{50} values of 37.5 µg/ml and 93.75 µg/ml respectively (Figure 4.2). This outcome corroborates with the previous results obtained during the screening process. Nonetheless, Molecule 8.1 EC_{50} was not determined due to the fact that the working concentration ranges used was not below 12.5 µg/ml. However, at a concentration of 12.5 µg/ml, Molecule 8.1 is inhibiting 54.85 % of HIV NL4-3 cells. Thus the concentration ought to decrease for the EC_{50} of this peptide to be determined. Molecule 8.1 EC_{50} could be less than 12.5 µg/ml. Furthermore, this result demonstrates the significance of mutating amino acids

residues in this putative anti-HIV AMP sequences, to either increase their biological activity or change their binding orientation with their receptor; if we want to compare the EC_{50} of Molecule 8 and his mutated peptide, Molecule 8.1 and the effect of substituting an amino acid in the parental peptide. The use of positively charged amino acid in increasing the binding affinity of the AMP to their receptors and to ultimately increase the biological activity of AMPs to their target pathologies needs to be evaluated and confirm the significance of using amino acids of this class (Lee *et al.*, 2011). The same can be applicable to the hydrophobic amino acid residues, which are said to ameliorate the binding and biological activity of AMPs (Chan *et al.*, 2006).

The lack of inhibition observed during the treatment of NL4-3 by Molecule 1.1 despite the substitution of a short R-group (phenylalanine) with a long R-group (tryptophan) could be due to the fact that the replaced amino acid residue was of the same charged as the previous residue. The length of the R-group did not contribute to any increase binding affinity of the mutated AMP to its receptor, HIV gp120 protein as it was initially extrapolated during the physicochemical characterization and the parameters to consider for the mutations (Biro, 2006). Rather than reducing the space between the interaction of Molecule 1.1 with HIV gp120, and to generate a higher binding score during the docking, the binding score was reduced due to the length addition of a five-carbon ring group, increasing the distance between the two compounds; thus the reduction in the binding score (Chapter Two). This observation could be the major contributing element that has affected the negative activity generated by Molecule 1.1 after the treatment of HIV NL4-3, with this compound (Figure 4.1). The EC_{50} of Molecule 10 could not be determined despite the fact that Molecule 10 concentration used for the HIV treatment was up to 150 µg/ml. The use of an increase concentration for the peptide was not considered for treatment as this increase might lead to increase toxicity to the host cells.

Overall, the experimental validation of the activity exhibited by these AMPs highlights the necessity to utilize suitable predictive tools for the design of compounds that would serve as a lead compound for the inhibition and/or prevention of infectious organisms. Hence, the anti-HIV assays confirmed and prove that the utilization of the HMMER algorithm to design and discover new putative AMPs, to either target HIV or any particular infectious disease, using experimentally validated

AMPs, aiding in the construction of predictive profiles would enable the search for novel AMPs, with activity against this pathology (Brahmachary *et al.*, 2004; Fjell *et al.*, 2007). Predictive tools that simulate peptide structures and their capacity to interact with other molecules have enabled the screening of potentially new compounds through their docking to selected receptors, making these computational tools essential and crucial for predictions as to make the right decision and selection during the screening procedure. Such techniques could, therefore, pave the way for more cost effective methods and reduce the time utilized during the biological search of the same lead compounds.

After determining the anti-HIV activity of the AMPs, the need to establish their cytotoxicity to human cells was imperative. Even though the EC_{50} seem to be of a high dosage to inhibit viral growth, it has to be taken into account that the various cytotoxicity concentrations (CC_{50}) of Molecule 7 and Molecule 8 could still not inhibit 80 % of T cells, at a concentration up to 150 µg/ml (Figure 4.3). This experiment further establishes the selective toxicity of these peptides. The AMPs would thus not disturb the immunological capacity of T cells in the human defence system, to properly stimulate other cells to regulate the bodies defence mechanism since no toxicity to these cells was observed when treated by the various anti-HIV AMPs. These AMPs further prove their ability to prevent the binding and attachment of HIV gp120 to CD4+ of T cells, macrophages/monocytes, and dendritic cells, without damaging these cells function to defend the human immunological capacity. However, these peptides rather demonstrated and affirmed their anti-HIV activity and their selective toxicity toward HIV NL4-3. Additionally, establishing the CC_{50} of these AMPs would help determine their therapeutic index (TI) or selective index (SI = CC_{50}/EC_{50}), which could be within an acceptable range (Becker, 2007). The therapeutic ability of Molecule 7 and Molecule 8 would be able to inhibit the viral replication and could prevent the invasion of healthy T cells and macrophages/monocytes by directly blocking gp120 contact and interaction with the CD4+ of these cells (Chan *et al.*, 1997; Kowalski *et al.*, 1987).

4.6. Conclusion

The reduction of HIV pandemic would only be achieved if the fight against the HIV pathology leads to a considerable decrease in the progression of the disease through increased research to identify adequate therapeutic compounds that would prevent the virus replication and clear the body from various sources of reservoirs. Several putative AMPs were tested in this study, where the peptides were rationally designed and developed based on their ability to block the binding between HIV gp120 protein and the human CD4 surface protein. Conclusive results have shown that three AMPs (two parental anti-AMPs and one mutated AMP) inhibit HIV-1 NL4-3 in an *in-vitro* experimental procedure (Figure 4.2), therefore reinforcing the idea that these AMPs could block the interaction of HIV gp120 to CD4 surface of T cells, macrophages and monocytes. Additionally, this *in-vitro* results also showed that anti-HIV toward HIV-1 NL4-3 was achieved with less cytotoxicity to the T cells, thus strengthening the implementation of these AMPs for human utilization. The overall data prove that these AMPs could serve as a lead compound for the development of peptide-based drugs such as microbicide and intravenous cocktail, to prevent further infection of healthy human cells, even with an infected individual.
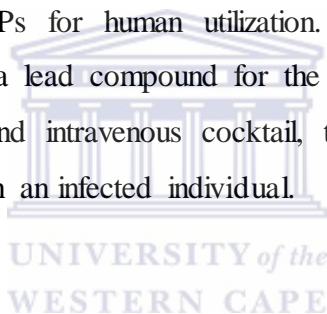
CHAPTER FIVE: ANTI-BACTERIAL ACTIVITY OF PUTATIVE ANTIMICROBIAL PEPTIDES

5.1. Introduction

The human body is equipped with a defence mechanism, which enables it to eradicate foreign bodies and/or pathogenic organisms (Du Pasquier and Flajnik, 1999; Goldsby *et al.*, 2003). However, infection of the human body with the HI Virus is a major concern since such invasion has added additional stress to the body immune system. The attack of T lymphocytes, macrophages, monocytes and dendritic cells by the virus using the CD4+ surface molecule has prevented the human immune system from fighting back. Such attack has also caused the human defence mechanism to fail in the presence of other pathogens, and this failure is due to the fact that virus replication takes place in the same cells that are meant to protect the body from any microbial attack (Chinen and Shearer, 2002). The inability of the human defence system to fight back, followed by the virus evasion of the immune system will ultimately result in complete immunity breakdown, hence give way for the entrance of other pathogenic organisms into the body (Smith, 2008; Cheung *et al.*, 2005). This stage of HIV progression is termed Acquired Immune deficiency Syndrome (AIDS).

With progression to AIDS, other diseases and infectious pathogens will gain access into the body. Such infectious pathogens may include *Staphylococcus aureus*, *Candida albicans*, Herpes simplex, *Mycobacterium avium complex* (MAC), *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* just to name a few (Kaplan *et al.*, 2000). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two examples of bacteria that have serious clinical and medical implications in immunocompromised individuals and accounts for the major causes of nosocomial infections worldwide (Gould, 2006; Ferroni *et al.*, 1998). In addition, both pathogens have been cited as the major causative agents for many infections around the globe. These have contributed to broad range infections including skin infections, respiratory infections, and other major illnesses. In some instances, these infections can lead to life-threatening infections such as pneumonia, meningitis, toxic shock syndrome and

bacteremia (Ferroni *et al.*, 1998; Balcht and Smith, 1994; Curran and Al-Salihi, 1980).

Besides the fact that the immune system of an HIV-infected individual is at a critical point of failure, the currently available antibiotics used to eradicate these pathogenic microbes are ineffective. Such ineptitude of the new antibiotics was encountered due to microbial resistance towards these therapeutic molecules. Furthermore, immunocompetent individuals infected with *S. aureus* and/or *P. aeruginosa* has also demonstrated low susceptibility to these drugs due to antibiotic resistance genes (Jevons, 1961; Poole, 2004; Johnson *et al.*, 2001; Hiramatsu *et al.*, 1997).

The lack of effective anti-bacterial antibiotics to inhibit the infectious diseases and to stop the ability of these microorganisms to replicate have encouraged microbiologists, bacteriologists, and clinical pathologists to embark on a journey in search of alternative remedies to treat such microbial infections. The antimicrobial peptide has proven to be a good candidate as a potential source of anti-bacterial activity (Ngai *et al.*, 2006; Andersson *et al.*, 2003; Steiner *et al.*, 1998; Niyonsaba *et al.*, 2002; Pütsep *et al.*, 1999; Kubo *et al.*, 1996). A number of them have been commercially developed and are available on the market. Such examples include FDA approved Polymixin B-Collistin-Colomycin (prodrug) and Daptomycin (Cubicin), and which are used to treat skin infections (Gupta *et al.*, 2009; Shoemaker *et al.*, 2006).

In addition to their anti-HIV activity, the main objective of this chapter was to determine the potential anti-bacterial activity of putative antimicrobial peptides against Methicillin Resistant *Staphylococcus aureus* (MRSA ATCC 33591) and Methicillin sensitive *Staphylococcus aureus* (MSSA ATCC 25923) as well as *Pseudomonas aeruginosa* (ATCC 10145).

5.2. Methods

5.2.1. Preparation of samples

5.2.1.1. Bacterial strains

The anti-bacterial activity of the putative antimicrobial peptides was carried out against *Staphylococcus aureus* spp. and *P. aeruginosa*, obtained from the American Type Culture Collection (ATCC). The *S. aureus* spp. were comprised of ATCC 25923

and ATCC 33591 strains where the first strain is susceptible to methicillin and many antibiotics, and the second strain is resistant to methicillin. The culture isolates were grown in Tryptone Soya agar (TSA) and Tryptone Soya Broth (TSB) (Oxoid Ltd, UK). The microtiter broth dilution method was used to determine the minimum inhibitory concentration (MIC) of the various AMPs.

5.2.1.2. Antimicrobial peptide compounds

The seven AMPs utilised for the antibacterial assay, included five putative anti-HIV AMPs taken from our previous work (Tincho *et al.*, 2016) and two mutated AMPs, which was found to bind gp120 as demonstrated in Chapter two. The selected AMPs were chemically synthesized by GL Biochem Ltd. (Shanghai 200241, China) using the solid-phase method and they were purified to > 98 % by reverse-phase High-Pressure Liquid Chromatography and the AMPs were shipped in a lyophilized form.

5.2.1.3. Preparation of antimicrobial peptide and positive control concentration

After the purchase of the AMPs, the lyophilized AMPs were stored at -20 °C for long storage. The AMPs were dissolved in sterile distilled water (dH$_2$O) and the various AMP working concentrations of the microtiter broth dilution assay were prepared in two-fold serial dilutions starting at a concentration of 500 μg/ml to 31.25 μg/ml. Ampicillin was utilized as the positive control in this assay and a working solution of 100 μg/ml was prepared in which lyophilized ampicillin was dissolved in distilled water and subsequently filter sterilized.

5.2.2. Antibacterial susceptibility activity of the antimicrobial peptides

Various methods have been employed to determine the susceptibility of pathogenic microbes to antimicrobial peptides or on other therapeutic compounds. Whilst these methods vary from the disk diffusion method, the gradient diffusion method and the microtiter also called microdilution method; the most recommended method has been the micro-dilution method because it offers a more quantitative result of the bacterial susceptibility, rather than a qualitative result that is offered by the other methods (Jorgensen and Ferraro, 2009).

5.2.2.1. Microtiter broth dilution method

The microtiter broth method employed to measure the antibacterial activity of the AMP's was performed according to the standards and guidelines as stipulated in the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012). MRSA ATCC 33591, MSSA ATCC 25923 and *P. aeruginosa* isolates were cultured on TSA agar and incubated to grow for 24 hours. Following incubation, purified isolates of ATCC 33591, MSSA ATCC 25923, and *P. aeruginosa* were inoculated into TSB and were grown overnight at 37 °C. The following day, 1 ml of overnight bacterial cultures were transferred into 9 ml of TSB and was incubated at 37 °C. In a 96 well flat bottom plate, 100 µl of each of the various bacterial strains were seeded once the turbidity was in accordance to a McFarland standard. AMPs were serially diluted in sterile distilled water and 100 µl of the compound was added to each well. A control with the bacterial inoculum and Ampicillin was included in the experiment. Each experiment was run in triplicate. The test plates were sealed and incubated in a shaking incubator at 37 °C for 24 hours. After 24 hours incubation, 40 µl of INT was added to each well and incubated again for three hours. Absorbance readings were taken at 620 nm on a microtiter plate reader, 3 hours after adding INT, then after every 6 hours subsequently. Absorbance results were exported into an Excel file, where they were transformed into a percentage, in a process called Normalizing.

5.2.2.2. Statistical analysis

The normalized results from microtiter dilution assays were analysed using the statistic algorithm GraphPad Prism Software, version 7 (GraphPad software, San Diego, CA, USA). The data were expressed as mean ± SD (Standard deviation) of the normalized values from the three experiments.

5.3. Results

Since the antimicrobial peptides and the antibiotic Ampicillin completely soluble in distilled water, incorporating it into TSB was not a problem. The microtiter dilution assay yielded some promising preliminary results.

5.3.1. Peptide inhibition of *P. aeruginosa* using microtiter broth method

The inhibition activity of the AMPs on *P. aeruginosa* showed that Molecule 3 and Molecule 7 resulted in complete inhibition of this bacterium after 24 hours of treatment, with AMP concentrations ranging from 0.5 mg/ml to 0.03125 mg/ml. No growth was observed 48 hours post treatment (Figure 5.2 and Figure 5.3). In the contrary, Molecule 1, Molecule 8, Molecule 10 and Molecule 8.1 could only inhibit a range of 10 % to 30 % of *P. aeruginosa* after 24 hours treatment, with AMP concentrations ranging from 0.5 mg/ml to 0.03125 mg/ml. The same inhibition ranges were observed when the treated plates were examined 48 hours post treatment (Figure 5.1, Figure 5.4, Figure 5.5 and Figure 5.6). However, no inhibition was observed when *P. aeruginosa* was treated with Molecule 1.1, even at the highest peptide concentration of 0.5 mg/ml, over the period of 48 hours. Thus Molecule 1.1 does not have anti-bacterial activity against this microbe (Figure 5.7).
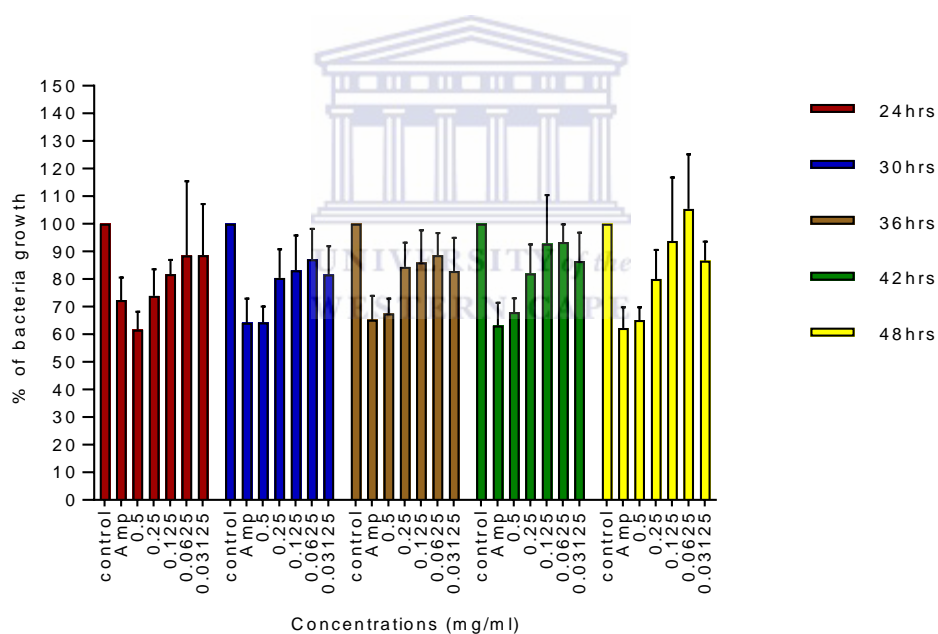
Figure 5.1: The effect of Molecule 1 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
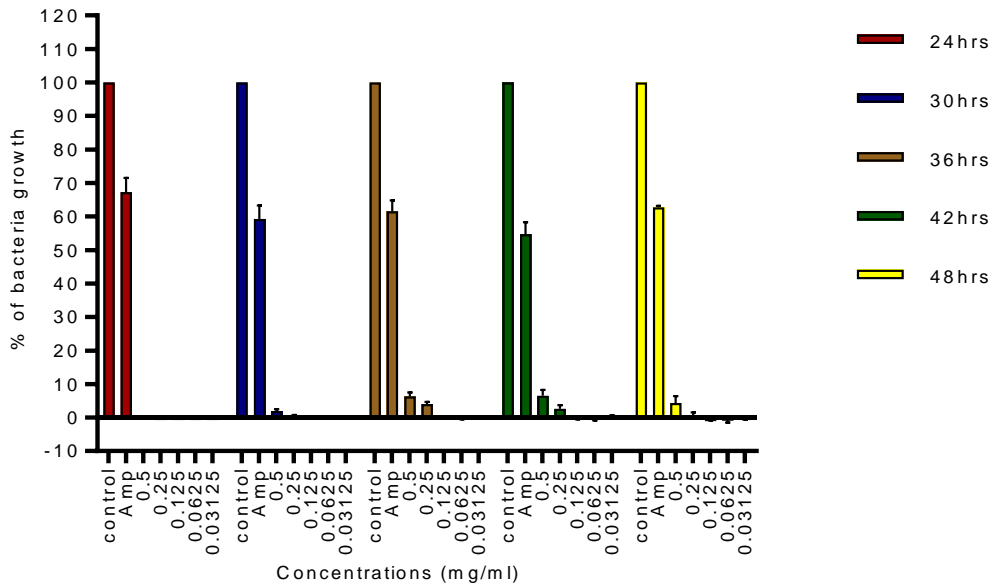
Figure 5.2: The effect of Molecule 3 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
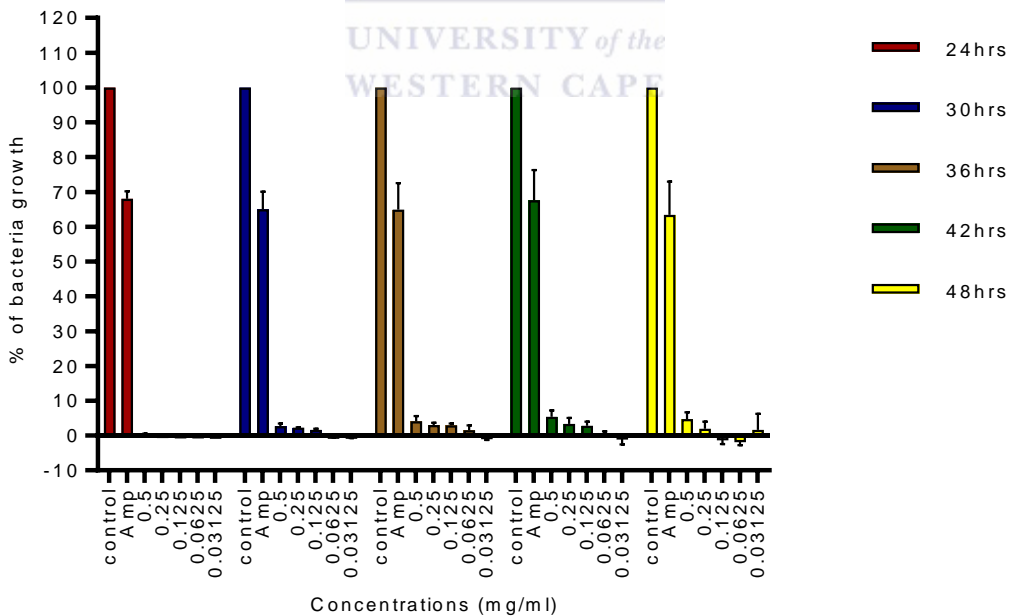
Figure 5.3: The effect of Molecule 7 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

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Figure 5.4: The effect of Molecule 8 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

Figure 5.5: The effect of Molecule 10 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

Figure 5.6: The effect of Molecule 1.1 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

Figure 5.7: The effect of Molecule 8.1 on the growth of *P. aeruginosa* after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

5.3.2. Peptides inhibition of *MRSA* using microtiter broth method

The microbial susceptibility of these peptides was also determined for MRSA and MSSA. Since MRSA is a leading cause of hospital-acquired infections, treatment of MRSA with the various peptides showed that the bacterium was mostly resistant to these peptides. Whilst, almost no inhibitions were observed for Molecule 1, Molecule 8 and Molecule 1.1, even with the highest concentration of 0.5 mg/ml (Figure 5.8, Figure 5.12 and Figure 5.13); slight inhibitions were however, observed for Molecule 3, Molecule 7, Molecule 10 and Molecule 8.1 with concentration ranging from 0.5 to 0.25 mg/ml, with a percentages inhibition ranging from 20 to 40 % (Figure 5.9, Figure 5.10, Figure 5.11 and Figure 5.14). It should also be mentioned that the treatment time has no effect on this bacterium, as the percentages of live bacteria remained constant across the timeline of treatment.

Figure 5.8: The effect of Molecule 1 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
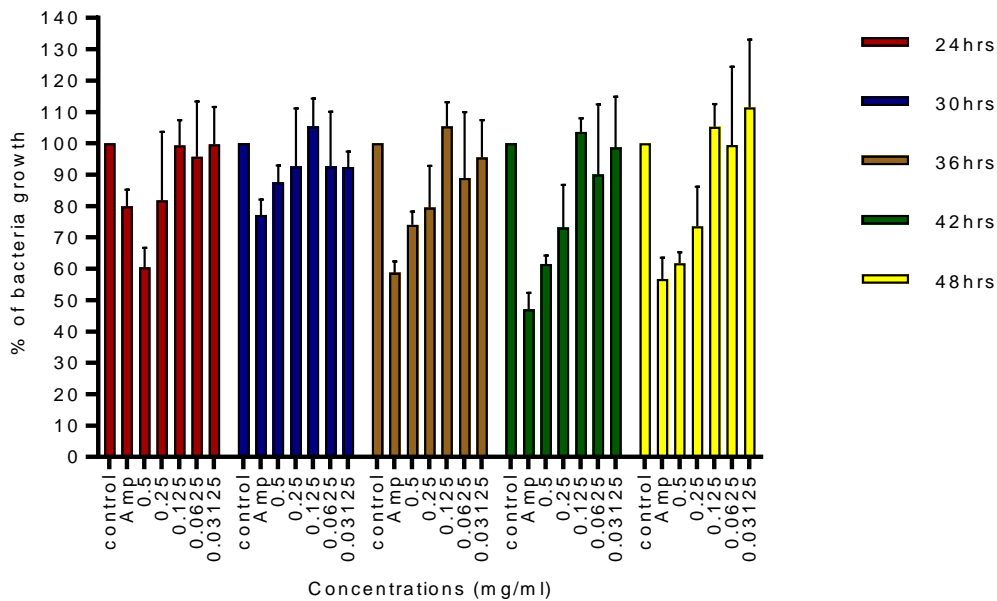
Figure 5.9: The effect of Molecule 3 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
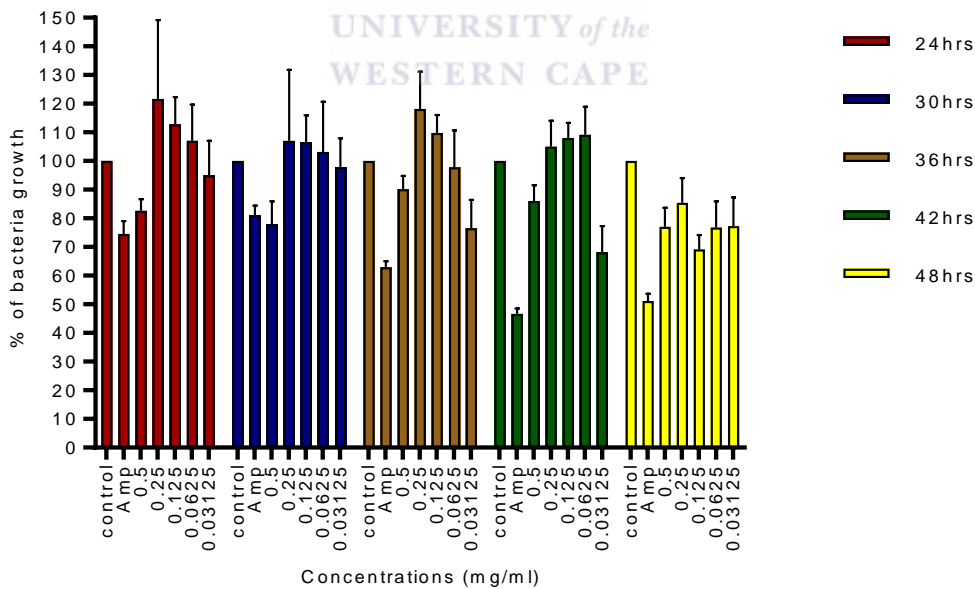
Figure 5.10: The effect of Molecule 7 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
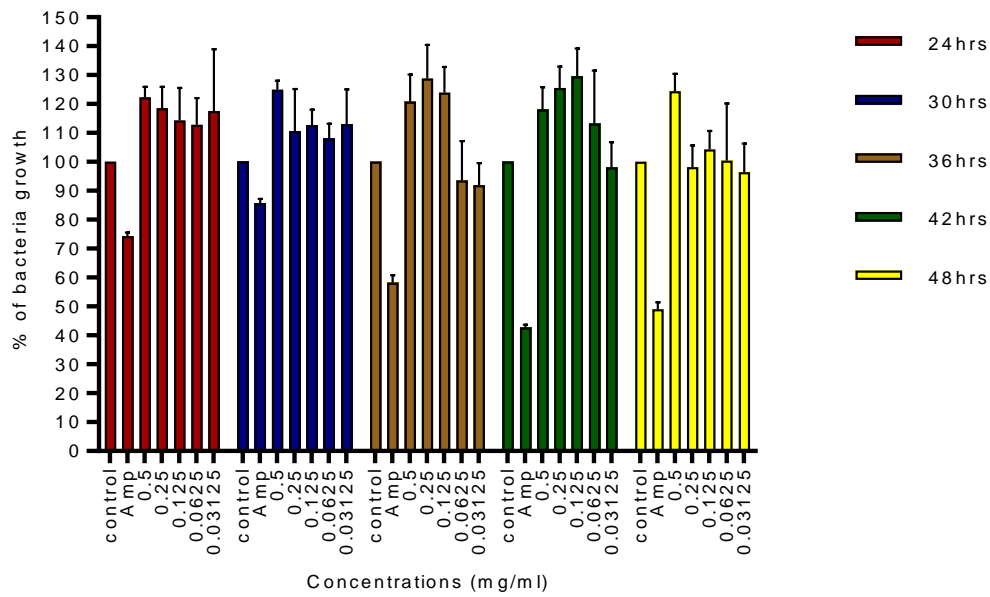
Figure 5.11: The effect of Molecule 8 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
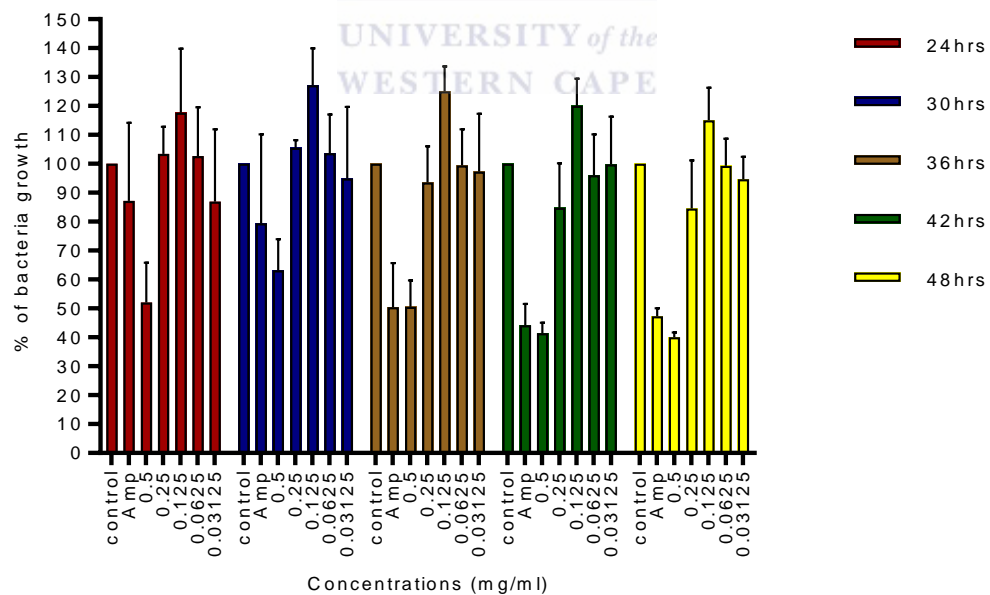
Figure 5.12: The effect of Molecule 10 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
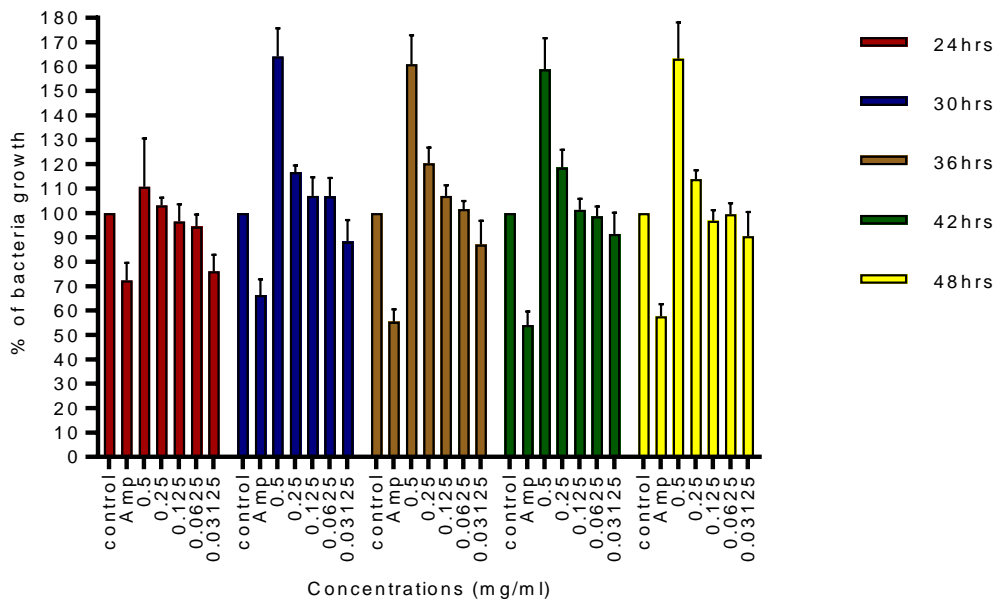
Figure 5.13: The effect of Molecule 1.1 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
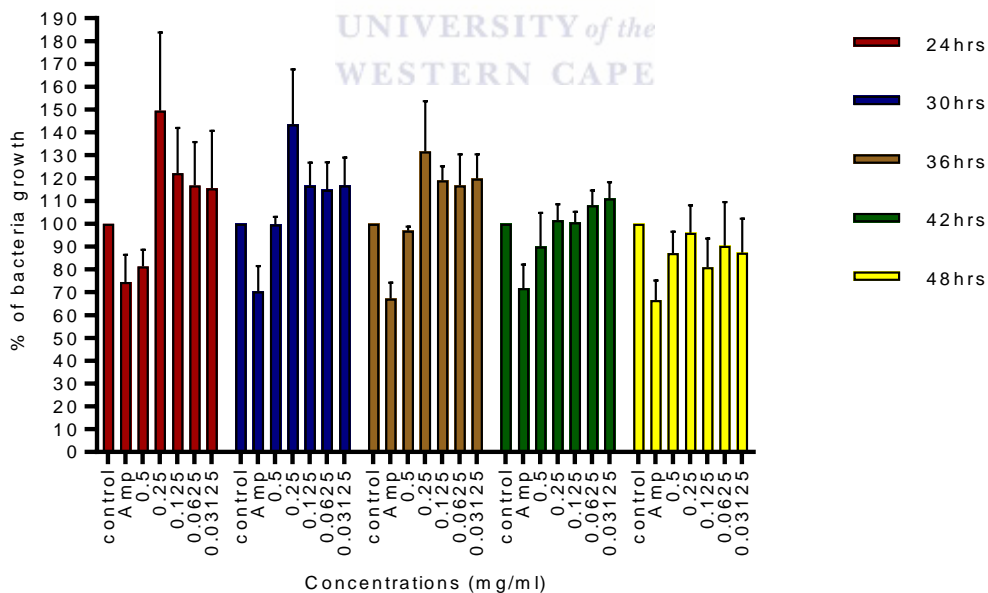
Figure 5.14: The effect of Molecule 8.1 on the growth of MRSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

5.3.3. Peptides inhibition of *MSSA* using microtiter broth method

The ability of the peptides to inhibit MSSA was not much different from that of the resistant strain of *Staphylococcus* i.e MRSA. The antibacterial activity of the AMPs indicated that only Molecule 1, Molecule 3, Molecule 7, Molecule 8 and Molecule 10 would inhibit MSSA growth at a peptide concentration of 0.5 mg/ml, with a percentage growth inhibition of 37 %, 50 %, 25 %, and 45 % respectively. However, no inhibition was observed when the peptide concentrations were reduced from 0.2 to 0.03125 mg/ml (Figure 5.15, Figure 5.16, Figure 5.17, Figure 5.18 and Figure 5.19). Bacterial inhibitions were noticed when the MSSA was treated with the mutated peptides, Molecule 1.1 and Molecule 8.1 (Figure 5.20 and Figure 5.21).

As observed for MRSA, the time response treatment of the bacterium by the various peptides did not really slow the bacterial growth. Thus, the percentage growth for each peptide treatment remained constant after 24 hours and 48 hours of treatment.
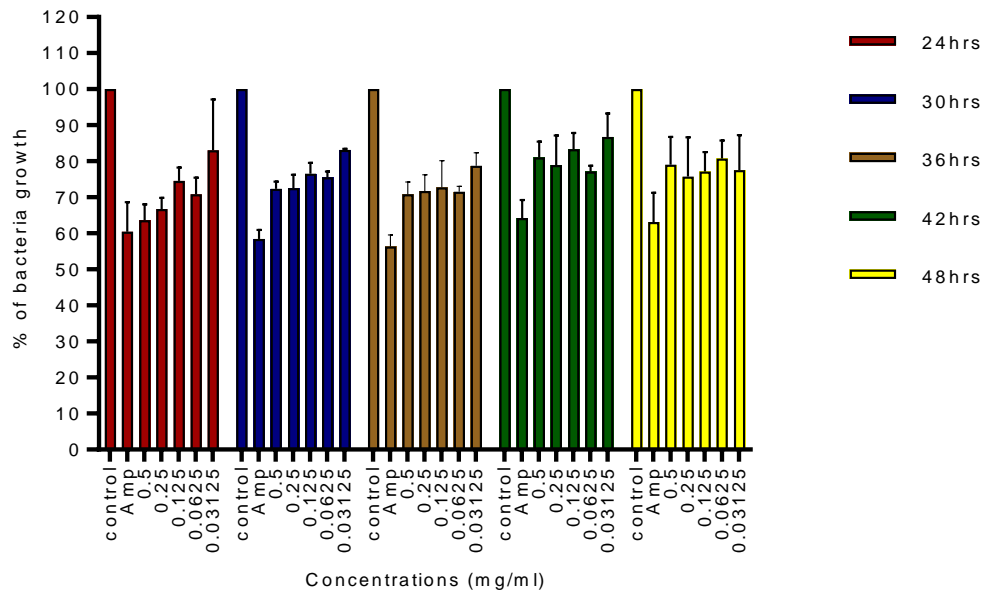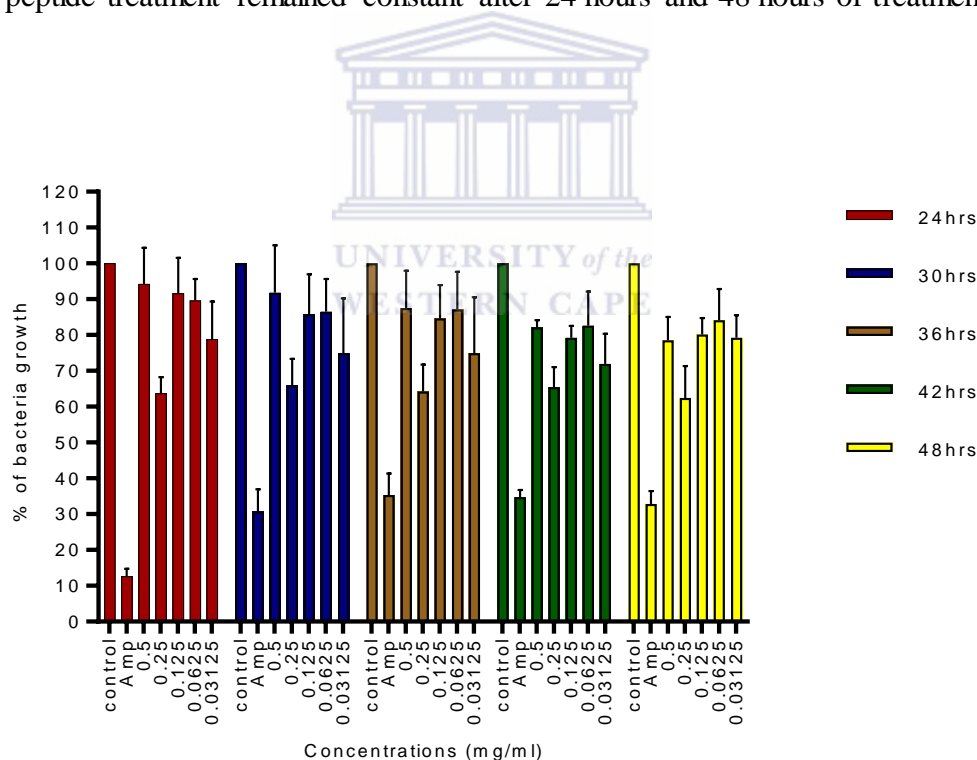

Figure 5.15: The effect of Molecule 1 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
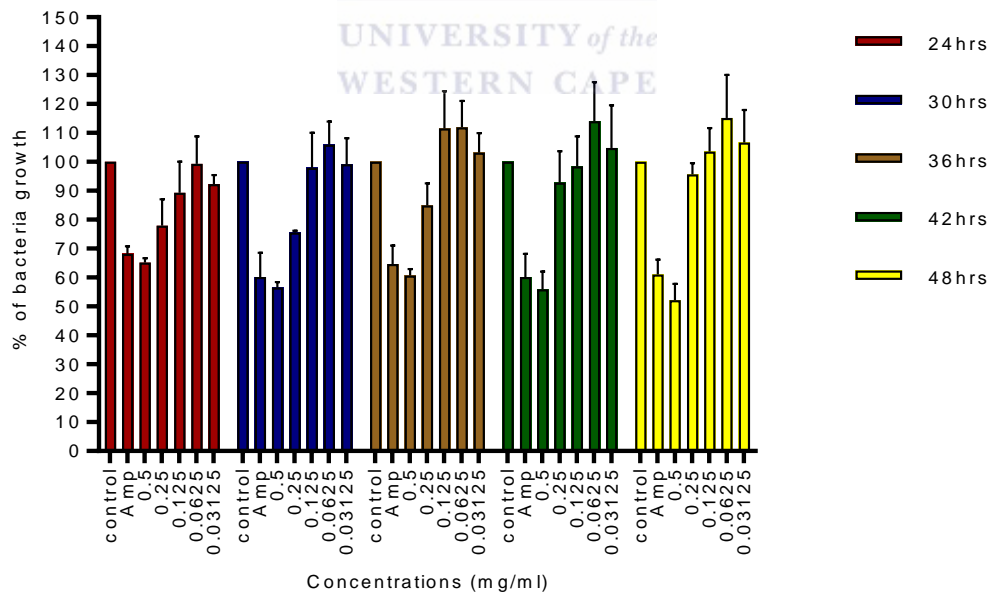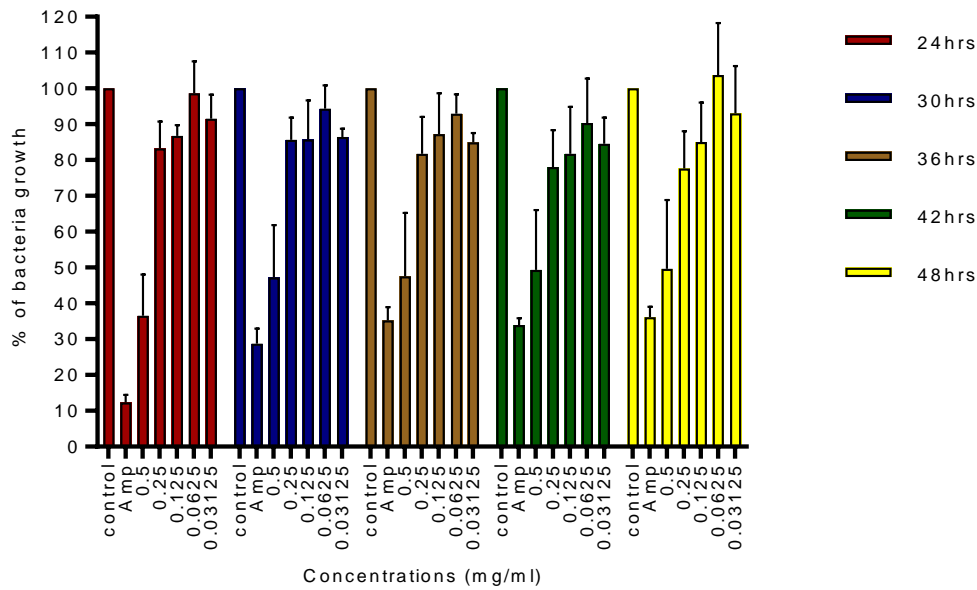
Figure 5.16: The effect of Molecule 3 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
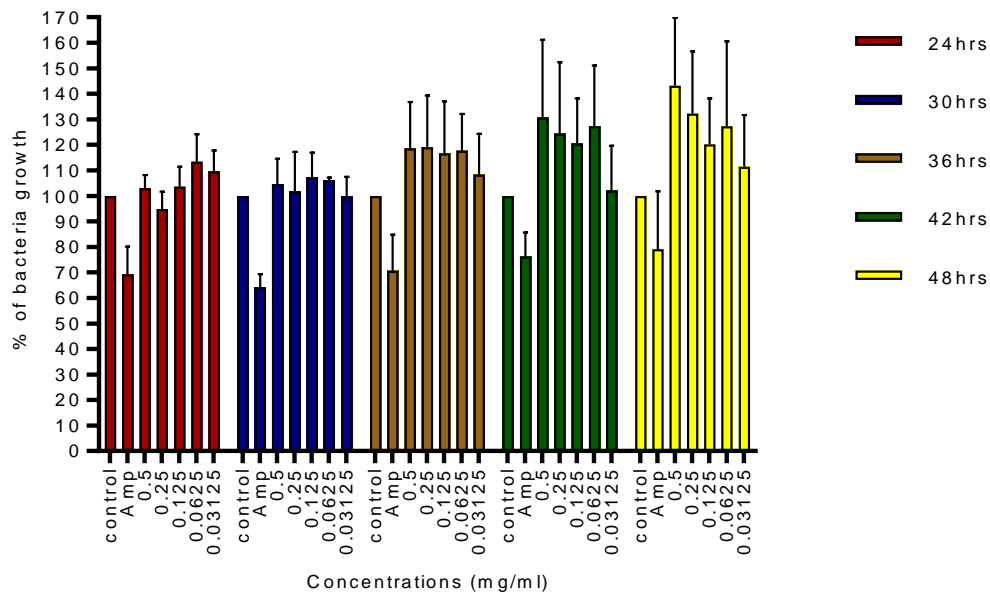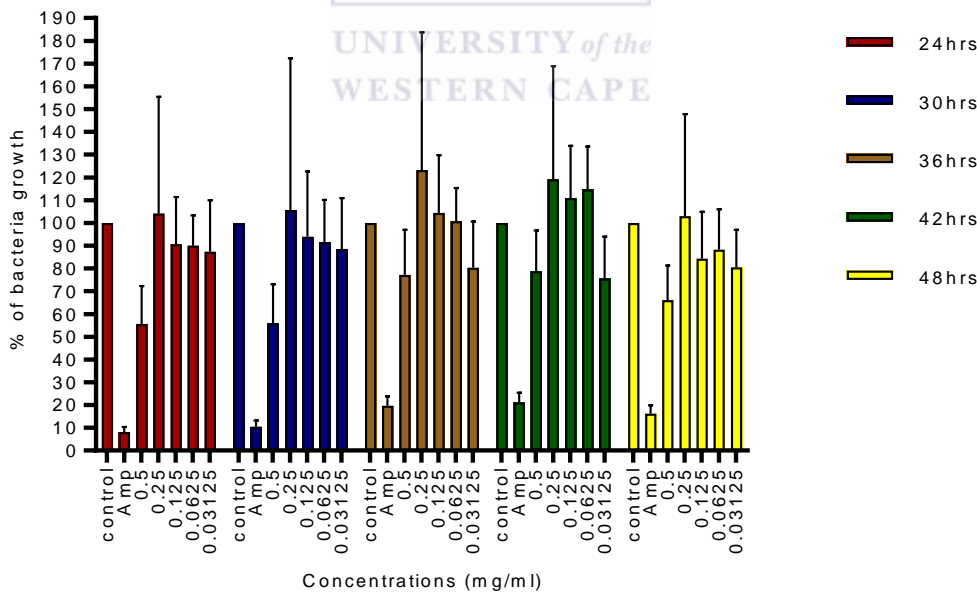

Figure 5.17: The effect of Molecule 7 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
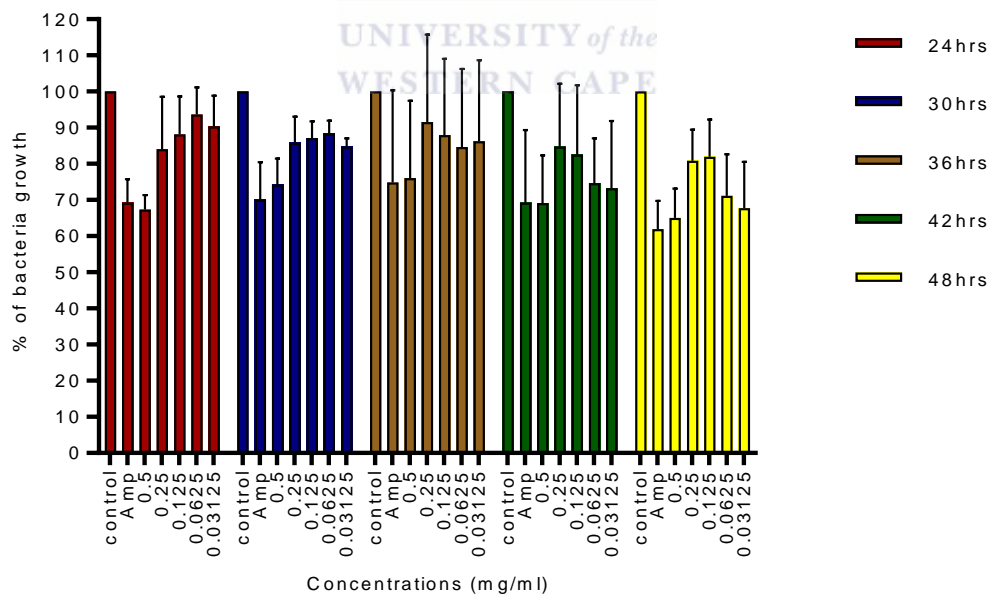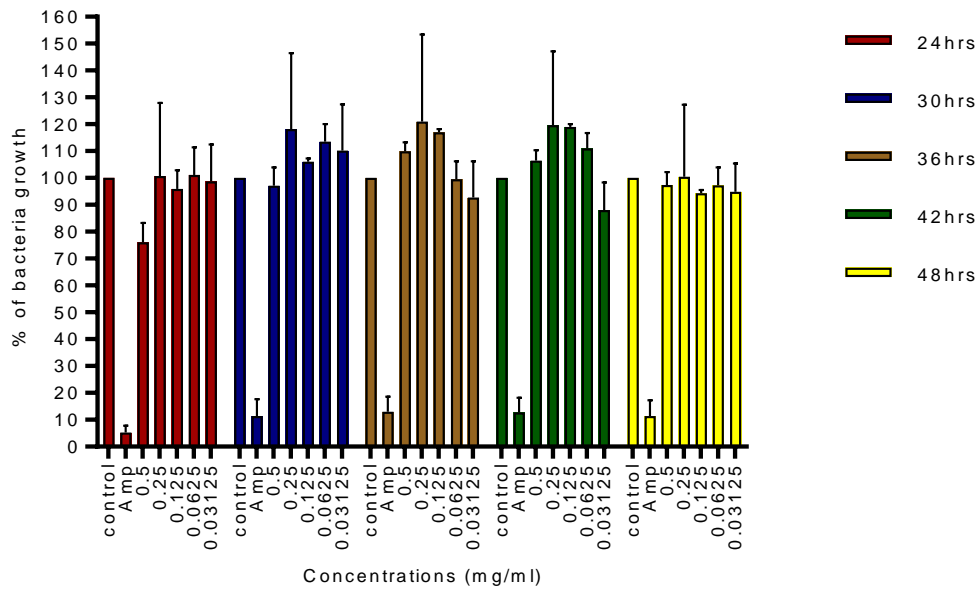
Figure 5.18: The effect of Molecule 8 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
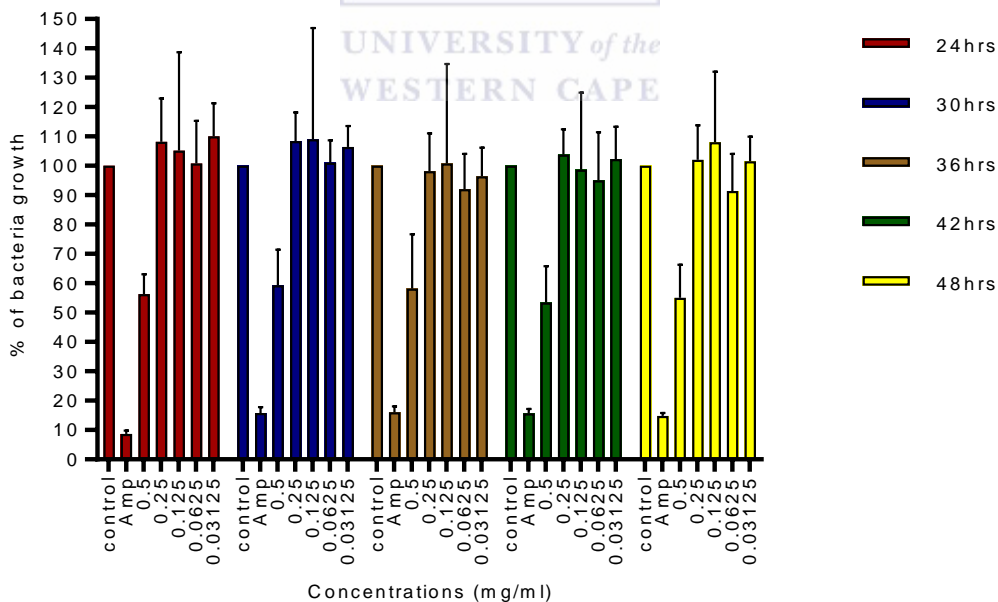
Figure 5.19: The effect of Molecule 10 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
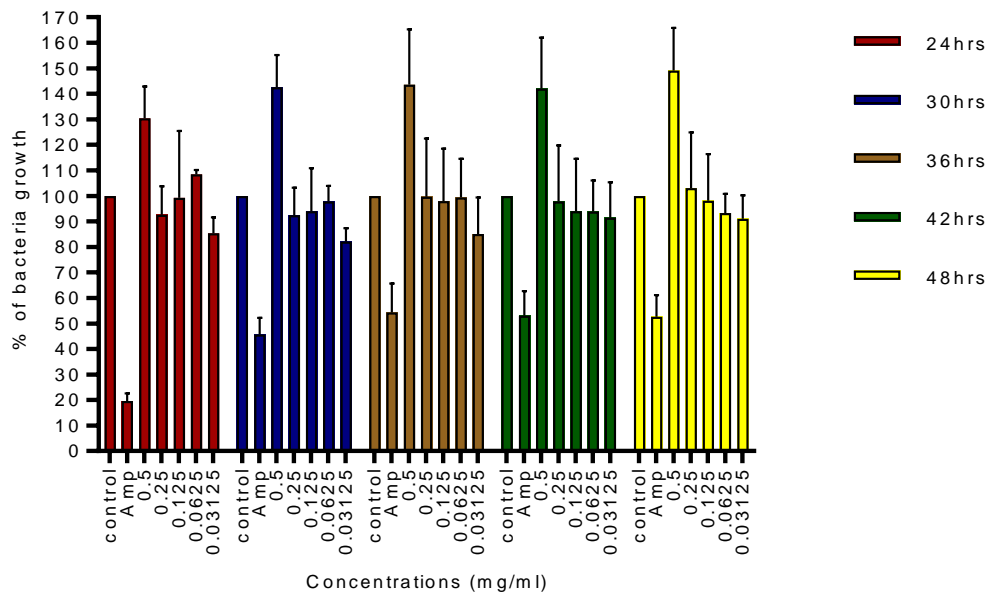
Figure 5.20: The effect of Molecule 1.1 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.
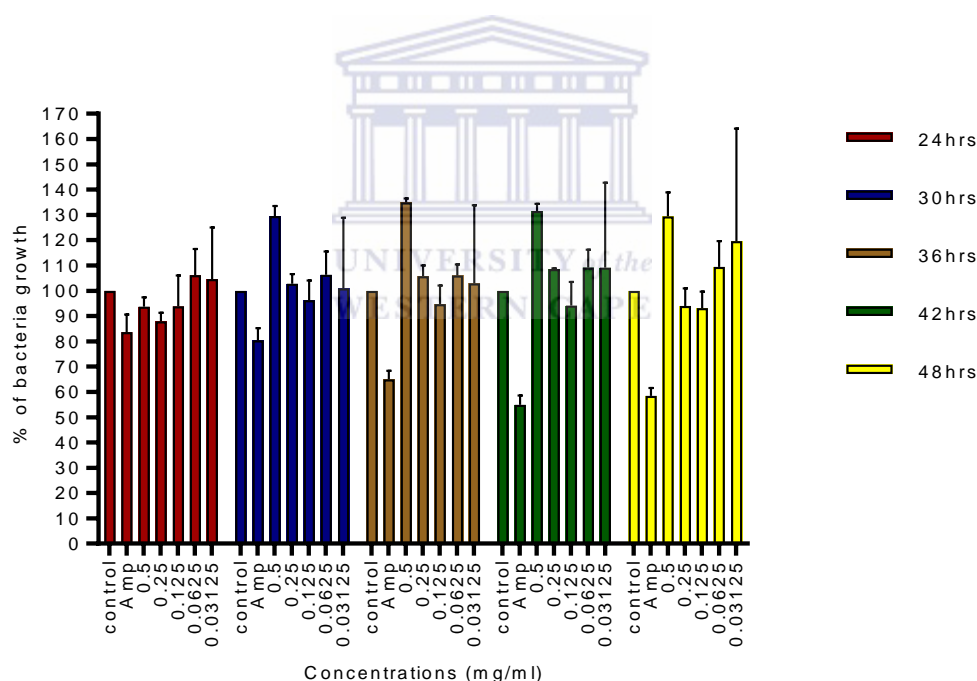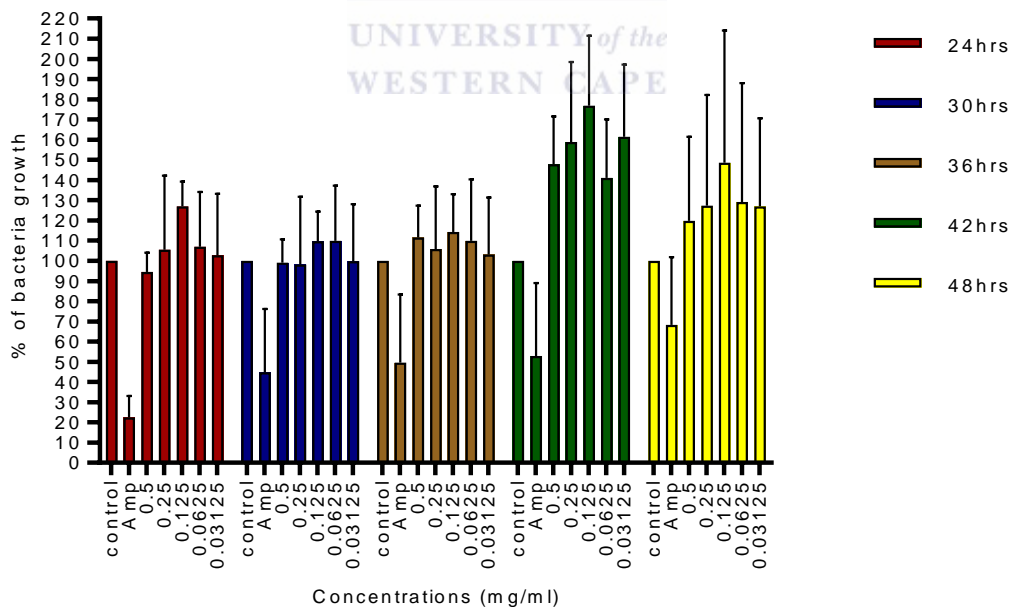

Figure 5.21: The effect of Molecule 8.1 on the growth of MSSA after dose and time response treatment detected by the microtiter assay. The error bars represent the standard deviation (±SD) of triplicates.

5.4. Discussion

The medical and pharmaceutical industry is in the race to develop and discover novel drugs that can serve as potent antimicrobials. Time is a major factor since few new antibiotics have been developed in the past decade, and the problem is compounded by the increase in bacterial resistance to available antimicrobials. Whilst the bacterial resistance may be due to misuse of these antibiotics, concern has also been raised on the fact that there is no correlation at the speed at which amount of molecules to fight these pathogens are developed and the number of microbial pathogens being discovered. Due to these shortcomings, the only option is to develop more antibiotics molecules to counter the increase in antibiotics resistant attacks of the pathogens.

The experimental procedures for screening for potent anti-bacterial AMPs have not yielded promising anti-bacterial results using the microtiter dilution method. Whilst concern might be raised on the choice of using this method to screen for anti-bacterial activity, the disc-diffusion method lacks the ability of being a quantitative method because the disc-diffusion method only tells us if the bacterium is susceptible to a particular compound but does not tell us the percentage of bacterium killed in the process, as compared to the initial input bacterium (Jorgensen and Ferraro, 2009). Additionally, the microtiter dilution method is the most appropriate and standard method to test for susceptibility on fast growing bacteria such as *Staphylococcus* spp. and *P. aeruginosa* (CLSI, 2012).

Since the AMPs were soluble in distilled water, it was not necessary to use other solvents to check the solubility of these peptides. Unlike solvents such as DMSO, ethanol, methanol, n-butanol, chloroform, ethyl-acetate, acetone, water does not have any negative impact on the experiment procedure nor does it affect the growth of bacteria (Dahiya and Purkayastha, 2012; Jastaniah, 2014).

Although the quest for novel antibiotics is an urgent matter, only two antimicrobial peptides were able to completely inhibit the growth of *P. aeruginosa*, even at the lowest AMP concentration of 0.03125 mg/ml (31.25 µg/ml) with the microtiter dilution method. Various concentrations of the seven AMPs, ranging from 0.5 to 0.03125 mg/ml were not able to inhibit up to 50 % of *Staphylococcus* spp. even for the methicillin-sensitive *Staphylococcus aureus*. Nonetheless, we noticed that Molecule 1, Molecule 8, Molecule 10 and Molecule 8.1 were able to inhibit growth of

Staphylococcus aureus by 10 % to 30 % after 24 hours treatment, with AMP concentrations ranging from 0.5 mg/ml to 0.03125 mg/ml (Figure 5.1, Figure 5.4, Figure 5.5 and Figure 5.6).

Inhibitions of MRSA by Molecule 3, Molecule 7, Molecule 10 and Molecule 8.1 was shown to inhibit around 20 to 40 % of MRSA 24 hours post-treatment with AMPs concentration ranging from 0.5 to 0.25 mg/ml (Figure 5.9, Figure 5.10, Figure 5.11 and Figure 5.14). On the other hand, only Molecule 1, Molecule 3, Molecule 7, Molecule 8 and Molecule 10 could inhibit 37 %, 50 %, 25 % and 45 % MSSA respectively, with a peptide concentration of 0.5 mg/ml 24 hours after the treatment with these peptides (Figure 5.15, Figure 5.16, Figure 5.17, Figure 5.18 and Figure 5.19).

The addition of more peptides into each well after 24 hours could inhibit the bacterial growth; however, the higher peptides concentration ranges could have toxic effects (Chapter Four). Many antimicrobial peptides have been proven to have anti-bacterial activity, on selected pathogenic microbes (Kubo *et al.*, 1996; Steiner *et al.*, 1998; Pütsep *et al.*, 1999; Niyonsaba *et al.*, 2002; Andersson *et al.*, 2003; Ngai *et al.*, 2006; Cao *et al.*, 2012), hence the current work yields promising anti-bacterial activity despite having two of the peptides showing anti-bacterial activity out of seven peptides tested. This result was excepted since the AMPs were developed based on HIV receptor (Tincho *et al.*, 2016). Nevertheless, testing the anti-bacterial activity of these peptides was to examine the possibility of the AMPs having additional biological activity besides the anti-HIV. Dual biological activities have been demonstrated by certains AMPs (Pan *et al.*, 2007; Pan *et al.*, 2009; Tharntada *et al.*, 2009; Shang *et al.*, 2009; Chen *et al.*, 2011; Cao *et al.*, 2012; Wang *et al.*, 2012), thus the results obtained here is conviencing and promising.

The mechanism of action in which Molecule 3 and Molecule 7 used to exhibit their anti-bacterial activity has not yet been established. It could be speculated that their activity is carried out using the Barrel-stave mechanism, the carpet mechanism (Giuliani *et al.*, 2007), or the toroidal pore mechanism (Brogden, 2005). The barrel-stave mechanism could be the appropriate mechanism of choice since most of our peptides are α-helical, β-sheet peptides, extended with α-helical structure, extended with β-sheet structure; and it has been found that most α-helical or β-sheet AMPs use

this mechanism to exert their activity on pathogens (Breukin and Kruijff, 1999). However, this mechanism of action needs to be established so as to confirm the real route in which these peptides inhibit the bacterial growth.

5.5. Conclusion

The mixed results obtained in this search for potential antibiotics remains a challenge in the field of drug development and discovery. The microbial screening of novel AMPs against *Staphylococcus aureus* spp. (*S. aureus* MSSA ATCC 25923 and MRSA ATCC 33591) and *P. aeruginosa* revealed that Molecule 3 and Molecule 7 could individually inhibit the growth of *P. aeruginosa* at the lowest concentration of 0.03125 mg/ml thus could form a good platform to develop a potent antibiotic drug. However, these peptides were not developed for this purpose but the preliminary data suggests that we may have a potential compound, which can be used to develop a potent antibiotic.

The future work should focus on determining the MIC that inhibits 50 % of *P. aeruginosa* 24 hours after treatment with the peptides. Furthermore, more drugs resistant pathogens should be tested against these anti-bacterial peptides, to expand the list of bacteria that are inhibited by the peptides. In conclusion, the exact inhibitory mechanism of action of these peptides must be determined as it could add more knowledge about the way these AMPs function in order to destroy susceptible and resistant bacteria.

CHAPTER SIX: CYTOTOXICITY ABILITY OF PUTATIVE ANTIMICROBIAL PEPTIDES WITH POTENTIAL ANTI-CANCER ACTIVITY

6.1. Introduction

The world has always been confronted with many deathly pathogenic microbes including viruses, bacteria, fungi and many other infectious microbes that have decimated our populations in a rapid and exponential rate (Pacini, 1854; Gallagher, 1990; Kuhnke, 1990; Khaled, 1993; Bruns, 2000; Marr and Cathey, 2013; Miller, 2005; Pike, 2007; WHO, 2008; UNAIDS, 2010). The high mortality rates following these epidemics could either be explained by the inability of the human immune system to fight the microbial invasions or due to the lack of adequate medications, to suppress the pathogens. Whilst the scientific community is more concerned with the development of tools to fight the infectious pathologies we face daily, more deathly, non-infectious or non-communicable diseases are making their way into our societies at a slow rate but in an irreversible manner (Manton, 1988; WHO global report on non-communicable diseases, 2014).

Non-communicable diseases in this category may include pathologies such as obesity, diabetes, cardiovascular diseases, chronic respiratory diseases and cancers. Although the first five non-communicable diseases were not major problems in the past decades, the incidence of these diseases are rising as a result of our changing lifestyles, infrastructure development, poor quality diets and the lack of physical activity (Popkin *et al.*, 1995; Popkin, 1998; Popkin *et al.*, 1996; UN, 1999; WHO, 2009). These conditions might have tremendous effects on how the human body's innate immune system will respond to the invasion of other infectious pathologies. However, the conditions may be reversed either by implementing certain measures such as changing our eating practices and lifestyle; or by the use of medicaments and medical procedures (Popkin *et al.*, 1995; WHO, 1998).

Whilst the first four non-communicable diseases listed above might be managed and reversed, via several methods, another non-communicable disease such as cancer could also be developed due to lifestyle, some types of cancers may occur due to the presence of communicable diseases related to viral infections. Well known illustrated examples of cancer that can be acquired as a result of communicable diseases include: cervical cancer, which develops as a result of 90 % HPV infection in females (Kumar *et al.*, 2007); and liver inflammation or viral hepatitis, caused by viral infections such as Herpes simplex virus, Yellow fever, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus (Xiong, 2010; Okano and Gross, 2012; Anderson *et al.*, 1994). Furthermore, most cases of liver inflammation may result in liver cancers and this situation may not be possible to reverse, as it is the case with obesity and being over-weight or diabetes, which can be managed.

Cancers can be combatted more efficiently if these diseases are diagnosed at an early stage and the disease progression can be remediated through the use of different treatment regimens/methods including chemotherapy, radiotherapy and surgery. Whilst radiotherapy and surgery seem promising, there is a need for the patient to seek specialized treatment. Hence, the patients ought to visit specialized facilities, which are mostly located in urban areas. However, with the few facilities being available in developing countries, the waiting list become long and the patients die even before their first visit to the hospital, thus the high mortality rate in underdeveloped countries (Kent, 2010).

Due to the shortcomings encountered with a shortage and availability of highly specialized equipment and facilities to treat cancer patients, a better resolution will be the use of chemotherapeutic drugs. However, these drugs are rare, less effective and have many side effects such as anaemia and neutropenia. The most effective chemotherapeutic drug used for the treatment of many types of cancers include Cisplatin (Perilongo *et al.*, 2012; Waggoner, 2003; US FDA, 2006); therefore, the quest for additional drugs is eminent, to suppress the cancer onset and progression. Antimicrobial peptides have been proven to be potent anti-cancer compounds (Chen *et al.*, 2009; Lin *et al.*, 2009; Lin *et al.*, 2010; Hsu *et al.*, 2011; Wang *et al.*, 2012; Huang *et al.*, 2013) and many anti-cancer AMPs have advanced to clinical trial phase and are implemented as potent cancer drugs (Jemaa *et al.*, 2010; Shore and Cowan, 2011; Denmeade *et al.*, 2012; Engel *et al.*, 2012). Thus, this chapter was to determine

whether the putative AMPs shown to have anti-HIV and anti-bacterial activities as describe in previous chapters, could also be utilized as potential anti-cancer compounds. And if the cytotoxicity on cancers cells could not be obtained as expected, the end purpose of this chapter was to confirm the specificity of these putative AMPs, to harbour HIV activity, a function that has been demonstrated in chapter four of this thesis.

6.2. Methods

6.2.1. Human cell lines utilized

The cell-lines utilized to determine the anti-cancer activity of the putative antimicrobial peptides included the CHO (Chinese Hamster Ovary) cells, MCF-7 (breast cancer cell line), HeLa (human cervix adenocarcinoma cell line) and HT-29 (Caucasian colon adenocarcinoma grade II human cell line). MCF-7 was obtained from the American Type Culture Collection (ATCC), the CHO cell lines was kindly provided by Prof Jasper Rees (Sir William Dunn School of Pathology, Oxford University, United Kingdom). HeLa and HT-29 were kindly provided by Prof Denver Hendricks (Department of Clinical and Laboratory Medicine, University of Cape Town, South Africa). The MCF-7, HeLa and HT-29 cell lines were maintained and grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, and 1% penicillin–streptomycin in a 37 °C humidified incubator with 5 % CO_2 saturation. However, CHO lines were maintained and grown in Hams F-12 Nutrient Mixture, containing 10 % foetal bovine serum, and 1 % penicillin–streptomycin in a 37 °C humidified incubator with 5 % CO_2 saturation.

6.2.2. Antimicrobial peptides compounds

The AMPs used in this chapter are the same as already mentioned in chapter three, chapter four and chapter five. These peptides are composed of five putative anti-HIV AMPs: Molecule 1, Molecule 3, Molecule 7, Molecule 8 and Molecule 10 (Tincho MSc thesis, 2013) and the two mutated AMPs (Molecule 1.1 and Molecule 8.1), which was found to bind gp120 as demonstrated in Chapter two, In brief, these peptides were chemically synthesized by GL Biochem Ltd. (Shanghai 200241, China) using the solid-phase method and they were purified to > 98 % by reverse-phase High-Pressure Liquid Chromatography and the AMPs were shipped in a lyophilized form.

6.3. Cell culture

6.3.1. Cell thawing and seeding

Vials containing cell lines were stored at -150 °C in a freezer. The vials were placed in a 37 °C water bath until just thawed. Cells were then removed from the vials transferred to a 15 ml conical tube containing 5 ml complete media (containing serum and antibiotics). Cells were then centrifuged for 2 minutes at 800 x g to pellet them, and the cell pellets were resuspended in 5 ml of their respective complete media and transferred to a 25 cm^2 cell culture flask. Cells were incubated at 37 °C in a humidified incubator at 5 % CO_2.

6.3.2. Media replacement

Since all the cell lines used were adherent cell lines, spent media was simply removed after 48 hours by aspiration and discarded. Fresh complete media was added to cells before additional incubating at 37 °C in 5 % CO_2.

6.3.3. Morphological analysis

Throughout the growth and treatment process, the cells morphology was monitored with the use of a Nikon microscope at 20 X magnifications, fitted with a Leica digital camera.

6.3.4. Sub-culturing of cells

Once the cell cultures reached confluency, they were detached by trypsin digestion. Trypsin (1 X) was added to the cells CHO, MCF-7, and HeLa; and each flask was incubated for 2 minutes at 37 °C in 5 % CO_2. However, HT-29 cells were trypsinised by incubating them in 2 X trypsin and incubated for 3 minutes at 37 °C in 5 % CO_2. The cells were then transferred to an appropriately sized sterile tube and centrifuged at 800 x g for 3 minutes to pellet cells. The cell pellets were then re-suspended in complete media and sub-cultured in 25 cm^2 culture flasks.

6.3.5. Cryopreservation of cells

Once trypsinised, cell pellets were re-suspended in complete media containing 10 % DMSO, cell suspensions representing each cell line were transferred to cryo-vials at volumes of 1.5 ml per vial. These vials were then stored at -150 °C freezer until further use.

6.3.6. Cell count

The Countess^{TM} automated cell counter from Invitrogen was used for accurate cell counts, using the manufacturer procedures to perform the count.

6.4. Cytotoxicity assays

6.4.1. Preparation of antimicrobial peptide and positive control concentration

After the purchase of the AMPs, the lyophilized AMPs were stored at -20 °C for long-term storage. The AMPs stock solutions were prepared by dissolving the respective AMPs in sterile distilled water (dH$_2$O) since the peptides were soluble in this medium. Various working concentrations of the AMPs for use in the MTT assay were prepared in a two fold serial dilution starting at a concentration of 100 µg/ml going down 25 µg/ml. DMSO (at a concentration of 6 % v/v) was utilized as the positive control for the MTT assay.

6.4.2. Measurement of anti-cancer activity using the MTT assay

The *in-vitro* anti-cancer activity of the AMPs was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay as described by Freimoser *et al*., 1999. Once the various cell lines reached confluency in a 25 cm^2 flask, they were trypsinised and were seeded in a 96 well plate, with each cell lines seeded at 3 x 10^4 cell/well for CHO, HeLa, and HT-29; and 5 x 10^4 cell/well for MCF-7. Each cell line was seeded in triplicate and the cytotoxicity of each peptide toward each cell line was repeated three times. The plates were placed in an incubator at 37 °C in CO$_2$. Upon reaching 80-90 % confluency, various concentrations of the peptides (25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml), were prepared and were made up in either complete DMEM for MCF-7, HeLa and HT-29, or complete Ham F-12 for CHO. A 6 % DMSO was also prepared which served as the positive control

for the experiment. A total of 100 µl of the peptide solution, as well as the positive control, was added to the wells containing the cells. The plates were placed incubated at 37 °C, in 5 % CO_2 for 24 hours. A negative control for this experiment consisting of cells left untreated for 24 hours was also included.

Following treatment with the peptides, the plates were stained with 5 mg/ml MTT tetrazolium dye (20 µl per well) to evaluate the efficacy of the putative AMPs on the cell lines, and the plates were incubated again for three hours in an incubator with 5 % CO_2 at 37 °C. After, the media was removed and dimethyl sulfoxide (DMSO) (100 µl per well) was added to the cells. The plate was again incubated in a shaker at 37 °C, 5 % CO_2 incubator for 10 minutes. The absorbance for all the wells in the plates was determined using a multi-plate reader (Omega® POLARstar BMG Labtech, USA) at 570 nm, 600 nm and 630 nm. The final absorbance of the treated cells was done by subtracting the background absorbance of the multi-well plate at 630 nm and subtract from the 570 nm measurements. The percentage cell viability was calculated using the formulae:

$$\text{Cell viability (\%)} = \frac{(OD_{570} - OD_{630})(Treated.sample)}{(OD_{570} - OD_{630})(Untreated.control)} \times 100$$

Absorbance results were exported into an Excel file, where they were transformed into a percentage, in a process called Normalizing. The IC_{50} (Concentration inhibiting 50% of cancer growth) values of each putative AMP were determined using GraphPad Prism software (GraphPad software, San Diego, CA, USA).

6.3. Results

CHO, HeLa, HT-29 and MCF-7 cells were treated with increasing concentrations (25, 50, 75 and 100 µg/ml) of the AMPs (Molecules 1, 1.1, 3, 7, 8, 8.1 and 10) to determine the effective concentration that inhibits 50 % of the cell growth (EC_{50}) and to evaluate the toxicity of the peptides towards mammalian cells.

All 7 peptides induced a dose dependent decrease in the viability of all four-cell lines tested in this study. However, only a moderate decrease in cell viability was observed for most of the peptides. This study could only determine the EC_{50} for Molecule 1 and Molecule 8, since these were the only peptides that inhibited 50 % of cell growth in at least one of the cell lines. Figure 6.1 (A) shows that the treatment of HeLa cells with 25, 50, 75 and 100 µg/ml of Molecule 1 resulted in cell toxicity of 10 %, 41 %, 44 % and 51 %, respectively. Figure 6.1 (E) shows that the treatment of HeLa cells with 25, 50, 75 and 100 µg/ml of Molecule 8 resulted in cell toxicity of 22 %, 28 %, 40 % and 48 % respectively. Moreover, treatment of HeLa with Molecule 1.1 with 25, 50, 75 and 100 µg/ml peptide was also able to inhibit 5 %, 37 %, 39 % and 42 % of HeLa respectively. However, Molecule 1, Molecule 1.1 and Molecule 8 did not significantly affect the viability of the other cell lines (CHO, HT-29 and MCF7) since the viability of the cells varied between 90 % at the lowest dose and 80 % at the highest dose (Figure 6.1 (C), Figure 6.1 (D), Figure 6.1 (F) and Figure 6.1 (G)).

(A)

(B)

(C)

(D)

(E)

(F)

(G)

CHO
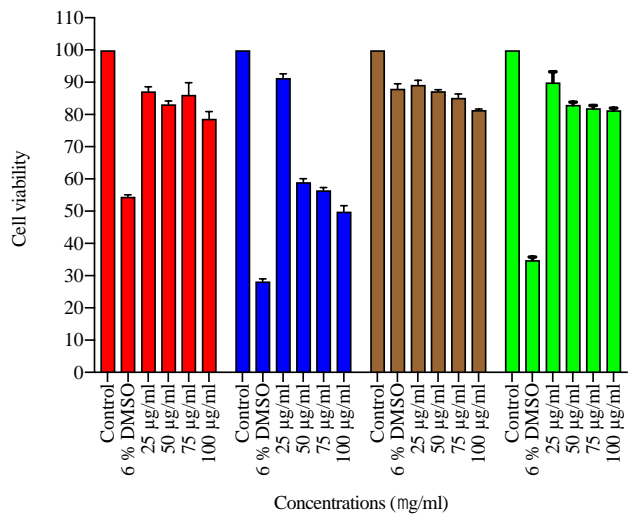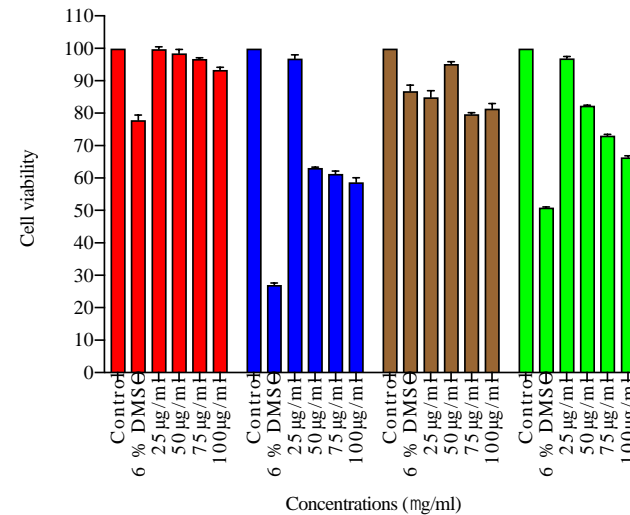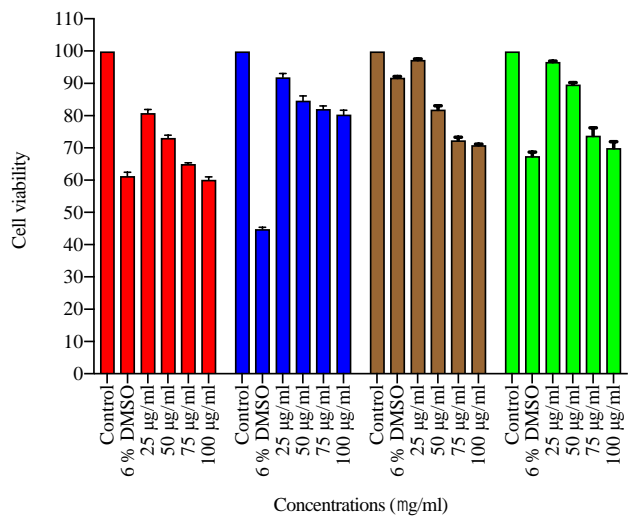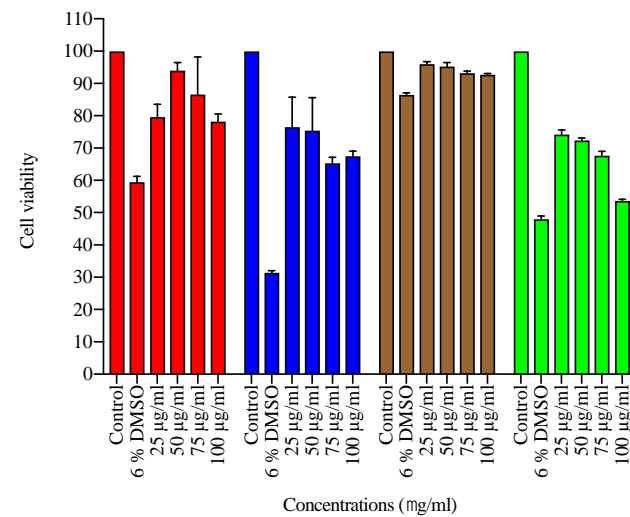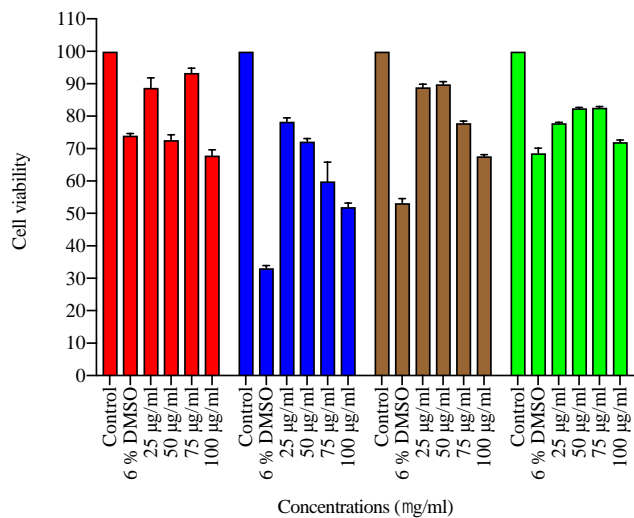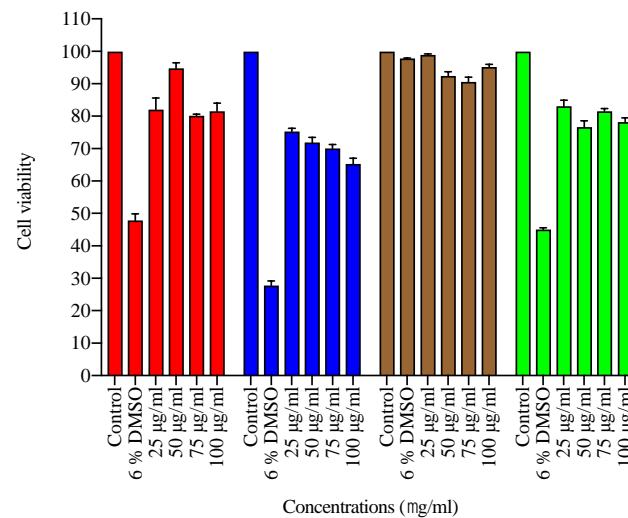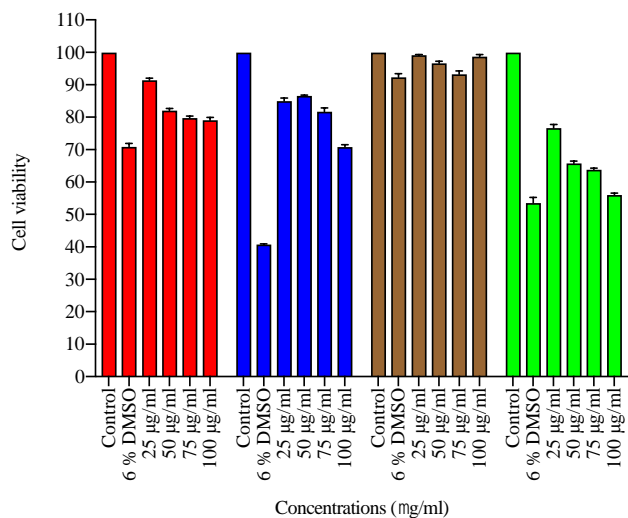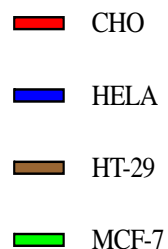
HELA

HT-29

MCF-7

Figure 6.1: The effect of the putative AMPs on CHO, HeLa, HT-29 and MCF-7 cell lines after dose treatment, detected by the MTT assay. The error bars represent the standard deviation (± SD) of triplicates. (A): Molecule 1; (B): Molecule 1.1; (C): Molecule 3; (D): Molecule 7; (E): Molecule 8; (F): Molecule 10; (G): Molecule 8.1

The analysis of the cancer cell lines CHO, HeLa, HT-29 and MCF-7 treatment with 100 µg/ml of the various putative AMPs (Molecules 1, 1.1, 3, 7, 8, 8.1 and 10) enabled the determination of the effective concentration that could cause the inhibition of 50 % of cell growth (EC_{50}) of certain putative AMPs since it was impossible to determine the EC_{50} at the lowest peptide concentrations. This concentration also enables the evaluation of the peptides toxicity towards mammalian cells.

The decrease in cell viability was observed for most of the cancer cell lines at the fix peptide concentration of 100 µg/ml, for all the AMPs (Molecules 1, 1.1, 3, 7, 8, 8.1 and 10). As such, it was possible to obtained as much as 20 % cells inhibition across the 4 cancer cell lines (CHO, HeLa, HT-29 and MCF-7) for Molecule 1, Molecule 3, Molecule 8 with the dose of 100 µg/ml AMPs. Nonetheless, Molecule 7, Molecule 10, Molecule 1.1 and Molecule 8.1 at 100 µg/ml AMPs concentration could only inhibit the CHO, HeLa and MCF-7 cell lines with 20 % toxicity (Figure 6.2). However, observation showed that the treatment of HeLa and CHO with 100 µg/ml of all the AMPs resulted in cell toxicity of 20 % of these cancer cell lines (Figure 6.2), thus the AMP could be considered as moderately cytotoxic for the various cancer cell lines.
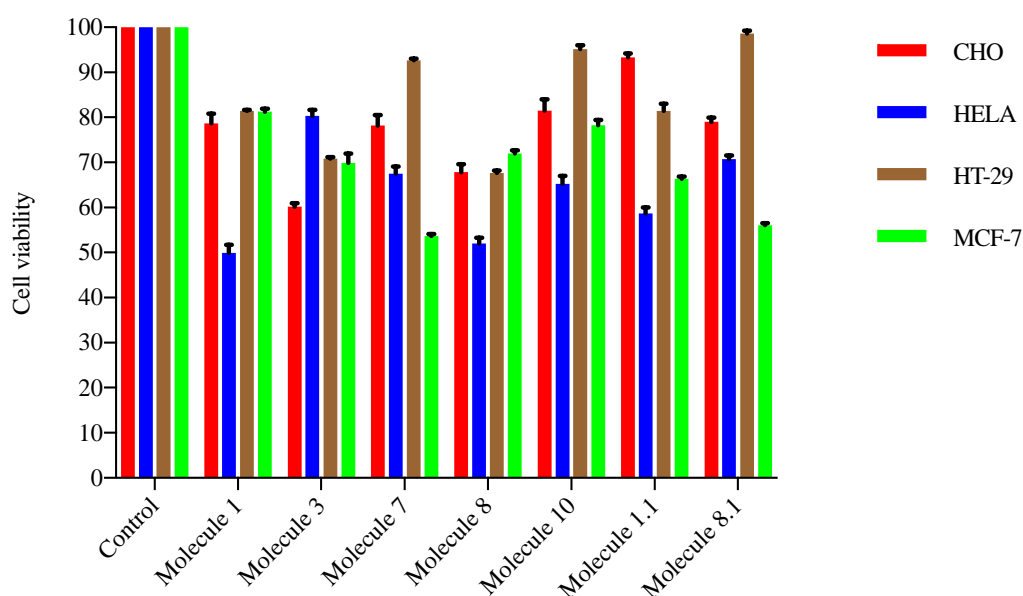
Figure 6.2: The toxicity effect of all the putative AMPs against CHO, HeLa, HT-29 and MCF-7 cell lines, after 100 µg/ml treatment, during the MTT assay. The error bars represent the standard deviation (± SD) of triplicates.

6.4. Discussion

The exploration of additional and new medications to combat many diseases, especially cancer is the ultimate goal of most research facilities and/or pharmaceutics companies around the world. Whilst this journey is a time-consuming process, demands a lot of funds, and sometimes end up being rejected at the clinical trial stages, due to numerous side effects, it becomes imperative that the lead molecule utilized for the development of these drugs are properly designed towards a specific disease receptor, that when the lead compound binds to this receptor, it should influence the progression of the disease. Nevertheless, some lead compounds have been proven to have additional activities and/or functions different from the initial function they were designed to execute (Pan *et al.*, 2009; Tharntada *et al.*, 2009; Shang *et al.*, 2009; Wang *et al.*, 2012), therefore prompting our evaluation of the potential anti-cancer activity of the putative AMPs.

The AMPs designed in this study were developed for applications in the treatment of HIV/AIDS. Specifically, the peptides were selected based on their capacity to bind the HIV gp120 receptor and thus prevent the binding of the HIV to CD4 cell surface receptor on human T cells, macrophages/monocytes, and dendritic cells. The biological activity of these putative AMPs was demonstrated to inhibit HIV pseudotype NL4-3 with EC_{50} varying between of 37.5 µg/ml and 93.75 µg/ml for Molecule 7 and Molecule 8 respectively (Tincho *et al.*, 2016). Ideally these AMPs should not be toxic to mammalian cells if the AMPs were going to be used as a therapeutic agent, which block the interaction between HIV gp120 and CD4. It was therefore also important to investigate the general toxicity of the AMPs to mammalian cells. It was already shown that these peptides at a concentration of ± 150 µg/ml could only cause around 80 % toxicity in normal human T cells (Tincho *et al.*, 2016) and should therefore not be toxic to normal human T cells at the EC_{50} of between 37.5 µg/ml and 93.75 µg/ml for Molecule 7 and Molecule 8 respectively.

In the current study, the cytotoxic effects of these peptides (Molecules 1, 3, 7, 8, 10, 1.1 and 8.1) were investigated on other mammalian cells (CHO, HeLa, HT-29 and MCF-7). Three of these cell lines are human cancer cell lines and one is a non-cancerous murine cell line. In general, the results show that the peptides are not cytotoxic to the four cell lines tested. Molecule 1, Molecule 1.1 and 8 were the most

cytotoxic peptides and the Hela cell line was more susceptible to the cytotoxic effects of the peptides compared to the other cell lines. The EC_{50} for Molecule 1, Molecule 1.1 and Molecule 8 was determined to be around 100 µg/ml in Hela cells. The reason of having different cell viability across the cancer cell lines used could be due to the fact that the cancer cell lines are all from different anatomical regions of the body. Moreover, the cancer cell lines might have been subjected to genetic mutations and have different gene expression patterns. Additionally, the differential responses of the treatments and the toxicity of Molecule 1, Molecule 1.1 and Molecule 8 could be due to the hydrophobic nature of these peptides that is within the recommended range (Giuliani *et al*., 2007), the presence of Cysteine amino acid residues and their positive charges (Table 2.3 and Table 2.4). These characteristics are major factors that favour the toxicity of AMPs, mostly the presence of Cysteine residue that aid in the peptide folding and avoid proteolysis of the peptides (Scott *et al*., 2008; Wang *et al*., 2010; Wang *et al*., 2011).

In general, Hela cells were more susceptible to the effects of the peptides and the non-toxicity of the other peptides could be explained by the reasons stated above. This study provides some preliminary data that suggests that the AMPs (in particular Molecules 1.1, 3, 7, 8.1 and 10) will not have significant cytotoxic effects on human cells. Nevertheless, the current study could have some limitations since the cancer cell lines might have been subjected to mutations after repeated cycles of growing the cell lines, hence, changing the cancer cell lines original genetic material. Therefore, the peptides ought to be performed on non-cancerous cell lines and animal studies needs to be carried out to study the real toxic effects of the peptides.

The intention of these experiments was also to highlight the possibility that these peptides may have additional biological activity other than the one that it was designed to perform, that is anti-HIV activity. It is possible for a drug to have multiple biological activities. For example, Some AMPs used to develop cancer drugs or that have been shown to have cancer activities, were later found to have anti-bacterial activities (Pan *et al*., 2007; Pan *et al*., 2009; Tharntada *et al*., 2009; Shang *et al*., 2009; Wang *et al*., 2012). Similarly, the Kn2-7 AMP that developed as anti-HIV agent also showed anti-bacterial activity (Chen *et al*., 2011; Cao *et al*., 2012). The fact that some of the AMPs and in particular Molecule 1, Molecule 1.1 and Molecule 8

show cytotoxic effects in Hela cells suggest that these peptides may also be investigated further for anticancer activities.

6.5. Conclusion

The path for the development of potent therapeutic molecules to neutralize cancer propagation is still a long journey to complete. Such result could be achieved if theoretical measures are applied in the conception of a lead molecule, which will serve this purpose. This will demand the design of compounds that are directed toward a cancer specific receptor with interaction of the ligand (lead compound) with the cancer receptor affecting its progression and/or prevent cancer cell proliferation. Whilst the aim of this chapter was to evaluate the possible anti-cancer activity of these anti-HIV AMPs, modest toxicity was only observed for Molecules 1, 1.1 and 8 and most of the AMPs were not toxic to CHO, HeLa, HT-29 and MCF-7 cancer cell lines and should therefore be safe to use as a anti-HIV treatment. Though the peptides were particularly designed to bind HIV gp120 protein and stop HIV infection, the cancer toxicity result might seem negative due of its initial design. However, an AMP might have activity against a specific target, the same AMP could have a different activity against many pathogenic organisms. Thus, the second activity displayed by this AMP does not discredit the main activity for which the AMP was designed to display, but could also show additional activity of the same AMP.

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSION

7.1. Introduction

The scientific community has embarked on a journey for the development of proper therapeutic molecules to fight various diseases. To this end, scientific research has facilitated the development of many drugs that could combat HIV at various stages of the virus lifecycle, from the binding of the virus to human cells, fusion of DNA and production of viral proteins (Pang *et al.*, 2009; Hare *et al.*, 2010; Volberding and Deeks, 2010). Though used in an individual treatment regimens, these therapeutic molecules could however, be more effective if they are to be used in combination with other molecules and this method of therapeutic regimens has helped reduce HIV replication.

Even though the combined therapies are effective, individual molecules still need to be developed. A well-suited example of such an anti-HIV drug would be a molecule that would prohibit viral entrance into the human cells and to reduce reservoir pockets in an infected patient. This new class of HAART was termed entry inhibitors or fusion inhibitors due to their action. Enfuvirtide is the only anti-HIV peptide-based drug of this class of HAART, which has received approval from the FDA (Dwyer *et al.*, 2007). Although other peptide-based drugs are either FDA approved (Kilby *et al.*, 2002) or are under clinical trials (Dwyer *et al.*, 2007), additional AMPs should be screened for to develop additional potent anti-HIV entry inhibitors to stop HIV ability to enter cells and help formulate new therapeutic regimens.

In the quest to search for novel therapeutic compounds, previous work has enabled the design of putative AMPs which could block the attachment of the HI Virus to the T cells, macrophages, monocytes by preventing HIV gp120 protein binding to CD4 surface protein (Tincho *et al.*, 2016). Thus the current project is aimed to confirm the activity of the previously identified peptide against HIV pseudotypes, after optimising the performance of the parental AMPs using *in-silico* site-directed mutagenesis, so as

to increase the binding affinity of the mutated AMPs to the HIV gp120 target. Furthermore, the anti-HIV activity and additional biological functions of these peptides were examined using various molecular methods.

7.2. *In-silico* optimisation of putative anti-HIV peptides via side-directed mutagenesis (Chapter two)

Whilst the need to design additional compounds for the development of HIV regimens are imperative; these compounds could be optimised so as to increase their biological function and make these compounds more specific and accurate towards their target molecule(s). The parental peptides identified to bind HIV gp120 protein, at the area where gp120 interacts with CD4 of T cells, macrophages and monocytes were subjected to site-directed mutations after "hotspot" residues for these peptides were determined. Knowing that these "hotspot" residues are very crucial to maintain the ligand on the receptor, discrete site-directed mutagenesis were performed on "non-hotspot" residues so that the mutated AMPs position on the HIV gp120 protein will not change significantly during the interaction process (Darnell, *et al.*, 2007; Zhu and Mitchell, 2011).

The inclusion of different R-groups in the respective AMPs during the site-directed mutagenesis process showed that the physicochemical properties of the mutated AMPs did not change significantly except for Molecule 7.1 and Molecule 8.1 which net positive charge increased to a + 1 thus affecting their hydrophobic ratio (Table 2.3 and Table 2.4). Furthermore, the substitution of a phenylalanine residue to that of a tryptophan amino acid residue allowed the introduction of a α-helical conformation to the mutated AMP, Molecule 1.1 (Figure 2.1). The same change was observed for Molecule 8.1, where the substitution of phenylalanine to histidine residue added a β-sheet to the mutated peptide (Figure 2.2). These conformational changes had a dramatic effect on the AMPs interaction with the HIV gp120 protein. Hence, only Molecule 1.1 and Molecule 8.1 were able to bind HIV gp120, at the point where HIV gp120 interacts with CD4 of human cells demonstrated using *in-silico* docking studies (Figure 2.5 and Figure 2.8). The binding score of gp120 bound to Molecule 1.1 was diminished as compared to the binding score of gp120 bound to Molecule 1. Conversely, the binding score of gp120 bound to Molecule 8.1 increased as compared to the binding of gp120 to Molecule 8 (Figure 2.6). Despite the opposite results on the

binding score of the mutated peptides, only these two AMPs will be included in the list of peptides to be tested for anti-HIV activity. It should be noted that the result obtained in Figure 2.6 was later confirmed during the anti-HIV activity screening process (Figure 4.1).

7.3. Binding capacity of selected putative anti-HIV AMPs to HIV protein gp120 (Chapter three)

This chapter was aimed at expressing HIV gp120 protein and demonstrate that identified putative AMPs and their mutated counterparts could bind to recombinant HIV surface protein gp120. The first part was carried out utilising an *in-silico* method for the design and optimisation of the gene responsible for expressing the HIV gp120 protein. The success of using this technique was justified by the expression of the fusion protein, with its size corresponding to that of the expected fusion protein size (Figure 3.5 to Figure 3.12).

Whilst the purification of the fusion protein seems problematic, it could not be attributed to the method utilised to design the construct, but this failure could rather be explained by the fact that the host utilised to express this protein was not able to express the full protein. The phenotype of the bacterium did not allow for post-translational modification, thus, the host could not express the sugar moieties that make up the protein backbone. The presence of N-Linked glycosylation sites are responsible for keeping the protein in its native conformation (Matthews *et al.*, 1987; Leonard *et al.*, 1990), hence the loss of these sugar moieties could be the elements responsible for destabilizing the protein structure, causing its cleavage hence preventing purification. The use of detergents to solubilise the protein could not enable the purification of this protein because of the host utilised, hence the inability to obtaining a pure recombinant protein. Nevertheless, the deglycosylation of HIV gp120 protein has not been affected by the ability of the protein to bind CD4 of T cells, macrophages and monocytes (Matthews *et al.*, 1987; Leonard *et al.*, 1990). However, this evidence was in opposition with other research, which proved that the sugar moieties are essential for the proper binding of gp120 and CD4 surface molecule (Li *et al.*, 1993).

Despite the problems encountered during the protein purifucation, a commercially available recombinant gp120 protein was utilised to conduct the binding study, to

determine if the putative AMPs could compete with gp120 for its interaction with the CD4 surface protein. The work carried out in this chapter was able to generate reasonable answers to demonstrate that certain peptides could block the interaction of gp120 and CD4 by utilizing a lateral flow platform. Results showed that this blocking was possible only when CD4 was on the membrane and gp120 were on the conjugate side (Figure 3.14 and Figure 3.15). Therefore, results illustrated in Figure 3.17 proved that the peptides were able to prevent gp120 protein binding to CD4 protein, demonstrating that these peptides could be used as potent entry inhibitors to stop HIV spreading to healthy cells, even in the case of an infected individual.

Although the results seem to be inconsistent when the peptides were conjugated, the two peptides (Molecule 1 and Molecule 7), which were conjugated to gold nano-particles successfully, proved to still prevent the binding of gp120 protein to CD4 surface molecule, hence showing conclusive competitive binding of Molecule 1 and Molecule 7. These two peptides could exhibit excellent biological functions, to prevent the entry of HIV into human T cells, macrophages and monocytes. However considerable effort ought to be undertaken for the remaining five peptides to produce a proper conjugated peptide, either by looking at the buffer or at the stability of the peptides. Besides the conjugate problem encountered in this chapter, the strength of the interaction could not be measured and the results interpretation has to rely strongly on the signal generated during the interaction using a lateral flow platform. Nevertheless, other binding techniques such as SPR, ITC, CD and BLI could be used to confirm the current results and to measure the strength of the interaction.

7.4. Anti-HIV activity of putative antimicrobial peptides (Chapter four)

Demonstrating that the AMPs interact with the CD4 protein and prevent the binding of HIV gp120 to CD4 was the first step to show that these peptides could exhibit excellent biological functions, to prevent the entry of HIV into the human T cells, macrophages and monocytes. This however would only remain speculation unless the activity of the putative anti-HIV is examined and proven. In this regard, the preliminary screening of the putative AMPs against HIV-1 NL4-3 showed that these AMPs have anti-HIV activity. Further dosage response experiments demonstrated that the inhibition of the virus increased when the amount (concentration) of the peptide was also increased during the test, proving that the activity of the peptides functions

in a dose-dependent manner. Molecule 7, Molecule 8 and Molecule 8.1 showed potent anti-HIV activity using a dose-response, which was used to establish the EC_{50} of Molecule 7 and Molecule 8, which were found to be 37.5 µg/ml and 93.75 µg/ml respectively (Figure 4.2). Unfortunately, the EC_{50} of Molecule 8.1 was not determined since the concentration of 12.5 µg/ml could not inhibit 50 % of the viral cells hence the concentration of the peptide ought to be increased so that 50 % inhibition can be achieved.

Although not all the putative AMPs have been shown to possess anti-HIV activity as extrapolated from the *in-silico* method, that the peptides binds to gp120, where gp120 interacts with CD4 protein of T cells, macrophages and monocytes. The three AMPs with anti-HIV activity could prove to be the solution to preventing the interaction of gp120 and CD4, thus preventing the infection of healthy cells; and ultimately stop the replication of the virus in an infected individual.

Observations during the HIV testing showed that Molecule 1.1 activity was reduced as compared to the parental peptide Molecule 1, whilst an increase in activity for Molecule 8.1 was shown as compared to its parental peptide Molecule 8 (Figure 4.1). This result was corroborated by the *in-silico* method, utilised for the peptide optimisation, in which the binding score of gp120's interaction with Molecule 1.1 was reduced as compared to the binding of gp120 to Molecule 1; and the binding score of gp120 interaction with Molecule 8.1 was increased as compared to binding of gp120 to Molecule 8 (Table 2.6). These observations provides justification for the use of computational biology and bioinformatics as a key component for the evolution of molecular biology and structural biology to ease the workload and speed the results outcome to find solutions to the problems facing our humanity especially in the health sector.

7.5. Anti-bacterial and anti-cancer activities of putative antimicrobial peptides (Chapter five and Chapter six)

Whilst it will be reasonable for the AMPs to only exhibit anti-HIV activity since the peptides were designed to interfere with the interaction of gp120 with CD4 protein, and prevent possible entrance of HIV to the human cells (Tincho *et al.*, 2016), one would thus expect that these peptides should not have any other biological activities. Nevertheless, their anti-HIV activity does not mean that these putative AMPs would

exclusively harbour anti-HIV activity since evidences have shown that AMPs could possess multiple biological functions (Pan *et al*., 2007; Pan *et al*., 2009; Tharntada *et al*., 2009; Shang *et al*., 2009; Chen *et al*., 2011; Cao *et al*., 2012; Wang *et al*., 2012), thus the reason for looking for other biological activities that could be exhibited by these peptides. Seeking additional biological functions of these peptides could also be justified by the rising problem of drug resistance, which call for the search of new lead compounds that can serve as the backbone for the design of potent drugs. This chapter attempted to evaluate the possible anti-bacterial and anti-cancer activity these putative peptides may display.

The microtiter dilution method utilised to evaluate the anti-bacterial activity proved that Molecule 3 and Molecule 7 completely inhibit the growth of *P. aeruginosa,* at the lowest AMP concentration of 0.03125 mg/ml (31.25 µg/ml) (Figure 5.2 and Figure 5.3). However, less considerable inhibitions were observed when methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) were treated with the peptides. While this result might be minimised in view of the number of peptides that were tested, it should be noted that *S. aureus* and *P. aeruginosa* are infectious microbes, which have become resistant to many conventional antibiotics (Cornelis, 2008) and are mostly opportunistic microbes to pathologies such as AIDS.

Although the cytotoxicity of the putative AMPs to CHO, MCF-7, HeLa and HT-29 was not significant, the lack of anti-cancer activity could be justified by the specificity of the designed peptides. Nevertheless, the additional anti-bacterial activity proves that an AMP could display a secondary activity even though it was devised for a principal purpose.

7.7. Conclusion

The purpose of this project was to establish the anti-HIV activity of five putative AMPs, designed through machine learning and computational prediction. Whilst the computational site-directed mutagenesis has been achieved successfully selected peptides were tested after confirming that these peptides could still bind HIV gp120 molecule, at the point where the protein interacts with the human cells surface molecule CD4. Conclusive results demonstrated that out of the five parental AMPs, only two peptides showed potent anti-HIV activity. Moreover, the mutated AMP,

Molecule 1.1 that has a low binding affinity with gp120 protein showed lower anti-HIV activity than that of the parental AMP, Molecule 1. The same correlative result was observed for Molecule 8.1, which showed better anti-HIV activity than the parental peptide, Molecule 8, prompting the need for more mutations within these AMPs to increase HIV activity and screen for a more potent anti-HIV compound.

Furthermore, the question of exclusive anti-HIV activity was raised since these peptides were conceptualised and designed on a particular receptor, hence the need to test for other biological activities for these AMPs. It has been demonstrated that a particular peptide with a specific activity could exhibit an additional activity or function, different from the one it was intended to execute (Pan *et al.*, 2009; Tharntada *et al.*, 2009; Shang *et al.*, 2009; Chen *et al.*, 2011; Cao *et al.*, 2012; Wang *et al.*, 2012). This concept was proven when Molecule 3 and Molecule 7 were able to inhibit the growth of *P. aeruginosa* after 24 hours of treatment, indicating that these AMPs have additional anti-*Pseudomonas aeruginosa* activity, though low or non-susceptive activity was observed for both MSSA and MRSA strains in the study. However, considerable toxicity was not observed when cancer cell lines treated for 24 hours with these peptides. However, the ability of the AMPs to bind gp120 at the area where this protein interacts with CD4 surface protein still ought to be demonstrated via molecular techniques, to exclusively say that the concept and the design of these peptides were successfully executed.

7.8. Future work

The work accomplished to date for this project needs further experimentation so that the full function of these putative AMPs is demonstrated. Thus, the future work will include:

- ➢ Introduce further *in-silico* site-directed mutagenesis on these putative anti-HIV AMPs, to optimise and increase their binding potential to prevent HIV-1 gp120 protein interaction to CD4 surface protein.
- ➢ This simulation would be followed by an *in-vitro* validation of the anti-HIV activity of the mutated AMPs.
- ➢ Furthermore, the CC_{50} of all AMPs should be determined and their individual Therapeutic Index or Selective Index should be derived.

- The broad anti-HIV activity of these AMPs will be determined by carrying out anti-HIV testing on different HIV-1 pseudotyped viruses.
- Additionally, the mechanism of action of these AMPs will be established and will help to determine the application of the molecules, either as preventive/prophylactic drugs or therapeutic drugs or gels/films.
- Finally, the complex formed between gp120 and anti-HIV AMPs will be solved using structural biology, to validate the observations made by the *in-silico* binding study.

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neutralization epitope on HIV-1 gp120. *Nature*, **445** (7129): 732-737.

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APPENDIX A

Supplementary materials for Chapter TWO

>Molecule 1:

CLRYKKPECQSDWQCPGKKRCCPDTCGIKCLDPVDTPNPTRRKPGKCPVTYG
QCLMLNPPNFCEMDGQCKRDLKCCMGM

>Molecule 3:

RWKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK

>Molecule 7:

RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK

>Molecule 8:

CLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

>Molecule 10:

WNPFKELEKAGQRVRDAIISAKPAVDVVGQATAIIK

Table A.1: The parental sequences of the anti-HIV AMPs sequence obtained after the initial docking of the HIV gp120 protein and the putative AMPs, published in Tincho *et al.*, 2016.

>Mutated Molecule 1 or Molecule 1.1: F62W

CLRYKKPECQSDWQCPGKKRCCPDTCGIKCLDPVDTPNPTRRKPGKCPVTYG
QCLMLNPPNWCEMDGQCKRDLKCCMGM

>Mutated Molecule 3 or Molecule 3.1: V$_{28}$L

RWKLFKKIEKVGRNVRDGLIKAGPAIALIGQAKSLGK

>Mutated Molecule 7 or Molecule 7.1: W$_2$H

RHKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK

>Mutated AMP 8 or Molecule 8.1: F12H

CLKSGAICHPVHCPRRYKQIGTCGLPGTKCCKKP

>Mutated Molecule 10 or Molecule 10.1: V$_{25}$L

WNPFKELEKAGQRVRDAIISAKPALDVVGQATAIIK

Table A.2: Mutated AMPs sequence obtained after site-directed mutagenesis of parental anti-HIV AMPs, and the position of amino acid residues mutation.

Molecule 3   Molecule 7   Molecule 10
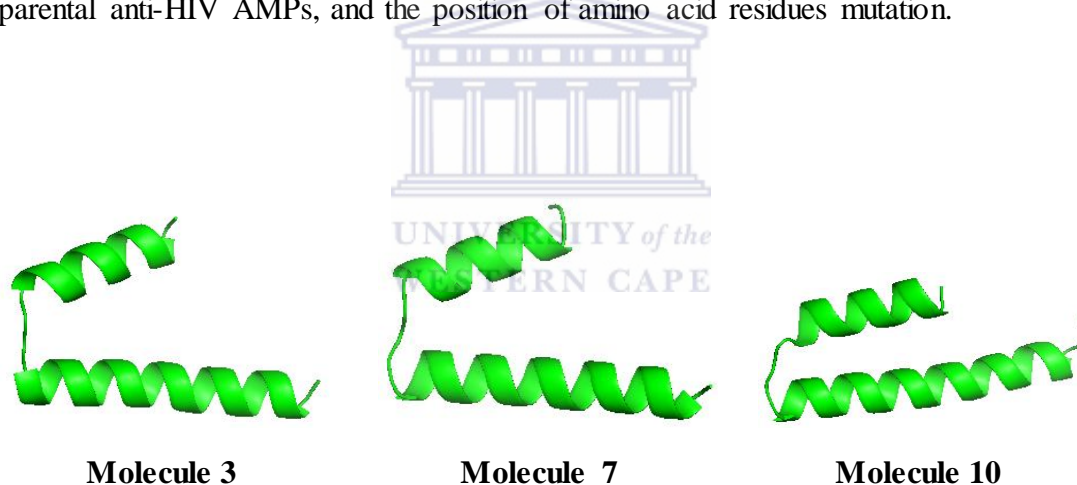
Figure A.1: The 3-D structures of the three alpha-helical parental anti-HIV AMPs Molecule 3, Molecule 7 and Molecule 10 predicted by I-TASSER server and represented in cartoon representation by PyMOL 1.3. Software

Figure A.2: gp120-Molecule 1 complex formation during anti-HIV-gp120 interaction. The cartoon representation in green colour is the HIV protein gp120 and the putative anti-HIV AMP (Molecule 1) is represented in light blue colour. T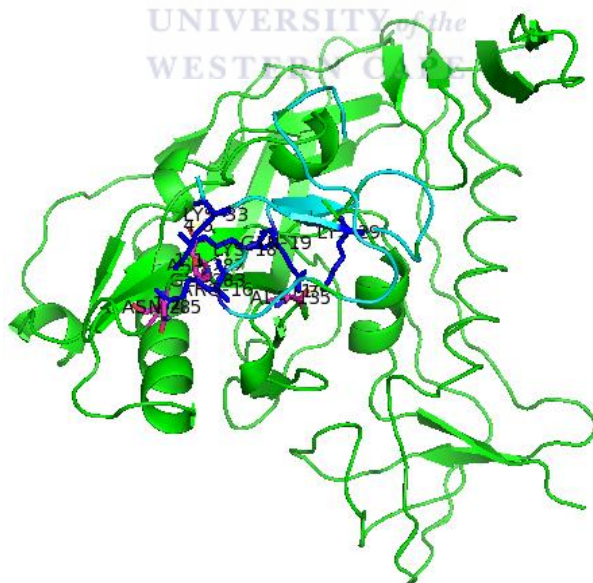he purple colour represents the stick representation of gp120 amino acids interacting with Molecule 1 amino acid stick representation in dark blue. Each amino acid is labelled with the position of their amino acid.

Figure A.3: gp120-Molecule 3 complex formation during anti-HIV-gp120 interaction. The cartoon representation in green colour is the HIV protein gp120 and the putative anti-HIV AMP (Molecule 3) is represented in light blue colour. The purple colour represents the stick representation of gp120 amino acids interacting with Molecule 3 amino acid stick representation in dark blue. Each amino acid is labelled with the position of their amino acid.

Figure A.4: gp120-Molecule 7 complex formation during anti-HIV-gp120 interaction. The cartoon representation in green colour is the HIV protein gp120 and the putative anti-HIV AMP (Molecule 7) is represented in light blue colour. The purple colour represents the stick representation of gp120 amino acids interacting with Molecule 7 amino acid stick representation in dark blue. Each amino acid is labelled with the position of their amino acid.
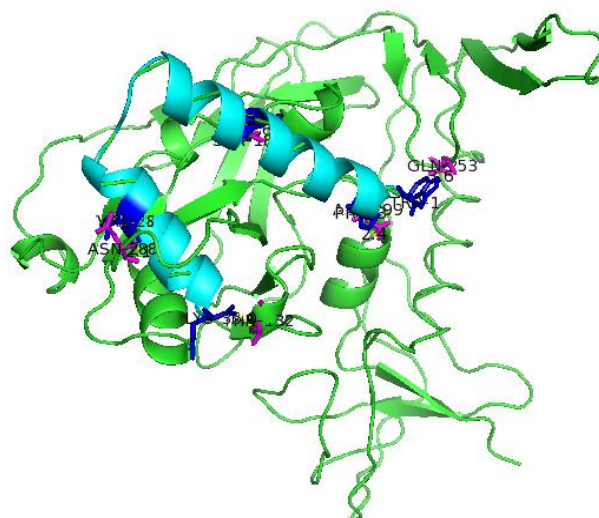

Figure A.5: gp120-Molecule 8 complex formation during anti-HIV-gp120 interaction. The cartoon representation in green colour is the HIV protein gp120 and the putative anti-HIV AMP (Molecule 8) is represented in light blue colour. The purple colour represents the stick representation of gp120 amino acids interacting with Molecule 8 amino acid stick representation in dark blue. Each amino acid is labelled with the position of their amino acid.

Figure A.6: gp120-Molecule 10 complex formation during anti-HIV-gp120 interaction. The cartoon representation in green colour is the HIV protein gp120 and the putative anti-HIV AMP (Molecule 10) is represented in light blue colour. The purple colour represents the stick representation of gp120 amino acids interacting with Molecule 10 amino acid stick representation in dark blue. Each amino acid is labelled with the position of their amino acid.

Table A.2: The area cover and the ACE's from the docking the gp120-putative-anti-HIV AMP using the PatchDock docking server. These interactions are the results generated from the previous work (Tincho *et al.*, 2016).

	gp120		
	Area (Å^2)	ACE	Transformation coordinates
Molecule 1	2433.90	281.93	-0.94 0.61 -2.68 14.27 19.05 2.11
Molecule 3	1926.00	410.28	-1.31 0.66 1.01 -5.19 -9.03 -16.06
Molecule 7	1906.00	295.00	2.31 1.12 2.99 8.08 -27.26 11.81
Molecule 8	1564.90	71.02	-1.23 0.07 1.76 9.50 5.77 -10.61
Molecule 10	1916.20	-34.06	-2.33 0.66 -2.29 9.00 14.84 -0.33

APPENDIX B

Supplementary materials for Chapter THREE

Table B.1: Chemical/Reagents and suppliers

Material	**Supplier**
Acetic acid	Merck
40 % 37.5:1 Acrylamide:bis-acrylamide	Promega
Agarose	Promega
Ampicillin	Sigma
Ammonium Persulfate (APS)	Sigma
Bacteriological agar	Merck
Boric acid	Merck
Bromophenol blue	Sigma
Coomassie Brilliant Blue R-250	Sigma
Dithiothreitol (DTT)	Roche
Disodium phosphate	Merck
Ethylene Diamine Tetra-acetic acid (EDTA)	Merck
Ethanol	Merck
Ethidium bromide (EtBr)	Promega
L-reduced Glutathione-S-Transferase	Sigma
Glycerol	Merck
Glycine	Sigma
Isopropanol	Merck
Isopropyl β-D-thiogalactopyranoside (IPTG)	Sigma
Lysosome	Sigma
Methanol	Merck

Monopotassium phosphate	Merck
cOomplete EDTA-free protease inhibitor	Roche
PageRuler™ Unstained Protein Ladder	Fermentas
Potassium chloride	Merck
Sodium acetate	Merck
Sodium Azide	Sigma
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide	Merck
Sodium dodecyl sulphate (SDS)	Merck
Urea	Merck
N, *N*, *N*', *N*'-Tetramethylethylenediamine (TEMED)	Sigma
Tris [hydroxymethyl] aminoethane (Tris)	Merck
Triton X-100 (iso-octylphenoxypoly- ethoxyethanol)	Sigma
Tryptone	Merck
Tween-20 (Polyoxyethylene [20] sorbitan)	Merck
Yeast Extract	Merck

Table B.2: Buffers and Solutions

2 X SDS Sample buffer:

62.5 mM Tris-HCl (pH 6.8), 2 % SDS, 25 % glycerol, 0.01 % bromophenol blue, 5 % β-mercaptoethanol.

10 X TBE

0.9 M Tris, 0.89 M boric acid, 0.032 M EDTA stored at room temperature.

10 X Tris-EDTA (TE)

10 mM Tris-HCl, 1 mM EDTA, pH 7.5

10 X Phosphate-buffered saline (PBS)

150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4.

DNA Loading buffer

0.25 % Bromo-phenol-blue, 0.25 % xylene cyanol and 30 % glycerol

Ampicillin

100 mg/ml ampicillin in distilled water; filter sterilized.

Sodium Chloride-Tris-EDTA/lysozyme (Lysis buffer)

10 mM Tris, pH 8, 150 mM NaCl, 1mM EDTA and 100 µg/ml lysozyme

Ammonium persulphate (APS)

A 10 % stock solution was prepared in deionised water.

Coomassie Brilliant Blue R-250 Staining Solution

0.25 g Coomassie Brilliant Blue R 250, 50 % ethanol and 10 % acetic acid

Cleaning buffer 1:

0.5 M borate buffer, (pH 8.5): 0.5 M NaCl

Cleaning buffer 2:

0.1 M acetate buffer, (pH 4.5): 0.5 M NaCl

Destaining solution

16.5 % ethanol and 5 % acetic acid.

Dithiothreitol (DTT)

A 1 M stock solution was prepared in 0.01 M Sodium acetate, pH 5.2. This solution was sterilized by filtration.

Ethylene diamine tetra acetic acid (EDTA)

A stock solution was prepared at a concentration of 0.5 M in deionised water, pH 8.0.

10 % Sodium dodecyl sulphate (SDS) 10 % SDS in distilled water.

<u>Elution buffer</u>

5 mM reduced glutathione, 50 mM Tris- HCl pH 9.0.

<u>Isopropyl β-D-thiogalactopyranoside (IPTG)</u>

A 1 M stock solution was prepared in deionised water. The solution was sterilised by filtration.

<u>Luria Agar</u>

14 g/l Bacteriological agar, 10 g/l Tryptone, 5 g/l Yeast Extract and 5 g/l NaCl

<u>Luria Broth</u>

10 g/l Bacto-tryptone, 5 g/l Bacto-Yeast Extract and 5 g/l NaCl

<u>Lysozyme</u>

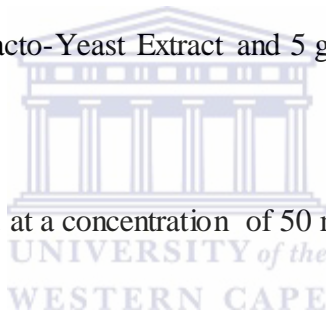A stock solution was prepared at a concentration of 50 mg/ml in deionised water.

<u>PBS-T</u>

150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 1 % Triton-X 100)

<u>Storage buffer</u>

2 M NaCl, 1 mM Sodium Azide

<u>U-Buffer</u>

8 M Urea, 50 mM Tris, 5 mM EDTA, 5 mM DTT and cOmplete EDTA-free protein inhibitor tablet

Table B.3: Equipment and suppliers

Equipment	Suppliers
211DS Shaking Incubator	Labnet
5415D Benchtop Microcentrifuge	Eppendorf
Centrifuge	Beckman Coulter
Tube Roller SRT9D_{SEP}	Stuart
UVP BioSpectrum Imaging System	UVP LLC
Mini-PROTEAN Tetra Cell	BioRad
CanoScan LiDE 120 electronic scanner	Canon

EVVLVNVTENFNMWKNDMVEQMHEDIISLWDQSLKPCVKLTPLCVGAGSC
NTSVITQACPKVSFEPIPIHYCAPAGFAILKCNNKTFNGTGPCTNVSTVQCTHG
IRPVVSSQLLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEINCTGAGHCNIA
RAKWNNTLKQIASKLREQFGNNKTIIFKQSSGGDPEIVTHWFNCGGEFFYCNS
TQLFNSTWFNSTWSTEGSNNTEGSDTITLPCRIKQIINMWQKVGKAMYAPPIS
GQIRCSSNITGLLLTRDGGNSNNESEIFRPGGGDMRDNWRSELYKYKVVKIE

Figure B.1: Protein sequence of HIV gp120, extracted from the complex of PDB ID 2NXZ (2NXZ: A|PDBID|CHAIN|SEQUENCE) deposited in the Protein Database Bank.

APPENDIX C

Supplementary materials for Chapter FOUR

Table C.1: Chemicals/Reagents and Suppliers

Material	Suppliers
Dimethyl Sulphoxide (DMSO)	Sigma
Roswell Park Memorial Institute medium (RPMI)	Lonza
Fetal Calf Serum (FCS)	Merck
Dulbecco's Phosphate Buffer Saline (DPBS)	Lonza
2.5 % Trypsin (10X)	Gibco
PEN-STREP	Lonza
2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrozlium-5-carboxanilide (XTT)	Sigma
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)	Sigma

Table C.2: Buffers and Solutions

RPMI Complete

50 ml Fetal Calf Serum and 5 ml PEN-STREP in 500 ml Roswell Park Memorial Institute Medium (RPMI), mix solution to obtain a homogeneous distribution.

XTT Solution

5 mg/ml XTT: Dissolve 5 mg of lyophilized XTT in 1 ml distilled water and filter sterilize.

MTS Solution

5 mg/ml MTS: Dissolve 5 mg of lyophilized MTS in 1 ml distilled water and filter sterilize.

APPENDIX D

Supplementary materials for Chapter FIVE

Table D.1: Chemicals/Reagents and Suppliers

Material	Suppliers
Tryptone Soya Agar CM0131 (TSA)	Oxoid
Tryptone Soya Broth CM0129 (TSB)	Oxoid
Iodonitrotetrazolium Chloride (INT)	Sigma
Ampicillin	Sigma

Table D.2: Buffers and Solutions

Tryptone Soya Agar (TSA)

40 g/l Tryptone Soya Agar CM0131 was prepared, and it contains a pancreatic digest of casein 15.0 g; enzymatic digest of soya bean 5.0 g; sodium chloride 5.0 g; agar 15.0 g.

Tryptone Soya Agar (TSB)

30 g/l Tryptone Soya Broth CM0129 was prepared, and it contains a pancreatic digest of casein 17.0 g; enzymatic digest of soya bean 3.0 g; sodium chloride 5.0 g; di-potassium hydrogen phosphate 2.5 g; glucose 2.5 g.

Iodonitrotetrazolium Chloride (INT)

A stock solution was prepared at a concentration of 4 mg/ml, by dissolving 4 mg of lyophilized INT with 1 ml distilled water and filter sterilized

Ampicillin

5 mg/ml lyophilized ampicillin in distilled water and filter sterilized.
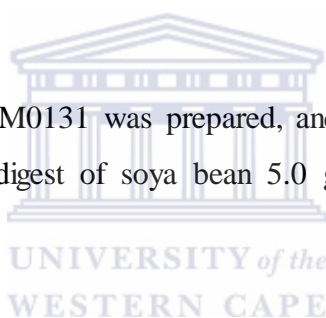
APPENDIX E

Supplementary materials for Chapter SIX

Table E.1: Chemicals/Reagents and Suppliers

Material	Suppliers
Dimethyl Sulphoxide (DMSO)	Sigma-Alrich
Dulbecco's Modified Eagle's medium (DMEM)	Lonza
Fetal Bovine Serum (FBS)	Merck Group
Dulbecco's Phosphate Buffer Saline (DPBS)	Lonza
2.5 % Trypsin (10X)	Gibco
Penicillin-Streptomycin (Pen-Strep)	Lonza
Hams F-12 Nutrient Mixture (Ham-12) 1X	Gibco
3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT)	Sigma-Alrich
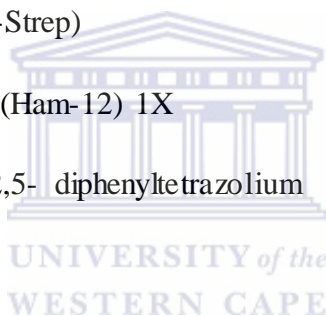
Table E.2: Buffers and Solutions

DMEM Complete

50 ml Fetal Bovine Serum and 5 ml Penicillin-Streptomycin in 500 ml Dulbecco's Modified Eagle's Medium (DMEM), mix reagents to obtain a homogeneous solution.

Ham F-12 Complete

50 ml Fetal Bovine Serum and 5 ml Penicillin-Streptomycin in 500 ml Hams F-12, mix regents to obtain a homogeneous solution.

MTT Solution

5 mg/ml MTT: Dissolve 5 mg of lyophilized MTT in 1 ml distilled water and filter sterilized.