Bioactivity of Metallodrugs

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Date: 11 December 2012

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ACKNOWLEDGEMENTS

I would to express my sincere gratitude to the following people/organisations/institutions for their contribution to the success of this work:

- God who is Father, Son and Holy Spirit for all the strength and courage.
- Prof. D Meyer, my supervisor, for her constant guidance, insightful criticism and motivation.
- The University of Pretoria (Biochemistry Department) for funding and resources.
- The Technology Innovation Agency (TIA) for funding the project.
- University of Johannesburg (Chemistry Department) for all the compound synthesis.
- All the staff at University of Pretoria's Student Health Services and donors for all blood donations.
  .
- My Postgraduate advisory committee, Dr P. Fonteh and Dr. P Burger, for all their feedback and constructive criticism.
- The HIV research team for all the motivation, laughs and companionship.
- My mother and sister for always believing in me and their continued support and prayers.
- My friend, Nothukela Mahambehlala for all the support, prayers and laughs.

PREFACE

A portion of this work was presented as a short talk at the 3rd SA TB conference which took place in June 2012 (ICC Durban). The talk was entitled: Novel metallodrugs inhibiting *Mycobacterium tuberculosis* and HIV. The accepted abstract for this presentation is attached at the end of this dissertation.

SUMMARY

BIOACTIVITY OF METALLODRUGS

By

Ntombenhle H. Gama

Supervisor: **Prof. Debra Meyer**

Department: **Biochemistry**

Degree: **MSc. Biochemistry**

Background: Decades after its discovery, the Human Immunodeficiency Virus-1 (HIV-1) remains a major threat to public health. Highly Active Antiretroviral Therapy (HAART) has been successful in the treatment of the virus; however these drugs are associated with a number of adverse effects often resulting in non-compliance by the patients and increasing an individual's susceptibility to opportunistic infections such as tuberculosis and cervical cancer. It is therefore crucial to continue the development of treatments that are less toxic but still provide significant control of viral infection allowing the immune system crucial recovery time.

Methods: Metals are known to possess medicinal properties and in this study the bioactivity of 12 gold, platinum and palladium based complexes was investigated for the ability to inhibit viral replication and/or selected opportunistic infections. The complexes (containing the metals) were compared to the associated ligands (non-metal containing precursors). Ligands and complexes are collectively called compounds. Direct inhibition of crucial HIV-1 enzymes was assessed and the effects of the compounds on cell proliferation were determined using flow cytometry and real-time cell analysis. The anti-tumour activity of the complexes was then measured on HeLa cells (a cervical cancer cell line) with compound specificity for tumour cell growth inhibition determined using Vero cells (a monkey kidney cell line). The complexes were further tested for their activity against *Mycobacterium tuberculosis* and the minimum inhibitory concentrations (MICs) for each compound determined.

Results: Three of the 12 complexes exhibited activity against HIV-1 protease, with the gold based complex **AE190** resulting in 71% enzyme inhibition at 10µM ($p = 0.002$). None of the compounds inhibited HIV-1 reverse transcriptase and 9 complexes had good chemotherapeutic activity, but low selectivity indices (SI values below 10). Only the bimetallic complex **AE177** was active against HIV-1 integrase. The palladium based complexes **AE188, AE189** and **AE190** had good activity against *M. tuberculosis* with MIC values of 1.56 µM for all three complexes and an SI value of 5.19 for **AE189**.

Conclusion: The metallic complexes presented in this report demonstrate potential for development into improved anti-retroviral therapies and in addition selected complexes demonstrated dual activity (not seen in HAART); where a single complex was active against two different targets in the HIV-1 life cycle (**AE177**) or against two separate infections (**AE190** active against HIV and *M. tuberculosis*). The low selectivity of a drug does not necessarily prevent its clinical use (e.g. cisplatin) but in this case, low selectivity will be used to recommend modified synthesis for improved activity. The ligands were universally inactive demonstrating the importance of the metal in the observed biological activity.

TABLE OF CONTENTS

LIST OF FIGURES

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LIST OF TABLES

IMPORTANT ABBREVIATIONS AND DEFINITIONS

ABST= 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADC= AIDS-defining cancers
AIDS= Acquired Immune Deficiency Syndrome
ART= Antiretroviral Therapy
CFSE= Carboxylflourescein diacetate succinimidyl ester
CNS=Central nervous system
Complex= The ligand with attached metal ion(s).
Compound= The ligand with no attached metal ion
CRT= Chemoradiation therapy
DNA= Deoxyribonucleic Acid
DS-DNA= Donor Substrate DNA
EI= Entry Inhibitors
HAART= Highly Active Antiretroviral Therapy
HIV= Human Immunodeficiency Virus
HPV= Human papilloma virus
IN= Integrase
INH= Isoniazid
INH= Isonicotinic acid hydrazide (Isoniazid)
INI= Integrase Inhibitors
MDR-TB= Multidrug resistant TB
MTS= [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenylo-2-(4-sulfophenyl)-2H-tetrazolium)
MTT= [3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide]
NADC= Non-AIDS defining cancers
NNRTIs= Non-nucleoside Reverse Transcriptase Inhibitors
NRTIs= Nucleoside Reverse Transcriptase Inhibitors
PBMCs= Peripheral Blood Mononuclear Cells
Pgp= P-glycoprotein
PI= Protease Inhibitor
PR= Protease
RNA= Ribonucleic Acid
RT= Reverse Transcriptase
RT-CES= Real Time Cell Electronic Sensor
SD= Standard Deviation
SEM= Standard Error of the Means
TB= Tuberculosis
TS-DNA= Target Substrate DNA
XDR-TB= Extremely drug resistant TB

1. INTRODUCTION

The first reports of an acquired immunodeficiency date back to the 1980s where a group of homosexual men showed symptoms of severe weight loss and fever which were evidence of infection with an opportunistic microorganism. All the patients exhibited depressed cell mediated immunity as well as poor *in vitro* antigen responses [1]. Since then, there has been remarkable development in the study of the acquired immunodeficiency syndrome (AIDS) with the Human Immunodeficiency virus (HIV) proven to be the etiological agent of AIDS. HIV infection remains a major health challenge with currently no cure or vaccine available. A reported 34.2 million people were living with HIV in 2011 and 1.7 million deaths globally were AIDS-related [2]. The virus attacks CD4 T-lymphocytes of the immune system through receptor specificity and there are two basic phases of the infection; the acute and the chronic phases. The acute phase is mainly asymptomatic while it is in the chronic phase that the infection becomes symptomatic. The chronic phase is characterized by rapidly decreasing CD4 and CD8 T-cells and increased viral load, making the patient susceptible to opportunistic infections [3]. Opportunistic infections (OI) include tuberculosis (TB) and cancers such as cervical cancer. In 2011 TB was reported to be the leading cause of mortality in HIV infected individuals [2] and studies have shown that decreased immunity associated with HIV resulted in greater rates of cancers associated with the human papilloma virus (HPV, Oliveira- Cobucci *et al.* 2012).

There have been great advances in the development of treatment against the viral infection. Highly active antiretroviral therapy has been successful in the reduction of AIDS-related mortality rates by lowering viral load and increasing the CD4 cell counts in infected individuals [5]. These drugs are however associated with a number of drawbacks. In a study on the pharmacovigilance of South African adults on HAART, 76.1 % of the patients went through regimen changes from first-line to second-line regimens and this was due to HAART-related toxicity. Major side effects included polyneuropathy, lipodystrophy and lactic acidosis [6]. This drug-related toxicity has also lead to the non-compliance of the patients, resulting in the emergence of resistant strains of the virus. This highlights the need for novel drugs that are less toxic but still effective in infection control.

Not only are there challenges in HIV treatment, but the treatment of associated OIs has also proven to be cumbersome. Over the years there has been the emergence of multi-drug resistant (MDR) and extremely drug resistant (XDR) strains of TB and these have been shown to be most severe in HIV-TB co-infected patients [7], [8]. Cervical cancer treatment using

concomitant chemotherapy and radiation therapy (chemoradiation therapy; CRT) has been shown to improve overall survival rates; however, there have been reports of increased acute toxicity in patients treated with CRT than with radiation therapy alone. In addition to this, there is still a number of patients that are not cured and this is due to tumour resistance [9]. Research therefore needs to evolve from just purely focusing on HIV treatment, to the simultaneous management of the virus and OIs such as TB and cervical cancer as well.

Herein, new metallodrugs were proposed for consideration as treatment that could satisfy the aforementioned need. Metals have shown great medicinal properties, ranging from the treatment of rheumatoid arthritis with gold, the chemotherapeutic properties of cisplatin against cancers such as ovarian cancer and the antimicrobial properties of silver against chronic ulcers [10]. Gold based compound have also been reported to inhibit the growth of malaria parasites (*Plasmodium berghei* and *P. falciparum*) as well as Gram-positive bacteria (*Bacillus subtullis*) [11]. This research group presented *in vitro* evidence for gold complexes able to inhibit HIV directly (Fonteh *et al.* 2009) and indirectly (Fonteh *et al.* 2012). Metal based compounds such as the zinc group metals and oxovanadium complexes have also been reported to possess potent anti-HIV activity (D'Cruz *et al.* 2003; Haraguchi *et al.* 1999; Bowman *et al.* 2008; Sun *et al.* 2007).

In research presented here the bioactivity of twelve gold, platinum and palladium based phosphine complexes were evaluated. The anti-HIV screening focused on the direct inhibition of the three major enzymes that are involved in the viral life cycle; HIV-1 reverse transcriptase (RT), protease (PR) and integrase (IN). None of the metal complexes exhibited RT inhibition; however, an outstanding observation was **AE177** which significantly inhibited the activity of both PR and IN. In addition to this the complex had moderate anti-cancer activity (IC_{50}= 6.16µg/ml; *P*-value= 0.05). The metal complexes were further tested for the inhibition of *Mycobacterium tuberculosis* and three palladium based complexes (**AE188**, **AE189**, **AE190**) showed remarkable activity. Palladium compounds have previously been reported to exhibit exceptional anti-tumour and anti-parasitic activity [11], [16]; however, there have been no reports of palladium based complexes active against *M. tuberculosis*. These results raise the possibility of dual action drugs that target different aspects of the viral infection and are effective against opportunistic infections as well. Indeed this research opens up possibilities of ultimately reducing the number of drugs to be taken especially in cases of co-infection with opportunistic microorganisms. This could contribute to increased drug compliance by patients and markedly reduce the emergence of drug-resistant strains.

The dissertation is outlined as follows; the literature review in the next section provides information on the viral life cycle and an in-depth look at the relationship between HIV and cervical cancer, as well as HIV and TB. Also included is how metallodrugs have contributed to microorganism inhibition and could potentially be beneficial against HIV. The literature review ends by providing a detailed hypothesis and the aims undertaken to achieve the main objective of this research (determination of the bioactivity of metal based complexes against HIV and the aforementioned OIs). The methodologies utilized precede the results and discussion which report on the novel activities of the complexes in relation to other authors' work in this field. Finally the overall conclusion presents the highlights of the work which is then followed by a comprehensive list of references. The appendix provides additional information not deemed part of the primary content of the dissertation and the draft article shows a part of this work already prepared for publication.

2. BACKGROUND AND LITERATURE REVIEW

2.1 Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) is a retrovirus that causes the acquired immune deficiency syndrome (AIDS) and it is transmitted sexually, perinatally or through direct contact with infected blood. HIV-1 exists as different subtypes designated A-K of which prevalence varies geographically. Ninety percent of all individuals living with HIV are infected with HIV-1 and a reported 34.2 million individuals worldwide were infected with the virus in 2011. South Africa is the most affected country with an estimated 5.6 million people living with HIV [2].

2.1.1 Structural Biology

Like all other retroviruses, HIV contains three major structural genes: *gag, pol* and *env,* as well as additional regulatory and accessory genes; *tat, rev, vpr, vpu* and *nef* which regulate viral protein synthesis (**Figure 1**). The overall genome is made up of two identical positive sense single stranded RNA molecules which are each associated with the reverse transcriptase enzyme [17] .

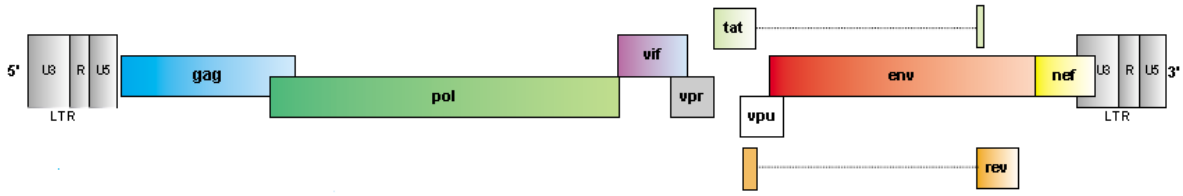
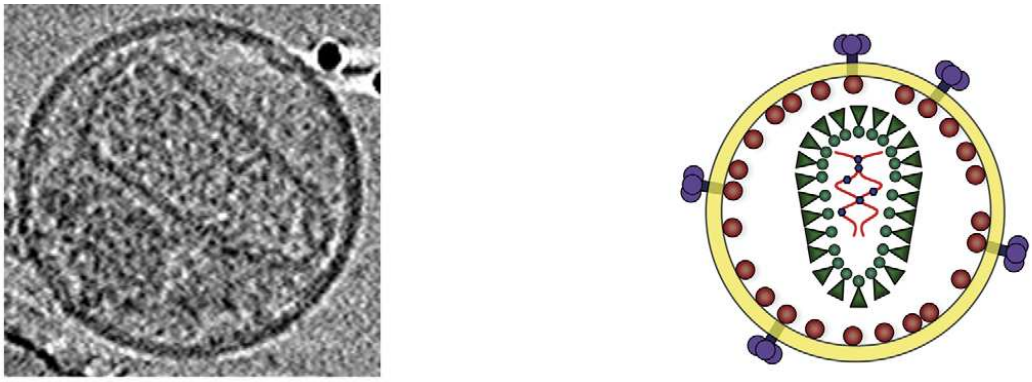


Figure 1: The genome of HIV-1. The whole genome codes for three main components of the virus that is important in its infection, replication and budding processes. From :Trkola 2004.

The virus attacks $CD4^+$ T-lymphocytes, monocytes, dendritic cells (DCs) and macrophages, which all have the CD4 antigen that acts as the main receptor for viral entry. The HI-virus has gp120 on its envelope (**Figure 2**) and interaction of this glycoprotein with CD4 results in the exposure of the chemokine receptors due to gp120 conformational changes. It was shown by Auwerx *et al.* that conformational changes to the gp120 are due to the reduction of intracellular disulphide bonds of the gp120 that occur when binding to the CD4 takes place. The exposed chemokine receptors now act as co-receptors for viral entry. The most common co-receptor used by HIV-1 is CCR5 (R5 viruses) and individuals who contain genetic mutations in this protein are relatively resistant to HIV-1 infection. Other variants of HIV-1 use CXCR4 as a co-receptor (X4 viruses). Binding of these co-receptors then leads to structural changes that result

in the exposure of the gp41 protein. This allows the insertion of the protein into the cell and facilitates the fusion of the viral envelope with the cell membrane [19].



(**A**) (**B**)

Figure 2: Mature HIV particles. (**A**) The viral particles as derived by electron cryotomagraphy and each particle is approximately 130 nm. (**B**) The scientific model of a mature viral particle depicting the core and the two RNA molecules in red associating with reverse transcriptase (blue). The gp120 proteins required for viral entry are shown in purple. From: Ganser-Pornillos *et al.* 2008.

2.1.2 Viral Life Cycle

HIV codes for three main enzymes; reverse transcriptase (RT), integrase (IN) and protease (PR). The main objective of these enzymes is to facilitate the effective transfer of all viral genetic material to the nuclear DNA of the infected cell, ultimately producing new infectious virus particles. The replication life cycle of the virus is represented below (**Figure 3**). Reverse transcriptase is responsible for the conversion of the viral RNA into a cDNA copy (proviral DNA). RT, along with all the structural proteins of HIV, is produced as part of the Gag-Pol precursor protein [20] . Viral integrase functions in the integration of the proviral DNA into the host genome [21]. Once integrated into the host genome, transcription of the viral genome occurs. This takes place through the action of the cellular machinery and cellular factors which bind to the 5'-long terminal repeats (5'-LTR). One such factor is the nuclear transcription factor κβ (NF-κβ), which plays a huge role in the initiation of HIV transcription. Interaction of NF-κβ with 5'-LTR results in the production of Tat, a viral protein that promotes transcriptional elongation [18].

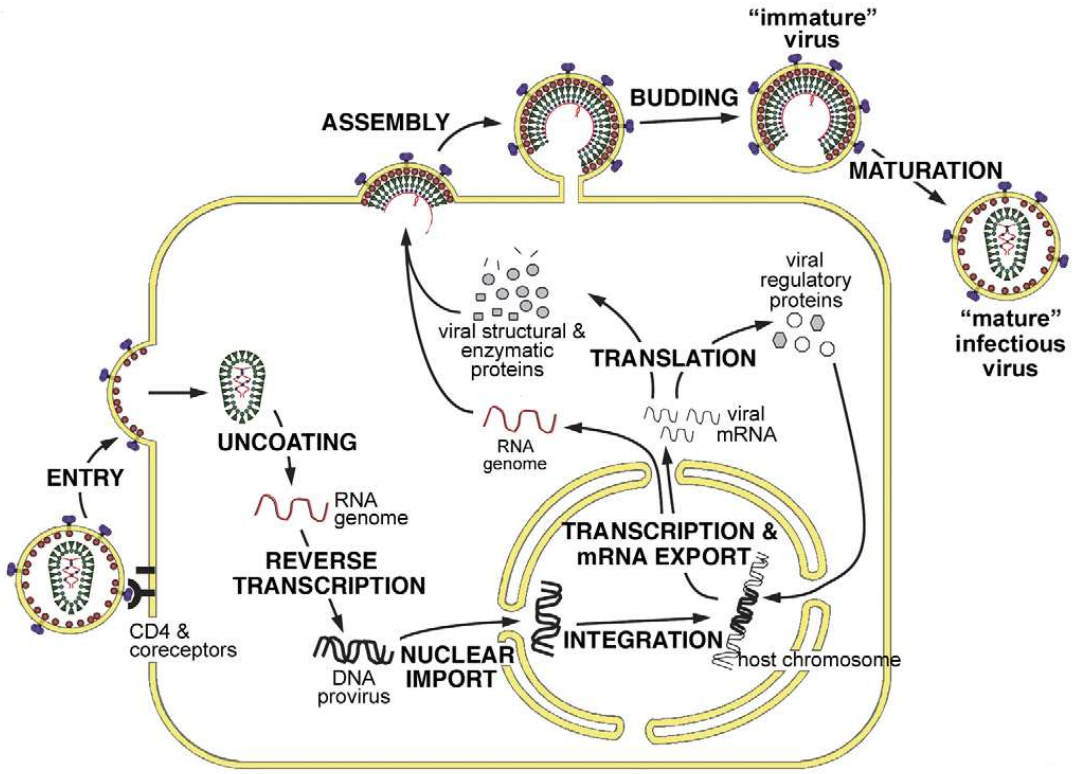


Figure 3: Summary of the HIV-1 replication cycle. CD4 receptors on cells and co-receptors CCR5 and CRCX4 facilitate the fusion of the virus into the cell and this is followed by a cascade of events, ultimately resulting in the production of more virus particles. From: Ganser-Pornillos *et al.* 2008.

Post-transcription, the HIV-1 specific transcripts generated are transported into the cell cytoplasm where assembly of new HIV particles begins. This is followed by budding out of the cell. The main proteins that play an essential role in the assembly of the new virions and their release from the cell are $p55^{gag}$ proteins, the main source of the structural components of the viral core. These proteins are trafficked to the cell membrane of the host and there they attach to the lipid bilayer [18]. The budding process is also influenced by *env* (a gene that codes for all the envelope proteins, gp120 and gp41), and involves the recruitment of two copies of the viral RNA genome as well as *vpr* (viral protein R, which interferes with the host cell cycle and arrests replication at G2 phase) [18, 22]. The budding process represents a second event of membrane fusion and this is followed by the viral maturation process.

PR is responsible for driving this maturation process through the cleavage of Gag and Gag-pol precursor proteins in a maturing capsid. This process results in the collection of RT, IN, Vpr and a condensed ribonucleoprotein complex that is made up of RNA coated with mature nucleocapsid proteins ($p24^{gag}$) [23].

Each step of the viral life cycle represents a potential area of inhibition. Inhibition of these enzymes could result in virions that are not able to infect any more cells, therefore either slowing down or halting the rapid spread of the virus throughout the whole body [24].

2.1.2.1 HIV-1 Reverse Transcriptase

HIV-1 RT is an asymmetric dimer consisting of a 66 kDa (p66) and a p66-derived 51 kDa (p51) subunit. The heterodimer is the biologically active form of the enzyme and monomeric subunits are devoid of polymerase activity [25]. There are two enzymatic activities performed by reverse transcriptase; one of these being a DNA polymerase that can replicate either DNA or RNA templates and the other RNaseH which acts in the cleavage of the RNA when it is part of a RNA/DNA duplex. These two activities function together in the conversion of viral RNA to double stranded DNA in the cytoplasm of the infected cell [26]. The structure of the enzyme and the different subunits into which it is divided is represented in **Figure 4** and the enzyme can be inhibited through the use of two types of inhibitors; nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

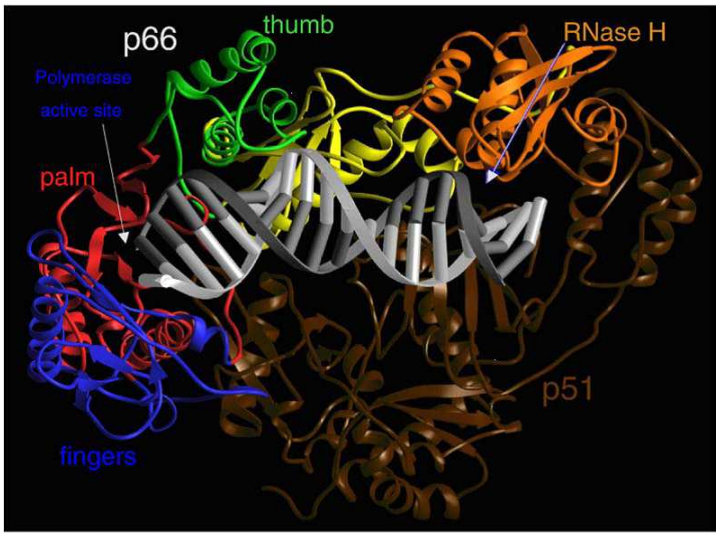


Figure 4: Structure of HIV Reverse Transcriptase. Here the enzyme is shown in complex with a nucleotide. The respective sub-domains of the p66 subunit are shown in blue (fingers), red (palm), green (thumb), yellow (connection) and orange (RNaseH). The light grey and dark grey are the template and primer DNA and the dark brown is the p51 subunit. From: Sarafianos *et al.* 2009.

2.1.2.2 HIV-1 Integrase

HIV-1 integrase functions in the integration of proviral DNA into the host genome [27]. This process is unique to and is essential for retroviral replication. The persistence of HIV can solely be attributed to the stable integration of the viral genome into the human genome [28].

The enzyme has low solubility and is prone to aggregation. This hampers atomic resolution structural studies. As a result, there is currently no full-length structure showing all the domains (N-Terminal, Catalytic Core and C-terminal) of the enzyme. Also lacking is the structure of DNA assembled in the catalytic site targeted by IN strand-transfer inhibitors (intasome). Insight was provided in early 2010 when the crystal structure of retroviral Prototype Foamy Virus (PFV) was published. The active site of PFV (**Figure 5**) has been shown to be virtually identical to that of HIV-1 IN [29].

The presence of divalent Mg^{2+} ions in the active site of the enzyme is important for enzyme activity. These act as cofactors and diketoacids which have been reported to be the most promising inhibitors of the enzyme are proposed to sequester to the ions, blocking DNA access [30].

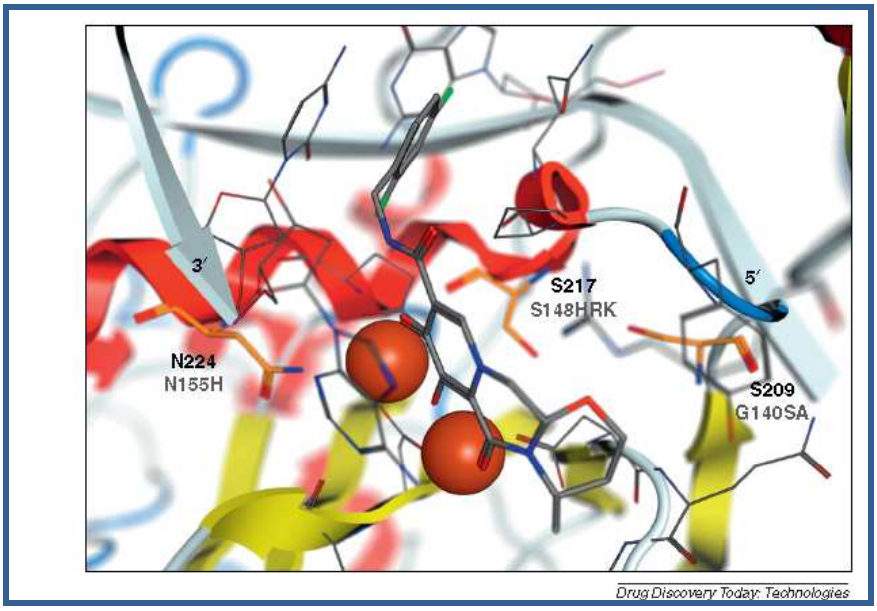


Figure 5: Structure of active site of Prototype Foamy Virus intasome complexed with dolutegravir, a promising second generation strand-transfer inhibitor. PFV integrase is a model for development of integrase inhibitors. The protein and DNA (3' and 5' indicated) are shown as cartoons. Residues corresponding to Raltegravir resistance are shown as orange sticks with residue numbers depicted in black (PFV) and grey (HIV-1). The inhibitor is shown to coordinate to two Mg^{2+} ions (brown spheres) sequestered in the active site. From: Demeulemeester *et al.* 2012.

There are basically three steps that take place during proviral integration. The first step is the assembly of the proviral DNA to the integrase. The second step is the endonucleolytic cleavage where 3' processing takes place and two nucleotides are removed from each end of the produced proviral DNA. The last step is strand transfer where the ends of the proviral DNA are integrated into the host genome through a number of trans-esterification reactions [31]. There have been proposed *in vitro* integrase inhibitors (INs), however, to date only one IN inhibitor has been FDA approved and that is Raltegravir [32].

2.1.1.1 HIV-1 Protease

The viral protease, a homodimer consisting of one active site (**Figure 6**), is coded for by the *pol* gene of the viral genome and it is responsible for the cleavage of large precursor core proteins into smaller core proteins during the internal assembly of newly released HIV particles [18]. The main proteins that are cleaved are the Gag and Gag-pol polyproteins, which are extremely important for viral replication. It is for this reason that HIV protease drug inhibitors are one of the most studied drugs involved in HIV treatment.

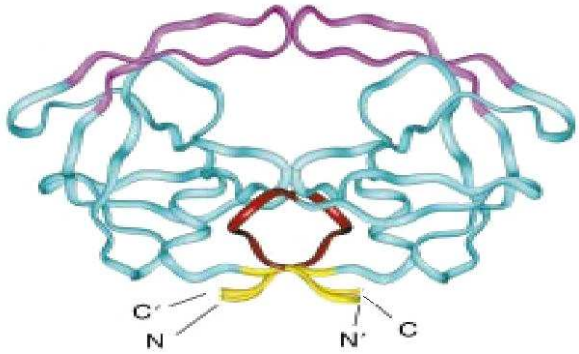


Figure 6: Structure of HIV Protease. The enzyme consists of two monomers that form the homodimer structure. The red indicates the autoproteolysis-sensitive loop, and the residues that form the interfacial four-stranded β-sheet are indicated in the yellow. The flap domain which covers the substrate-binding site is indicated in purple and the rest of the homodimer is in blue. From: Ishima *et al.* 1999.

HIV-1 protease has been shown to form part of the aspartic acid class of proteases. This has been confirmed by sequence homology, similar structural features and a similar mechanism of action. The enzyme, just like all other proteases in this class, has a conserved Asp-Thr-Gly sequence and mutation of any of these residues results in an inactive enzyme. The enzyme catalyses the hydrolysis of a peptide bond in a one-step process. One of the aspartic acid residues in the active site is thought to be deprotonated at the active pH range of the enzyme and this aspartic acid is associated with a nucleophillic water molecule. It is this nucleophillic water molecule that is responsible for the attack on the carbonyl group on the peptide bond

being broken [24]. The activity of the enzyme can be inhibited by a number of protease inhibitors (PIs) and crystal structure of the enzyme bound to an inhibitor exhibits substantial conformational changes [34].

2.1.2 Pathogenicity and treatment of HIV

There is currently no cure or vaccine for HIV and the infection can only be managed through treatment. In the absence of medication, HIV infection is associated with persistent virus replication. This results in chronic immune activation and this has been proposed as the major cause of lymphoid tissue damage and the deterioration of the immune system [35]. If still left untreated, the CD4 T-cell count of the patient drops to below 200 cell/µl of blood which is defined as AIDS. Here the patient becomes susceptible to a number of microbial infections and OIs. The individual presents symptoms such as neuropathy, thrush, weight-loss and lymphadenopathy and if there is no intervention the immune system continues to loose effect finally leading to death [36].

The main aim of available HIV treatment is to lower viral replication as much as possible while maintaining high CD4 T-cell turnovers. The early administration of highly active antiretroviral therapy (HAART) which is combination HIV chemotherapy consisting of two NRTIs and one NNRTI or a PI has proven to be successful in the control of HIV infection. Treatment has resulted in the decrease of hospitalization rates, opportunistic infections and HIV/AIDS related mortality. Indeed patients receiving HAART have been shown to experience lower rates of viral transmission and reduced rates of disease progression [37, 38].

2.1.2.1 Entry inhibitors

Viral entry inhibitors form part of the novel classes of antiretroviral agents. There are mainly three steps required for viral entry (**Figure 7**) and the first step is the attachment of the viral gp120 to the CD4 T-cell receptor. Secondly the gp120 attaches to the CCR5 or CXCR4 co-receptor. This then results in the fusion of the viral and cellular membranes, which is the final step of the entry process [32, 39].

There are currently two FDA approved entry inhibitors, Enfuvirtide (also known as T-20) and Maraviroc. Enfuvirtide is a synthetic peptide that interacts with gp41 blocking CD4-gp120 interactions [39, 40]. Maraviroc is a CCR5 antagonist that binds to a hydrophobic pocket in the CCR5 co-receptor inducing conformational changes (**Figure 7**). The drug is well tolerated and is associated with minimal side effects such as abdominal pain. Aplaviroc and Vicriviroc were also prescribed as entry inhibitors. Due to adverse reactions and poor antiviral activity, these drugs have been discontinued [41].

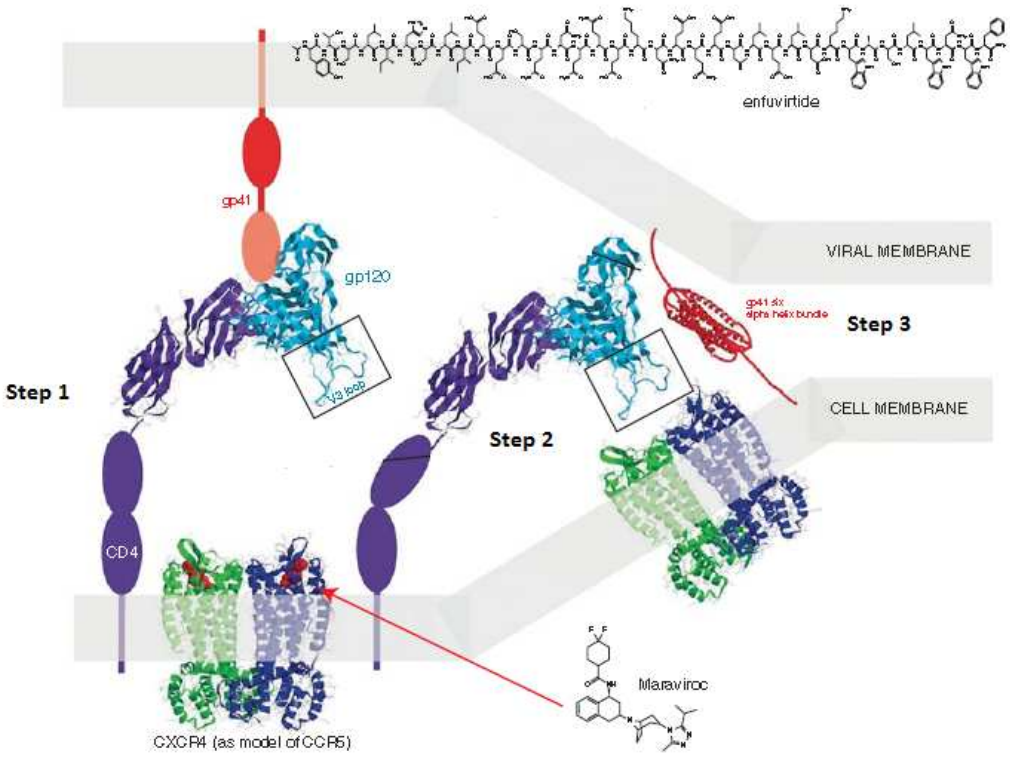


Figure 7: Inhibition of viral entry by Enfuvirtide and Maraviroc. Maraviroc inhibits the second step of viral entry; prevent the binding of gp120 to CCR5. In cases were the second step has occurred, Enfuvirtide binds to gp41 preventing the third step, which is fusion of the viral membrane with the cell membrane facilitated by gp41 From: Arts & Hazuda 2012.

Binding to CCR5 means that Maraviroc can only prevent infection by R5 viruses and not X4 viral strains that utilize the CXR4 co-receptor instead of CCR5 [43]. Inhibitors against this chemokine receptor have been developed (AMD3100 and AMD070), however their development has been discontinued due to adverse side effects as well as the fact that CXCR4 is required for a number of physiological processes [40].

2.1.2.2 Reverse transcriptase inhibition

As stated above, RT can be inhibited using two forms of inhibitors; NRTIs and NNRTIs. The drugs target the polymerase activity of the reverse transcriptase enzyme. NRTIs are structurally diverse analogues of the natural substrate of DNA synthesis (**Figure 8**) lacking the required 3'-OH. These therefore act as chain terminators for the elongation of newly synthesized viral cDNA [26].

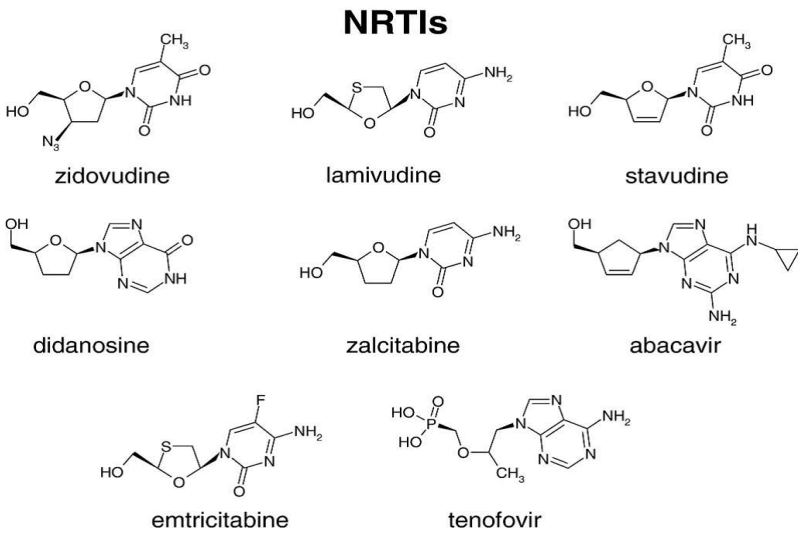
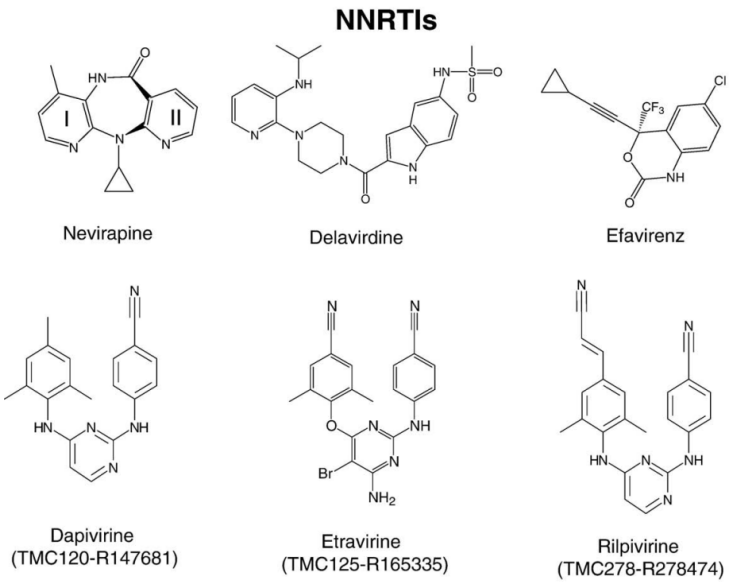


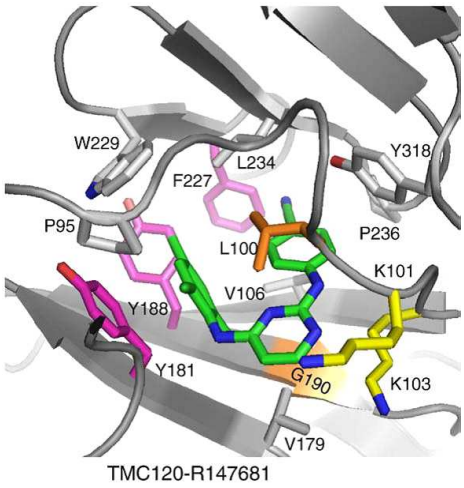
Figure 8: The structures of some NRTIs. These drugs are all approved by the United States Food and Drug administration and they are manufactured by various pharmaceutical companies all over the world. From: Sarafianos *et al.* 2009.

Triphosphates are not taken up by cells and these NRTIs are taken up as non-active agents which get activated in the cell through phosphorylation by host kinases into the active NRTI-triphosphates. It is this conversion that determines the activity and stability of these inhibitors and the level of these endogenous kinases is correlated to the effectiveness of the compounds [44]. The presence of catabolic enzymes can lower the concentration of these inhibitors in the blood stream, making them ineffective [26].

HIV-1 RT can also be inhibited through NNRTIs. A hydrophobic pocket called the NNRTI-binding pocket situated in the p66 subunit serves as a target for these inhibitors (**Figure 9B**). NNRTIs (**Figure 9A**) are a diverse group of lipophilic compounds made of more than 30 classes, specifically inhibiting HIV-1 RT and not HIV-2 RT [25]. Since these interactions are allosteric, NNRTIs can therefore be classified as non-competitive inhibitors of the enzyme as they do not hinder the binding of the dNTPs or the substrate of the enzyme during cDNA synthesis [26, 45]. Binding of NNRTIs at a site other than the active site results in the decrease in the catalytic rate by causing conformational changes in the 3-dimensional shape of the enzyme [46].



(**A**)



(**B**)

Figure 9: The structure of the FDA approved NNRTIs (A) and their binding pocket (B). Shown is the hydrophobic pocket with amino acid residues such as leucine (L100), tyrosine (Y188) and phenylalanine (F227) which all contain hydrophobic side chains. From: Sarafianos *et al.* 2009.

2.1.3.3 Integrase Inhibitors

The inhibition of HIV-1 integrase has been identified as one of the most attractive targets for antivirals, probably due to the absence of a human counterpart to enzyme which suggests potential lower toxicity of these inhibitors [27, 46, 47]. There have been a number of compounds with reported *in vitro* inhibition of HIV-1 integrase and the most promising inhibitors have been reported to be aryl diketoacids compounds. These inhibitors have a diketoacid subunit with an

aromatic moiety with either one or two arylalkyl substituents. The diketoacid subunit serves as a pharmacophore and interacts with the Mg^{2+} ions in the active site of the compound, blocking DNA binding. The aromatic moiety acts as a hydrophobic domain and is responsible for the selectivity to the strand transfer reaction [30].

Raltegravir (**Figure 10**) is the only FDA approved IN inhibitor and the compound was approved in October 2007 [32]. The drug has been shown to possess impressive potency with 10 day monotherapy resulting in a 2 $\log_{10}$ reduction in the viral load [49]. Raltegravir has exhibited low nanomolar *in vitro* enzyme inhibition with an IC_{50} value of 2.7 nM [32].

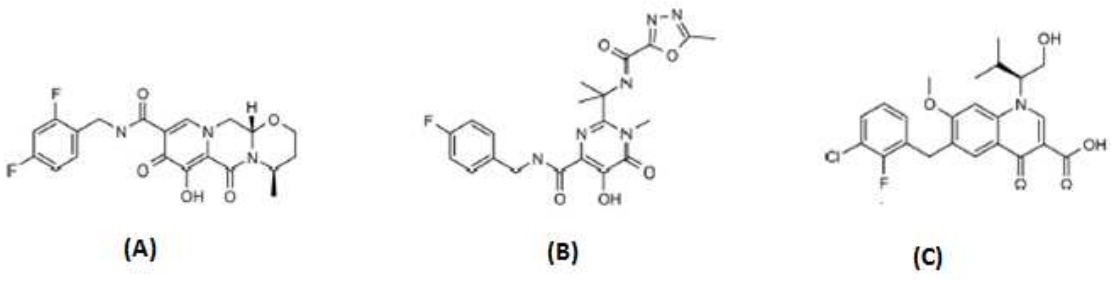


Figure 10: Structure of HIV-1 IN inhibitors. (A) Dolutegravir, **(B)** Raltegravir and **(C)** Elvitegravir. The compounds play a role in the inhibition of the strand-transfer reaction, resulting in no integration of the proviral DNA into the host genome. From: Hightower *et al.* 2011.

As of 2011, only two other compounds were in clinical development, Elvitegravir which was in the last stages of clinical development and dolutegravir (S/GSK 1349572) which was in phase 1 and two of clinical trials. There is already clinical evidence of the emergence of Raltegravir resistance and these novel drugs could help by-pass resistance [32].

2.1.3.4 Protease Inhibitors

As stated before, inhibition of HIV-1 protease results in the production of immature progeny virions that are incapable of infecting other cells. Due to this, the enzyme has emerged as one of the prime targets for HIV inhibition [51]. A number of drugs inhibiting HIV-1 protease (**Figure 11**) have been synthesized and these inhibitors basically form an enzyme-inhibitor complex, preventing it from performing its function [24].

Most protease inhibitors targeting the active site of the enzyme have been shown to possess hydroxyl groups which locate between Asp-25 and Asp-25', making favourable electrostatic interactions [24]. Others, for example peptidomometic inhibitors, have been shown to bind to the active site through a bridging structural water [52]. The effect of compounds binding to the active site is however short-lived and this is due to the prevalence of strains with mutations in

the active site. This has lead to investigations for alternative modes of inhibition and the dimerization interface of the enzyme (the most conserved region of the enzyme) has been identified as an attractive target for potential inhibitors. Such inhibitors provide a switch between the allosteric inhibition and dimerization interface inhibition of the enzyme [53].

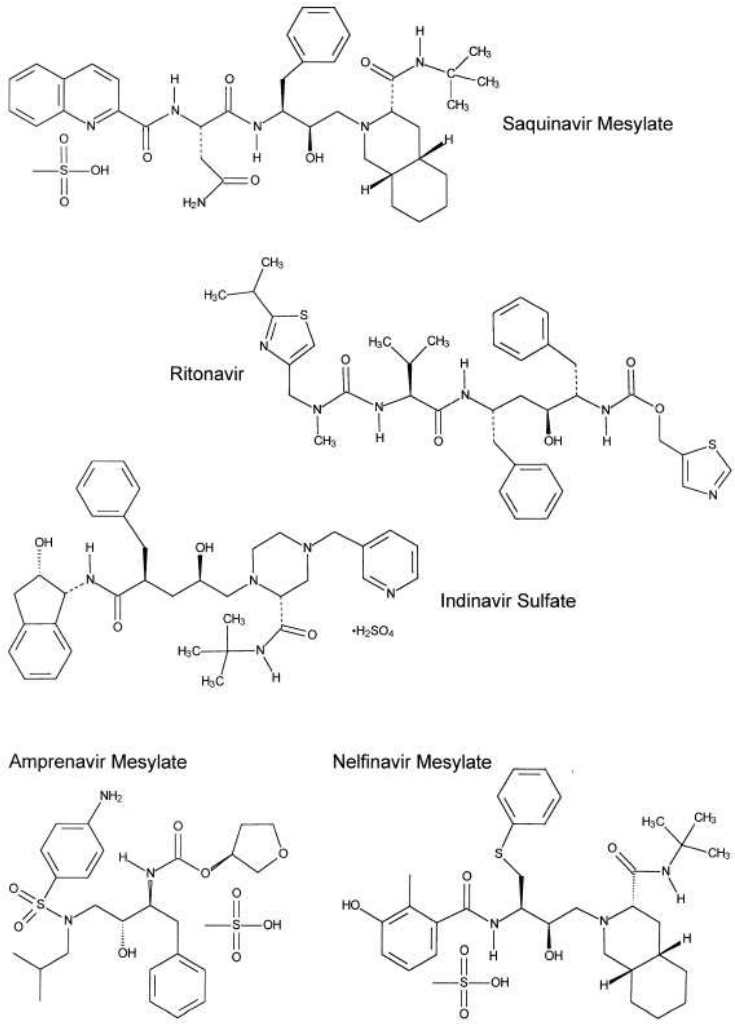


Figure 11: Structure of HIV-1 Protease inhibitors. Most of the effective inhibitors of the enzyme contain a core structure that mimics the transition state of the protease catalysis. From: G. C. Williams & Sinko 1999

All protease inhibitors, except Nelfinavir, have been recommended for combination use with ritonavir. This has been said to help increase PI serum levels, extend dosage intervals and therefore facilitate dosage, decrease the number of tablets and increase strength. All of this is also against strains with decreased sensitivity to PIs [55].

2.2 Opportunistic Infections

As stated above, untreated HIV infection leads to AIDS which is associated with a loss of immune-mediated control against diverse opportunistic pathogens [56]. These include bacterial, fungal and viral pathogens and examples of infectious particles are herpes virus-type 1, *Mycobacterium avium* and *M. tuberculosis* [57]. There are also neurological opportunistic infections and many of these are recognized as AIDS-defining conditions. These include central nervous system (CNS) cytomegalovirus, CNS tuberculosis, cryptococcal meningitis and cerebral toxoplasmosis [58]. This study focuses on two opportunistic pathologies/infections, cervical cancer and tuberculosis.

2.2.1 Cancer and HIV

There have been associations of HIV with different types of cancers and cancer is now one of the leading causes of death in immunosuppressed populations [4], [59]. In 2005, an estimated 40% of HIV-infected patients with AIDS developed cancer during the course of the disease. AIDS-defining cancers (ADC, which are cancers that would constitute an AIDS diagnosis in HIV positive patients) include Kaposi's sarcoma, non-Hodgkin's lymphoma and cervical cancer [60]. Non-AIDS defining cancers (NADC) include lung, Hodgkin's and skin cancer and both ADC and NADC pose higher risk in infected individuals than the general population [61], [62].

Cancer can be defined as uncontrolled cell growth due to defects in the cell regulatory circuits that govern normal cell proliferation [63]. One reason for the increased susceptibility to cancer observed in HIV positive patients is immune dysfunction associated with the infection. Impairment of the immune system could result in a decrease in general immune surveillance for malignancies [61]. In addition, immune suppression may accelerate the progression of different types of cancer in individuals that are already predisposed to these pathologies. Other reasons could be concomitant infection with oncogenic viruses [62]. One such oncogenic virus is the Human Papilloma Virus (HPV)

2.2.1.1 Cervical Cancer and HIV

Like HIV, HPV is a sexually transmitted virus and is the leading cause of cervical cancer (in women) as well as genital warts, penile and anal cancer in men [64]. Cervical cancer is the leading cause of female cancer mortality in eastern Africa and is the most prevalent cancer in HIV positive patients in sub-Saharan Africa [65]. These patients have been shown to possess substantially higher numbers of high-risk human papilloma virus (HR-HPV) particles and according to a study done in Côte d'Ivore, women with a CD4 cell count less than 200 cells per µl of blood were infected with a median number of three different oncogenic HPV strains (**Figure 12**) [66].

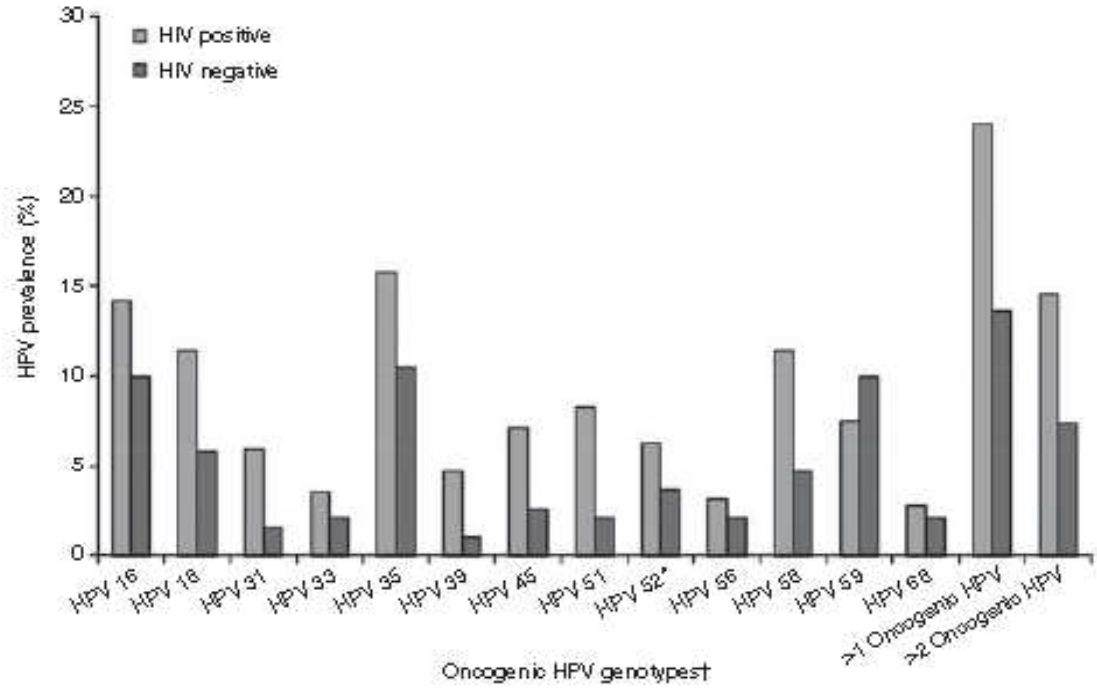


Figure 12: Risk of HIV infected women to high-risk HPV strains. HIV infection is associated with higher risk to HPV16 and HPV18 which are the most virulent and oncogenic strains of HPV which are detected in virtually all cases of cervical cancer. From: Jaquet *et al.* 2012.

Briefly, during infection with HPV, the oncogenes E6 and E7 induce malignant transformations resulting in changes in normal genetic and cellular functions. These infections are cleared in healthy women; however in HIV infected women, persistent infection with high risk HPV results in the accumulation of altered genetic material, leading to the development of cervical intraepithelial neoplasia and ultimately invasive cervical cancer [67].

2.2.1.2 Treatment for Cervical Cancer

The main course of death in women with cervical cancer is uncontrolled disease-state within the pelvis [68]. Therapy for cervical cancer includes surgery, chemotherapy, radiotherapy or a combination thereof [69]. The different forms of treatment for cervical cancer depend on the stage the patient is in. Radical hysterectomy and pelvic lymphadenectomy or radiation therapy can be used to treat patients with clinical stage IB or IIA cervical cancer [70]. Radical surgery alone as well as chemotherapy alone has been shown to achieve 5-year survival rates. Some studies have reported that treatment with a combination of chemotherapy and radiotherapy (chemoradiotherapy) can achieve better survival rates and decrease mortality than with chemotherapy alone. This may be due to the fact that chemotherapy regimens are cell cycle specific and therefore sensitize radiotherapy allowing the killing of cancer cells at different cell-cycle stages [69].

Apart from treatment, there are two vaccines against HPV which were approved in 2006 and these were targeted at women ranging between 9-26 years of age. The vaccines have further been extended to include the prevention of anal cancer in men and women in the same age group. A possible downfall for current vaccines could be the fact that they are targeted at only HPV 16 and 18, whereas HIV positive women presenting with cervical cancer are exposed to multiple high risk HPV infections and are generally much older than the specified age group eligible for the vaccines [65]. This therefore questions the ability of the vaccines to reduce disease in pre-exposed HIV-positive women, and therefore their cost effectiveness.

Although HAART helps in immune reconstitution, there have been reports of no significant changes in incidences of invasive cervical cancer when rates were compared between pre-HAART and post-HAART eras. In addition there have been speculations that cancer rates, especially lymphomas, will rise within areas with high availability of HAART [71]. This therefore necessitates new forms of treatment that can help control cervical cancer especially in HIV positive women.

2.2.2 Tuberculosis

The most common cause of mortality in HIV-1 infected individuals in sub-Saharan Africa is Tuberculosis (TB) [72]. Although almost a third of the world population is infected by *M. tuberculosis*, the impact of TB is most severe in populations with high rates of HIV infection due to the impaired cell-mediated immune responses associated with the virus. This results in greater susceptibility to TB, a more rapid progression of clinical symptoms and an often fatal outcome [73].

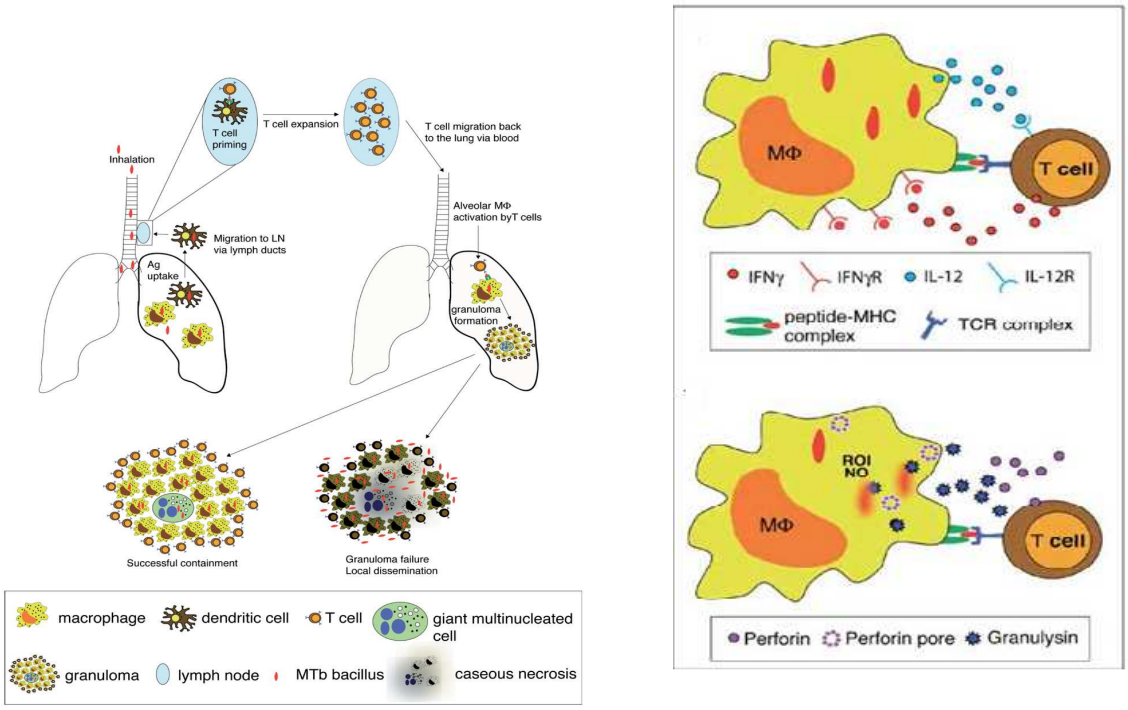
2.2.2.1 TB, HIV and the immune response

In order to understand the dynamics of HIV-TB co-infection, it is important to understand the immunopathogenesis of TB. During innate immunity in the lungs, *M .tuberculosis* interacts with a number of receptors such as complement receptor 3, which recognize components of the bacterium such as its lipoprotein. Binding of such receptors results in the activation of NF-κβ, and the activation of this natural factor promotes the production and secretion of cytokines and other pro-inflammatory mediators. This attracts natural killer cells, T-cells, dendritic cells and macrophages to the site of infection, resulting in the killing of intracellular mycobacterium [74]. However, as stated above, NF-κβ can bind to the 5'-LTR of HIV promoting viral replication [18].

HIV studies have indeed demonstrated that *M. tuberculosis* can increase HIV-Long Terminal Repeat (LTR) - driven transcription in T-cells and monocytic cells, resulting in increases in HIV

production. It has also been observed that there is a correlation between mycobacterium-induced HIV production and the secretion of cytokines such as TNF-α through NF-κβ induction. However, defences against *M. tuberculosis* do not only involve monocytic cells, but also CD4+ T-cells (infected macrophages and dendritic cells of innate immunity present antigens to T-cells in adaptive immunity) [75]. In addition, T-cells play an important role in containing the infection through the formation of granulomas which are classic structures that contain macrophages in the centre surrounded by T-cells (**Figure 13A**). Granulomas may result in the killing of the mycobacterium. This therefore means that reduction in the number of available CD4+ T-cells caused by HIV infection could hamper with granuloma formation resulting in the dissemination of the bacteria [76].

The above shows the importance of cytokines during HIV-TB co-infection and one such cytokine is interferon γ. It has been shown by the interferon γ release assay that the cytokine is necessary for protection against *M. tuberculosis* infection and this is when it is secreted from activated T-cells and natural killer cells. Interferon γ can activate macrophages and promote bacterial killing by allowing phagosomal maturation and producing antimicrobial reactive nitrogen and oxygen intermediates (**Figure 13B**). The loss of multifunctional CD4+ T-cells (due to HIV infection) capable of producing this and other cytokines therefore results in the exhaustion of the immune system and consequently death [74], [77].



(**A**) (**B**)

Figure 13: *M. tuberculosis* immunity. (**A**) Post infection with the *Mycobacterium*, macrophages and dendritic cells ingest the pathogen. These migrate to the lymphnodes where T-cells are primed to differentiate into antigen-experienced cells. T-cells then migrate to the lungs and activate macrophages, inducing the formation of granulomas. Successful formation and maintenance of these structures result in the containment of the pathogen (**B**). Upon recognition of the peptides presented on MHC molecules of macrophages by T-cells, the macrophage will secrete IL-12 which aids in the further activation of the cell. This result in the release of INF-γ, which is important for the activation of macrophages, triggering the production of factors such as reactive oxygen intermediates (ROI) and nitric oxide (NO), directly killing the pathogen. From: Hanekom *et al.* 2007.

2.2.2.2 Current TB Treatment

Over the years, effective Directly Observed Therapy Short course (DOTS) as well as Bacille Calmette-Guerin (BCG) vaccine have been available to combat of *M. tuberculosis* infection [78]. DOTS are a combination of drugs (**Figure 14**) consisting of Isoniazid (isonicotinic acid hydrazide (INH)), Rifampicin (RIF), Pyrazinamide (PZA) and Ethambutol. All these are administered over a period of two months, followed by a four month treatment with INH and RIF (Byrne *et al.* 2007)

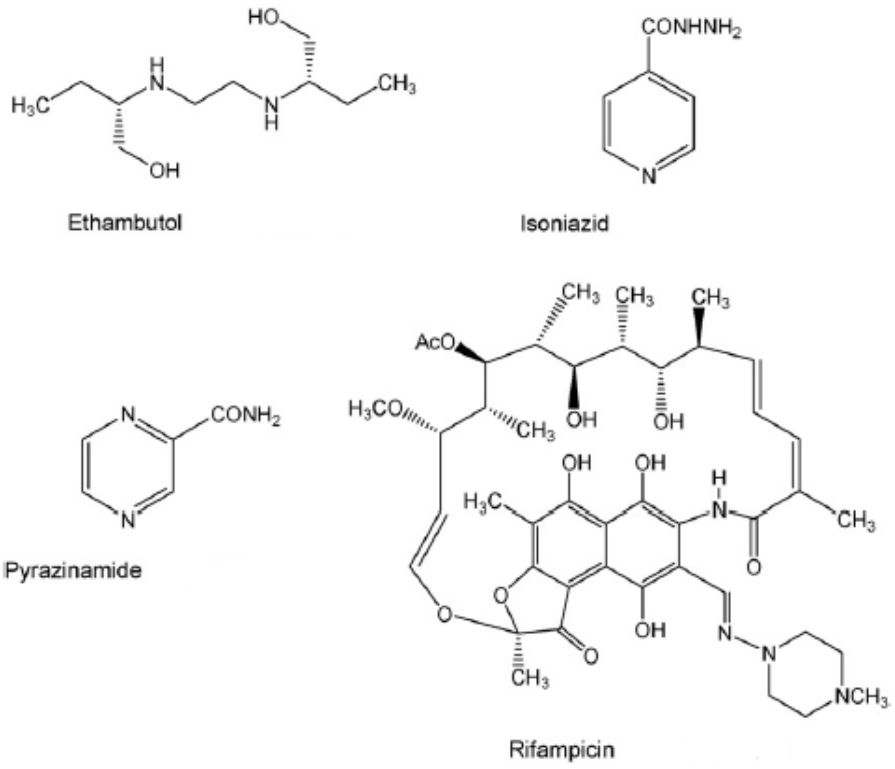


Figure 14: Structures of TB inhibitors. Isoniazid, Rifampin, Ethambutol and Pyrazineamide form part of the first-line agents against TB. These are the most effective agents and they are a necessary component of any short-course therapeutic regimen. From: Faria *et al.* 2010.

Although the available TB treatment has been seen to clear infection in most patients, there are a number of side-effects that are associated with the medication. The most effective therapy is a combination of Isoniazid, Rifampin and Pyrazinamide for 8 weeks, followed by Rifampin and Isoniazid for a further 4-7 months. Due to this lengthy period for full treatment, infected patients require close supervision. Creswell *et.al* (2010) have shown that there is a correlation between tuberculosis and non-communicable diseases such as diabetes, cardiovascular diseases and those related to smoking and alcohol abuse. Non-communicable diseases are a risk factor as they can have a negative impact on the host defence mechanism against the pathogen and therefore promote progression from infection to disease state. In addition, these diseases can complicate treatment e.g. alcohol addiction is one of the strongest risk factors for treatment interruption, leading to drug resistance. It is therefore crucial to develop therapies that require shorter periods for the full treatment of the pathogen [81].

2.2.3 Limitations of HIV and OI treatments

As mentioned above, treatment against HIV and its associated OIs (cancer and TB) is associated with a number of limitations. Here, adverse effects as well as the emergence of resistant HIV strains will be discussed.

2.2.3.1 Adverse effects

Up to 25% of patients on HAART discontinue their initial treatment regimen and this is due to the toxicity of the drugs as well as the inability of the treatment to suppress viral replication to below detection point (50 copies/ml). Some of the most significant side-effects include lactic acidosis, hyperglycaemia, bleeding disorders, osteoporosis and lipodystrophy [82]. Each HIV inhibitor is associated with its own side-effects (**Figure 15**) and these include haematological adverse reactions by AZT (anaemia and thrombocytopenia), central nervous system effects by Lamivudine (depression, neuropathy, insomnia, headaches and malaise), gastrointestinal effects by Stavudine (nausea, vomiting and anorexia), and hepatotoxicity by Zalcitabine [36].

Other side effects are naturally associated with HIV infection; however, treatment exacerbates these side effects. One such condition is severe myopathy which is associated with muscle weakness and muscle pain; muscle biopsies from affected patients reveal abnormal giant mitochondria [83]. Protease inhibitors have been associated with increased triglycerides and are therefore suspected of being the cause of lipodystrophy [84]. Lipodystrophy is a diverse group of disorders characterised by a number of physiological and metabolic alterations. Body changes include partial or complete loss of adipose tissue (lipoatrophy) or the pathological accumulation of adipose tissue in particular body compartments (lipohypertrophy). Metabolic abnormalities are characterized by insulin resistance and dyslipidaemia. Investigations into the pathological mechanism of the disorder also showed the implication of NNRTIs and NRTIs on changes in serum lipid concentrations, meaning that the condition is not caused by a single factor but is of multifactorial origin [85]. Therapeutically, the failure of CD4 cell reconstitution and adverse effects during receipt of virologically suppressive HAART is an indication of the need for alternative treatment options and the development of which, is one of the major aims of this study.

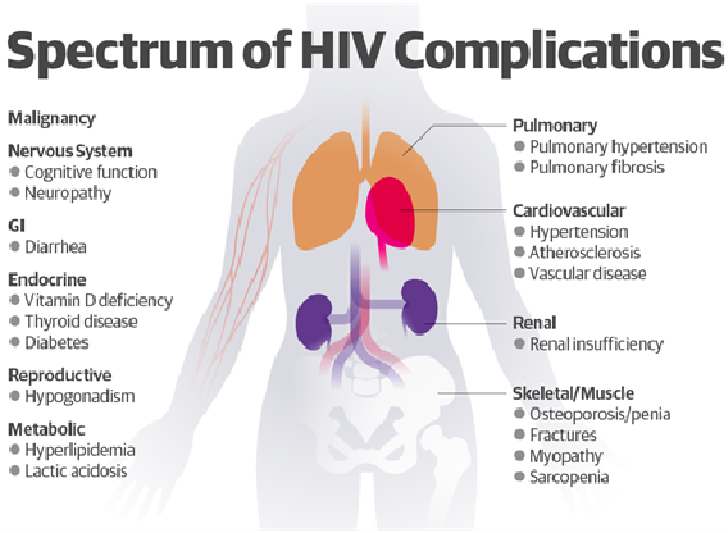


Figure 15: Adverse side-effects associated with HAART. Each HIV inhibitor has its own side effects and these side effects will result in physiological and metabolic changes. The severity of toxic side effects of the drugs often leads to the non-compliance of patients to treatment. From: www.aidsmap.com/HIV-and-TB-in-Practice-for-nurses-non-communicable-disease-HIV-and-TB/page/2367149/ [Accessed: 08/12/2012].

Cancer and TB treatment are also associated with their own adverse side effects that limit the effectiveness of these treatments. Major side effects of current cervical cancer treatments include vesico-vaginal fistula, recto-vaginal fistula and radiation fibrosis. Other toxicities include nephrotoxicity, skin erythema and rectal pain. Cisplatin-containing chemotherapy regimens have been shown to result in two-fold increase in gastrointestinal and haematological side effects [86]. Although not fully understood, Hart *et al.* (1999) have additionally reported statistically significant poor survival of African American women with stage II cervical cancer treated with brachytherapy and external beam radiation compared to their white counterparts [87].

Schaberg *et al.* (1996) have shown that hepatotoxicty is one of the major side effects associated with anti-mycobacterium treatment and that most patients develop exanthema (eruption of the skin as a symptom of disease) after the initial dose of pyrazinamide requiring the re-introduction of the drug at reduced doses [88]. Risk factors for drug-induced hepatotoxicity include old age, high intake of alcohol, chronic liver disease, malnutrition and viral hepatitis. Therefore, novel drugs with lower toxicity for patients at risk of intolerance are still required [89].

2.2.3.2 Emergence of Resistant Strains

The major cause of resistant strains is the non-compliance of patients to the treatment. This is true for HIV and TB drug-resistant strains. There has been an emergence of global resistant strains of *M. tuberculosis* to presently available treatment which are multidrug-resistant TB (MDR) as well as extensively drug-resistant (XDR-TB) strains. MDR is defined as a resistance to isoniazid and Rifampin, whereas XDR is resistance to the aforementioned drugs and at least three classes of second line drugs (flouroquinolones and either aminoglycosides or capreomycins, or both). In their study in TB-HIV integration in Kwa-Zulu Natal (South Africa), Gandhi *et al.* (2006) showed that 5% of all patients co-infected with both diseases had XDR-tuberculosis, and that the bacterial infection was rapidly and uniformly fatal [72].

HIV infected individuals have high levels of virus production and turnover, with an estimated 10^7 -10^8 productively infected cells in the lymphoid tissue of untreated individuals. This number is relatively stable and considering the short half-life of infected cells (1-2 days), new targeT-cells are infected at a very high rate in order to maintain this balance. This results in the production of 10^9- 10^{10} virions per day per person (FDA, 2007). This rapid transcription of viral RNA into DNA is prone to errors (1 in every 10 000 bases). Mutations that mostly occur are base substitutions, although duplications, insertions and recombinations are also possible. The constant introduction of mutations ensures that the patient has a diverse and complex mixture of viral quasispecies. Any viral transcript that has selective advantage over the others, such as resistance to a particular antiretroviral drug, will over time over take the others. However, complete resistance requires the gradual accumulation of additional mutations [26], [91]. Highly active antiretroviral treatment helps in the halt of this selection process and resistance can only occur if the replication of the virus continues to occur at drug levels that are insufficient to block the replication, therefore giving rise to a positive selection pressure on viral strains that are less susceptible to the drugs. As this continues, the development of viral strains that are resistant to all drug components of HAART begin to emerge, leading to situations were it becomes impossible to control the replication of the virus with available treatment [91].

Drug resistance has been observed in almost all the types of HIV inhibitors. Understanding the mechanism of action of the different classes of antiretroviral drugs provides insight into where in the HIV genome, mutations that confer resistance could occur. These are not limited to the site of action of the drug and could occur in the enzyme substrate (gag-pol cleavage sites for protease inhibitors) or another viral-encoded protein that exist in complex with a target protein (gp120 and gp41) (FDA, 2007). There have been more than 60 mutations associated with integrase strand transfer inhibitors which occur in the IN-coding region of the *pol* gene. High

levels of INI resistance *in vivo* have been shown to be complex and that treatment failure on INIs is much more common in patients infected with wild-type virus [92].

The most documented form of ART drug resistance is resistance to RT inhibitors. As stated above, nucleotide analogue inhibitors used currently are modified at the 3'-position of the sugar ring and are chain terminators. In their work in the isolation and crystallization of stalled covalently tethered complexes of RT with template and primer, Huang *et al.* (1998) concluded that point mutations occurring in the neighbourhood of the incoming nucleotide are sufficient to cause drug resistance. They also observed that the orientation of the incoming dNTP is affected by a set of protein side chain contacts and changes to these contacts can have an effect on the rate of nucleotide incorporation (the rate of phospholysis has been thought to contribute to AZT resistance) [93]. There are two basic mechanisms associated with NRTIs resistance (**Figure 16**) which are the impairment of the incorporation of the dNTP into DNA, and the removal of the analogue from the prematurely terminated DNA chain. This means that the enzyme has the ability to discriminate between the normal nucleosides and the NRTIs [91], [93].

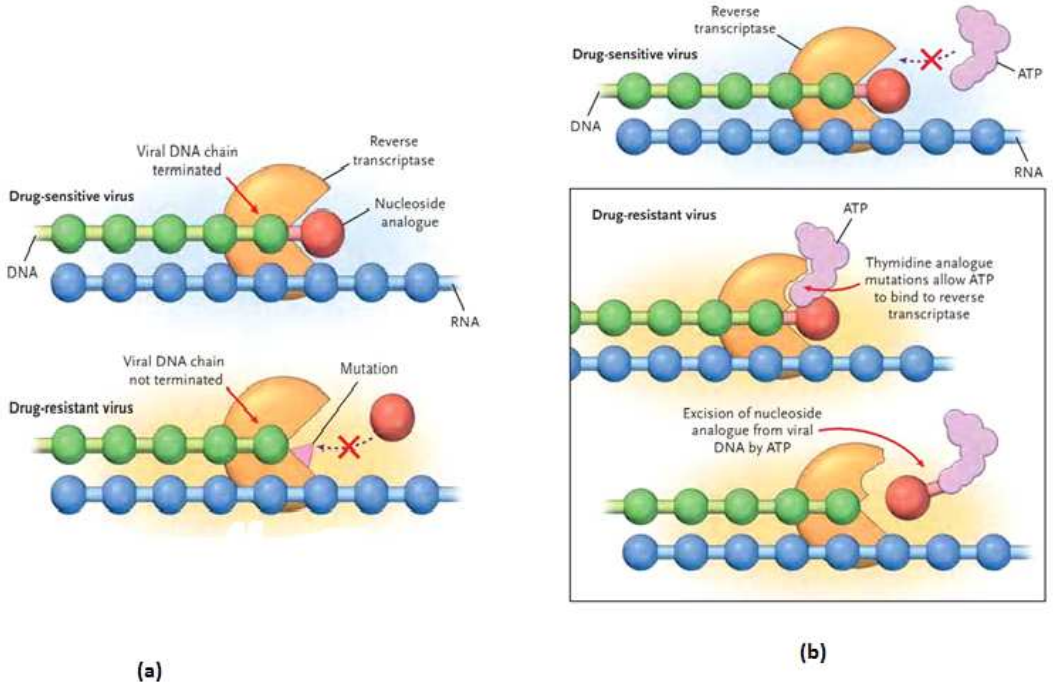


Figure 16: Resistance to nucleoside reverse transcriptase inhibitors. (**A**)The Incorporation of a dNTP analogue into drug-sensitive viral strains results in the termination of DNA elongation. However, mutations in drug-resistant strains prevent this incorporation resulting in viral DNA production even in the presence of chain-terminators. (**B**) In drug-sensitive viral strains, ATP does not have access to the reverse transcriptase; however, thymidine mutations allow ATP to bind to near the 3' end of the incorporated analogue into the DNA strand. This results in the excision of the dNTP analogue, allowing the reverse transcriptase to proceed normally). From: Clavel & Hance 2004 (Reproduced with permission from New England Journal of Medicine, Copyright Massachusetts Medical Society).

Resistance to NNRTIs is mainly due to mutations that occur in and around the NNIBP region (**Figure 17**). These mutations are usually of the amino acids found in the hydrophobic pocket that are responsible for making the pocket hydrophobic. This results in either the loss or the change in hydrophobic interactions. Other possible mechanisms of resistance are steric hindrance and pocket entrance mutations that change the shape of the pocket, making it difficult for the NNRTIs to enter the pocket [93].

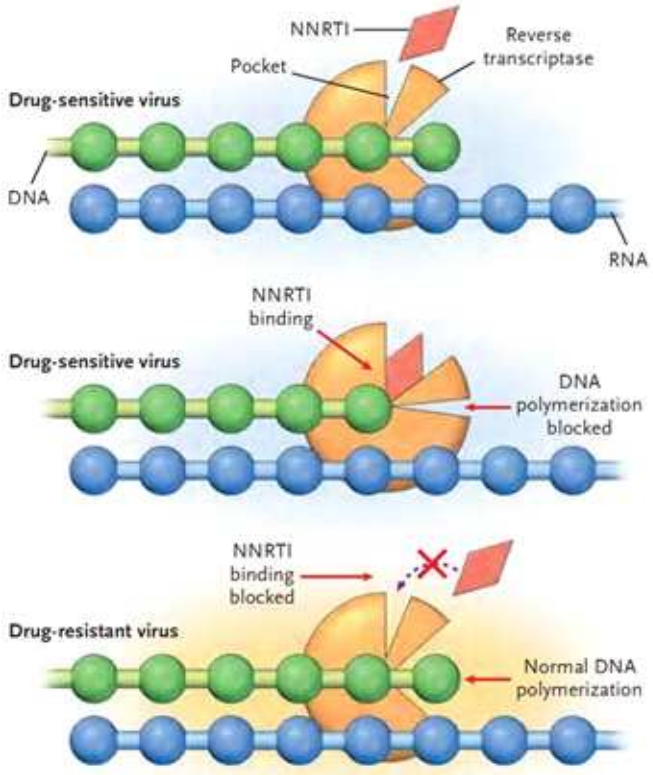


Figure 17: Resistance to non-nucleoside reverse transcriptase inhibitors. In drug sensitive viruses, NNRTIs bind into the adjacent hydrophobic pocket and inhibit polymerization. However, drug resistant viruses possess mutations that prevent the binding of NNRTIs; resulting in normal DNA polymerization. From: Clavel & Hance 2004. (Reproduced with permission from New England Journal of Medicine, Copyright Massachusetts Medical Society).

Changes in amino acids in the active site of the protease enzyme are the major source of resistance to protease inhibitors. These changes directly or indirectly result in the change in the number and nature of contact points between the enzyme and the inhibitors, resulting in reduced drug affinity to the enzyme [94].

Taken together, adverse side effects, emergence of drug resistant strains and the absence of treatment that permanently eliminates HIV infection (therefore requiring lifelong therapy) in order to prevent progression to AIDS, necessitates the development of novel inhibitors that are less toxic and can act against resistant strains. In addition, resistant strains of TB play a role in

the mortality of HIV infected individuals, therefore treatment that is active against both HIV and TB would be of great benefit in the fight against AIDS.

2.3 Metal complexes in medicine

In the past, the importance of metal ions was mainly seen as being that of essential nutrients, however, metal ions are becoming increasingly prevalent diagnostic and therapeutic agents for the study and treatment of a number of diseases and disorders. Metal complexes under analysis include the known essential ions zinc, manganese and copper, as well as ions that have previously been thought off as being poisonous such as selenium and molybdenum [10].

Cisplatin and Carboplatin are platinum based complexes and the most widely used chemotherapeutic agents in the world. These are however associated with a number of limitations including poor aqueous solubility (1 mg/ml) therefore requiring intravenous administration. Toxicity issues (nephrotoxicity, neurotoxicity etc.) and the resistance of tumour cells are also associated with these drugs. This has lead to the investigation of other transition metals, gradually shifting the focus beyond platinum based complexes [11]. This shift has lead to a number of metallodrug discoveries with interesting applications which include:

- anti-cancer activity of iron, copper and zinc through the inhibition of the enzyme ribonucleotide reductase
- inhibition of heme oxygenase by metalloporphyrins, resulting in decreased bilirubin accumulation
- treatment of bipolar disorder with lithium and
- the use of tri-arsenical drugs such as melarsoprol in the treatment of trypanosome mediated illnesses such as African sleeping sickness [95].

This study investigates the bioactivity of gold, platinum and palladium based complexes. The use of gold complexes for medicinal purposes dates back to 2500BC. Throughout prehistory, most major civilizations have recognized the medicinal effects of gold with administration of gold powder potions to ill patients [15 ,95]. The Chinese also used gold flakes or dust for therapeutic effects and Indians used gold to treat leprosy [97]. Monovalent gold cyanide complexes also showed cytotoxic effects on TB bacillus *in vitro*. Gold complexes have been used for the treatment of asthma, pemphigus (an autoimmune disease of the skin) and arthritis. In addition to this vast range of medicinal uses, gold complexes show potential as anti-cancer and anti-HIV therapeutic drugs [98]. The anti-tumour activity of these entities has been investigated for several years and research has been based on three rationales; (1) analogies between square planar complexes of Pt (II) and Au (III), of which both are d^8 ions, (2) analogy to the

immunomodulatory effects of gold(I) anti-arthritic agents and (3) complexation of gold (I) and gold (III) with known anti-tumour agents resulting in new complexes with enhanced activity [96].

2.3.1 Metal complexes and HIV

Observed anti-arthritic and potential anti-tumour activity of gold based complexes has resulted in the study of these complexes as anti-HIV agents. This major interest in gold as an anti-HIV agent was further accentuated by work done by Shapiro and Masci (1996) were a patient who was not receiving any anti-HIV drugs, but being treated with Auranofin for psoriatic arthritis showed an increase in the CD4 cell count [99]. After treatment with Auranofin, the patient showed remarkable clearance of her psoriatic lesions and her arthritis was fully treated. The patient did not show any opportunistic infections in 2 years of follow-up, she gained weight and her CD4 cell count was significantly higher than when she started with the treatment [99]. In addition to that, gold thioglucose (AuSTg) has been reported to be able to inhibit reverse transcriptase (RT) in cell free extracts [98]. More evidence of potential anti-HIV activity of gold is seen in the work done by Fonteh *et al.* (2008) where tetra-chloro-(bis-(3,5-dimethylpyrazolyl)methane)gold(III) chloride demonstrated *in vitro* inhibition of reverse transcriptase and protease. Development of such dual drugs can aid in the reduction of the number of drugs that need to be taken by HIV positive patients (incorporates both components of HAART) and perhaps contribute to compliance [100]. Bis(thioglucose)gold(I) has been found to inhibit the replication of one strain of HIV *in vitro*. This complex is thought to be active based on the interaction of gold(I) with a cysteine residue in a surface protein of the viral envelope. The complex was also shown to be active against reverse transcriptase *in vitro* [101].

In addition to gold based anti-HIV agents, a mixed-valent ruthenium-oxo oxolate complex has also been shown to possess potent activity against both R5 and X4 tropic HIV-1 with a cytoprotective activity toward HIV-1 infected cells. The compound inhibited HIV reverse transcriptase at an IC_{50} of 1.9 nM [102]. Mercury, cadmium and zinc complexes have been reported by Haraguchi *et al.* (1999) to inhibit the expression of HIV-1 antigens in C8166 cells with IC_{50} values ranging from 0.12- 13 µg/ml and zinc chloride was shown to inhibit the transcription of HIV-1 RNA at 100 µg/ml. This inhibition in transcription was proven to be specific to HIV-1 RNA transcription rather than cellular RNA transcription [13]. Lebon *et al.* (2002) reported copper complexes with ability to inhibit HIV-1 protease and inhibition was found to be in a time-dependent manner [103]. Recently nickel, copper, cobalt and zinc metal complexes with aminosulfonated and aminocarboxylated ligands were synthesized by Garcia-Gallego *et al.* (2012), and these complexes had moderate anti-HIV activity, with potential activity as fusion inhibitors. When these complexes where analysed post infection of cells with HIV, some were also capable of inhibiting HIV replication. This could possibly mean that the complexes could be targeting different stages of the viral life cycle, providing HIV infection

preventative and therapeutic behaviour in one single molecule. The authors went on to analyse synergistic effects of the complexes with AZT, and all the complexes were able to enhance the inhibition capabilities of AZT by up to 42 % [104].

The above is evidence that although metal based compounds have not yet been approved for the treatment of HIV, metallodrugs possess great potential as anti-HIV agents.

2.3.2 Metal complexes and Cancer

The discovery of the anti-cancer activity of the platinum based complex Cisplatin, and its analogues Carboplatin and Oxaloplatin (**Figure 18**) have been the driving force behind the investigation of the activity of other transition metals. Cisplatin is one of the extensively used anti-cancer metal complexes with activity against testicular, ovarian, bladder, head and neck cancers [104, 105]. The main target for these complexes is DNA and cisplatin covalently binds to DNA forming adducts that interfere with DNA replication and transcription [107]. The square planar tetra-coordination of platinum allows for its complexes to intercalate into DNA; gold (III) compounds have also been shown to possess this square planar tetra-coordination as their dominant coordination geometry [105]. In fact, these complexes have exhibited really good anti-cancer potencies, with gold (III) porphyrins demonstrating potent *in vitro* anti-cancer activity towards a number of relevant cell lines, including cisplatin and multi-drug resistant cancers [15].

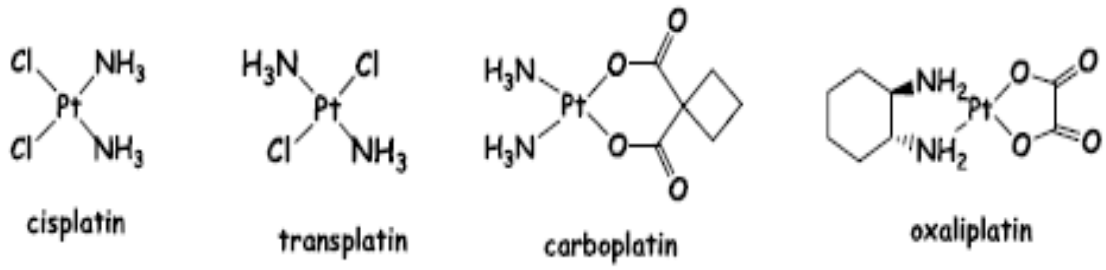


Figure 18: Platinum based anti-cancer drugs. Some of the most relevant platinum complexes in clinical use. The square planar geometry of these compounds allows for intercalation into DNA, hindering molecular processes. From: Casini *et al.* 2008.

Apart from gold (and platinum) there are other transition metals that have exhibited anti-cancer activity. Copper complexes have been reported to induce apoptosis, with 2-benzimidazolecarbamate complex copper(II) bromide showing activity against MCF-7 cells [106]. Harpstrite *et al.* (2007) reported a novel gallium(III) complex with differential cytotoxicity profiles on human epidermal carcinoma cells expressing P-glycoprotein (Pgp). Pgp is a plasma membrane protein and represents one of the best characterized barriers to cancer therapy.

Inhibition of Pgp therefore provides a means of increasing oral absorption of drugs with a reduction in the excretion of the drugs [109]. Such work shows the potential development of other targets (besides DNA) for chemotherapy. The ability of metal complexes to undergo ligand-substitution reactions with bio-molecular targets could result in the discovery of more such targets [107].

2.3.3 Metal complexes and TB

There have been a number of reports of metal based complexes with activity against bacteria and parasites. Silver and its salts have long been known for their antimicrobial activity and this is mainly due to the ability of silver to interfere with electron transport systems in cells. In addition, silver forms interactions with thiol groups on enzymes that are vital for bacterial growth [11]. Evidence of the anti-parasitic properties of metals has been reported by Wasi *et al.* (1987), with effects of various metal complexes on malaria parasites. Here the anti-malaria drugs primaquine and amodiquine were used as ligands to the metal ions [VO(II), Cr(III), Fe(III), Cu(II), Co(II), Ni(II), Zn(II), Cd(II), Hg(II), Rh(III), Pd(II), Au(III), Ag(I), Mn(II), Sn(II) and Pt(II)], and all the metal complexes had activity against *P. falciparum* to the same extent as the parental drugs [110]. The growth of *P. berghei* as well as two chloroquine resistant FcBI and FcB2 strains of *P. falciparum* has been inhibited by the gold-chloroquine complex $[Au(PPh_3)(CO)]PF_6$. Incorporation of the gold fragment resulted in a marked improvement in the efficacy of chloroquine [111].

The modern investigation on the antimicrobial activity of metals was sparked by the discovery of the bacteriostatic behaviour of $KAu(CN)_2$ on tubercle bacillus in the early 1900s. Recently anti-TB copper complexes with Schiff base ligands have been reported by Joseph *et al.* (2012). These complexes exhibited minimum inhibitory concentration (MIC) values ranging between 2 and 60nM and one of the complexes was found to be more active than the known inhibitor ethambutol [112]. Ferreira *et al.* (2012) have also reported a novel organometallic [Pd(C-bzan)(SCN)(dppp)] compound with an ability to inhibit the growth of *M. tuberculosis* at an MIC of 5.15 µM. The high antimycobacterial activity of this compound is said to be attributed to the chelating coordination of the 1,3-bis(diphenylphosphino)propane (dppp) as well as the presence of Ph_2P and N-benzylideneaniline (bzan) lipophilic groups [113].

Metal complexes provide great structural variety overall, with more diverse stereochemistry than organic compounds [114]. This study seeks to determine the activity of novel palladium, gold and platinum compounds, hoping to add to the already spectacular advances that have been made in the field of organometallic chemistry.

2.4 Drug discovery and screening

Before a particular drug can be administered to humans as a therapeutic agent, the product requires sequential rounds of optimization and screening. According to Peters *et al.* (2012), the main aim of the drug discovery process is to "identify drug candidates with the highest possibility of completing clinical trials, reaching the market, and establishing themselves as efficacious and well tolerated, safe medicines" [115].

In the 1970s, lead compounds were discovered by chance, clinical observation of side-effects or the screening of natural and synthetic product libraries which had already undergone considerable biological evaluation before being identified as a lead drug [116]. However, these processes were labour intensive and time consuming, and researchers developed methods with the aim of increasing the efficiency of the drug discovery process [117]. These include high-throughput screening (HTS) and computational structure-based drug design (*in silico* methodologies) [118]. HTS involves the *in vitro* screening of hundreds and thousands of compounds for lead determination. These *in vitro* assays include testing the absorption, distribution, metabolism, elimination and toxicity (ADMET) of the compounds. The main aim of such drug screens is to distinguish between good and bad compounds quite early in the discovery process, therefore saving costs that may arise due to the withdrawal of the product from the market because of adverse side effects. Another advantage of screening large numbers of compounds for a particular function is that a favourable compound can provide a core structure around which hundreds and thousands of variant compounds can be synthesized through structure based design and pharmacophore modelling [118, 119].

Structure-based design involves the prediction of the efficacy of binding of potential ligands to the target protein using molecular modelling which is followed by the subsequent synthesis and biological screening of the specific lead compound. The impact of this method on drug discovery pipeline is shown in **Figure 19**, and there is a large number of protein structure information available that promotes compound identification and optimization, as well as contribute to target identification and validation [117].

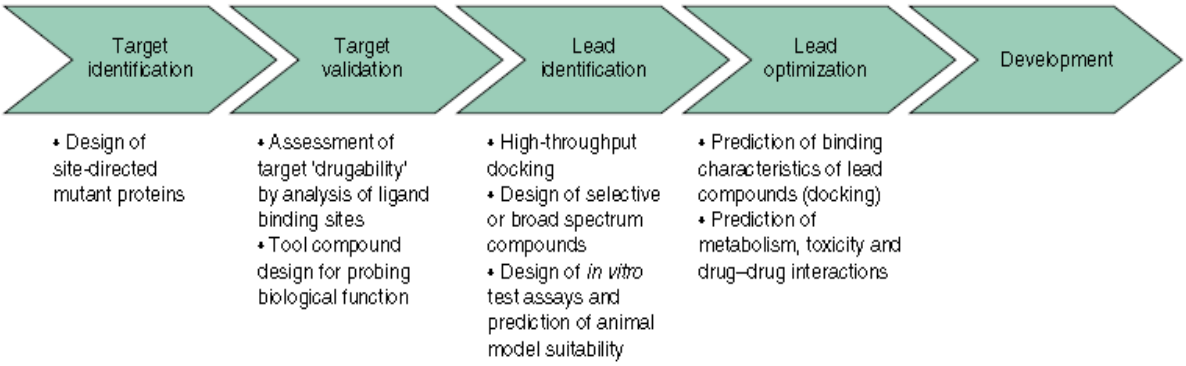


Figure 19: The drug discovery process and impact of structure based drug design. There are five basic steps involved in the identification and development of lead compounds that can enter clinical trials for further development. From: Hillisch *et al.* 2004.

This project is in the lead identifications stage. Available information on the life cycle of the virus has provided potential targets for these complexes and the existence of therapies (HAART) that are already active against the identified targets has proven their "drugability". Compounds presented here were partially subject to the lead optimization stage, where drug toxicity (hepatotoxicity) predictions along with solubility, plasma protein binding and blood-brain-barrier penetration were predicted using *in silico* ADMET predictors.

2.5 Hypothesis

Based on the above the hypothesis of this study was:

Metal complexes (containing palladium, platinum and gold) could serve as lead compounds against HIV and its associated opportunistic infections, in particular tuberculosis and cancer.

2.6 Aims

In order to prove the hypothesis, palladium, platinum and gold containing phosphine complexes were synthesized and characterized by collaborators. Answers to the following questions were then investigated:

- **Can the metal complexes inhibit the activity of HIV-1 RT, PR and IN?** These are the most important enzymes in the life cycle of the retrovirus and existing treatment are effective inhibitors of these enzymes but with severe toxicity and metal complexes with activities against some of these have been developed.

- **What are the cell cytotoxicity 50% (CC_{50}) values of these complexes on peripheral blood mononuclear cells (PBMCs) and TZM-bl cells?** Active complexes should preferably show enzyme inhibition at concentrations that are way below the CC_{50} value

of that complex. These cells are model cells for HIV research and therefore relevant to this study.

- **What are the effects of these complexes on cell proliferation and can this be observed through flow cytometry and real time cell analysis?** Ideal anti-HIV therapeutics should promote the proliferation of CD8 T-cells and cells providing protective immunity.

- **Can the complexes inhibit the growth of HeLa cells and if so, what is the selectivity index for each complex?** One of the major pathologies that are associated with HIV is cervical cancer. Metal complexes have been shown to be effective chemotherapeutic agents. HeLa cells are a cervical cancer cell line, inhibition of the growth of these cells could mean the potential chemotherapeutic activity of these complexes.

- **Do the compounds exhibit anti-*M. tuberculosis* activity, and if so, what is the minimum inhibitory concentration for the active complexes?** *M. tuberculosis* induced TB is the major cause of death in HIV infected individuals. A palladium based complex has been reported to possess activity against the bacterium.

- **What is the overall drug-likeness of each complex based on *in silico* ADMET analysis, and how do these compare to the current HAART?** ADMET predictions form part of the lead optimization stage of the drug discovery process. Active complexes should at least show improved drug-like properties in comparison to current HAART.

3. MATERIALS AND METHODS

All used reagents were of analytical and cell-culture grade. Please see **Appendix I** for the list of all reagents and company information. All used formulae are also presented in **Appendix I.**

3.1 Compound Information

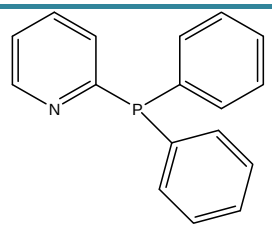
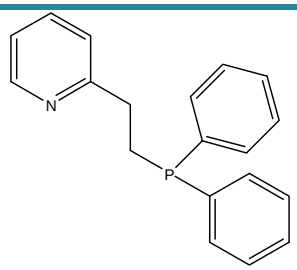
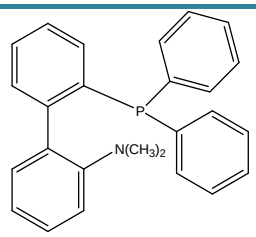
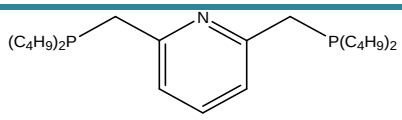
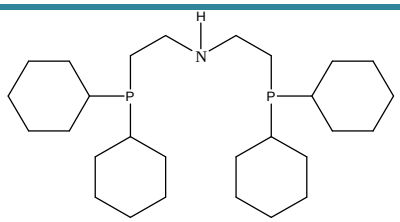
All the compounds were synthesized by Afag Elkhadir at the Chemistry Department of the University of Johannesburg under the supervision of Prof. J. Darkwa. A brief description of compound synthesis follows below. The ligands are those compounds without a metal atom (named **AEL1-6**), while complex is used to describe the ligand bounded to a metal ion(s). The word "compound" collectively includes the ligands and the complexes. The complexes that were tested in this study have been grouped according to the ligand types that were used in their synthesis.

Method: Briefly, during synthesis all manipulations were done by standard Schelnk techniques and all utilized solvents were of analytical grade. The Braun MB SPS 800 drying solvent system was used for drying the compounds. All the ligands were purchased and used as received (Sigma-Aldrich, Germany). Three types of metal complexes were synthesized, palladium, platinum and gold complexes. Palladium dichloride [$PdCl_2$] and potassium tetrachloroplatinate [$K_2(PtCl_4)$] were obtained from the South African Precious Metals and gold (III) chloride [$AuCl_3$] was purchased (Sigma-Aldrich, Germany). The starting materials; bis(acetonitrile)dichloro Pd(II) [$PdCl_2(NCMe)_2$], cis/transdichlorobis(dimethylsulfide)Pt(II) [$PtCl_2(SMe_2)_2$] and chlorotetrahydrothiopen gold(I) [Au(THT)Cl] were synthesized following literature procedures from Rülke *et al.* (1990) and Van Asslet *et al.* (1994). Details on the synthesis and structure confirmation are described in the thesis of Afag Elkhadir, University of Johannesburg (2013).

3.1.1 Compound structures

The table below (**Table 1**) represents all the ligands that were used to synthesize the metal complexes. All the ligands were phosphine ligands with the presence of at least one phosphorus ion.

Table 1: Structures of all the ligands used for complex synthesis

Sample Code and MW(g/mol)	Formula	Name	Structure
AEL1 263.28	$C_{17}H_{14}NP$	Diphenyl-2-pyridylphosphine	
AEL2 291.33	$C_{19}H_{18}NP$	2-(2-(diphenylphosphino)ethyl)pyridine	
AEL4 381.45	$C_{26}H_{24}NP$	2-(Diphenylphosphino)-2'-(N,N-dimethylamino)biphenyl	
AEL5 395.54	$C_{23}H_{42}NP_2$	2,6-Bis(di-t-butylphosphinomethyl)pyridine	
AEL6 465.67	$C_{28}H_{53}NP_2$	Bis[2-dicyclohexylphosphino)ethyl]amine	

Tables 2, 3 and **4** below show all metal complexes that were synthesized and tested in this study, and these have been classified based on the type of ligand binding to the metal ion. The metal ions used during synthesis were palladium, platinum and gold; except for complex **AE177** which was a palladium-gold bimetal.

Table 2: Diphenyl-2-pyridylphosphine (AEL1) complexes

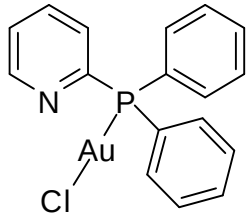
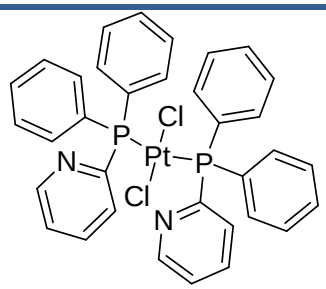
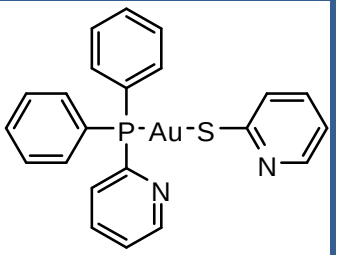
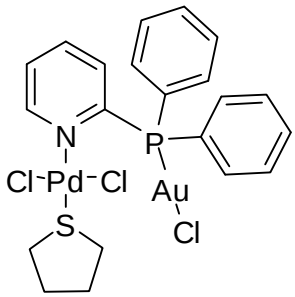
Sample Code and Molecular Weight (g/mol)	Formula	Full Name	Structure
AE20 495.69	$C_{17}H_{14}NPAuCl$ Au(L**1**)Cl	Diphenylphosphino-2-pyridylgold(I) chloride	
AE72 792.53	$C_{34}H_{28}N_2P_2PtCl_2$ $PtCl_2$(**L1**)$_2$	Dichloro-*bis*(diphenylphosphino)-2-pyridylplatinum(II)	
AE359 570.40	$C_{22}H_{18}N_2PAuS$	2-pyridinethiolgold(I)diphenylphosphino-2-pyridine	
AE177 761.19	$C_{21}H_{22}AuCl_3NPPdS$ $AuPdCl_3$(**L1**)(SC_4H_8)	*bis*-palladium(tetrahydrothiophene)-[(diphenylphosphino-2-pyridyl)]AuCl chloride	

Table 3: Complexes of the ligands 2-(2-(diphenylphosphino)ethyl)-pyridine (AEL2) and 2-(Diphenylophosphino)-2'-(N,N-dimethylamino)biphenyl (AEL4)

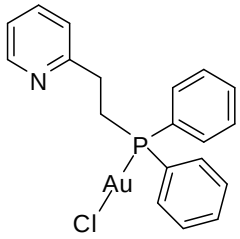
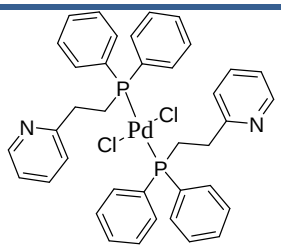
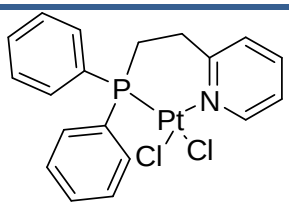
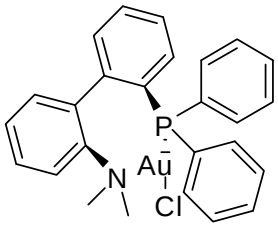
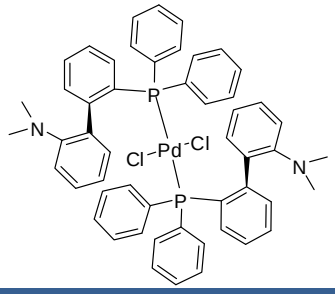
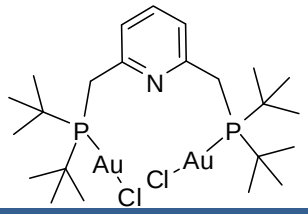
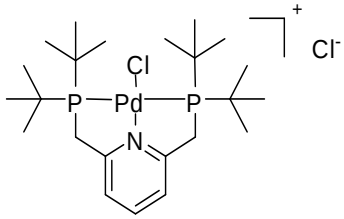
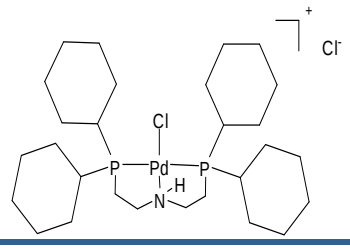
Sample Code and Molecular Weight (g/mol)	Formula	Full Name	Structure
AE76 523.75	$C_{19}H_{18}NPAuCl$ Au(L**2**)Cl	2-(2-(diphenylphosphino)ethyl)pyridylgold(I) chloride	
AE99 759.97	$C_{38}H_{36}N_2P_2PdCl_2$ $PdCl_2(L\mathbf{2})_2$	Dichloro-*bis*-2-(2-(diphenylphosphino)-ethyl)pyridylpalladium(II)	
AE98 557.31	$C_{19}H_{18}NPPtCl_2$ $PtCl_2(L\mathbf{2})$	dichloro-(2-(2-(diphenylphosphino)-ethyl)pyridylplatinum(II)	
AE125 613.87	$C_{26}H_{24}NPAuCl$ Au(L**4**)Cl	2-(diphenylphosphino)-2'-(N,N-dimethylamino)biphenylgold(I) chloride	
AE188 940.22	$C_{52}H_{48}N_2P_2PdCl_2$ $PdCl_2(L\mathbf{4})_2$	Dichloro-*bis*(2-(diphenylphosphino)-2'-(N,N-dimethylamino)biphenylpalladium(II)	

Table 4: Complexes of 2,6-*bis*(di-t-butylphosphinomethyl)pyridine (AEL5) and Bis[2-dicyclohexylphosphino)ethyl]amine (AEL6).

Sample Code and Molecular Weight (g/mol)	Formula	Full Name	Structure
AE205 860.38	$Au_2C_{23}H_{43}NP_2Cl_2$ Au_2(L**5**)Cl_2	2-6-*bis*(di-t-butylphosphinomethyl)-pyridylgold(I) chloride	
AE189 572.87	C23H43NP2Pd Cl2 PdCl2(L**5**)	2, 6-[(di-t-Butylphosphino)-methyl]pyridylpalladium(II) chloride	
AE190 572.87	$C_{28}H_{53}NP_2PdCl_2$ $PdCl_2$(L**6**)	dichloro*bis*[2-(di-Cyclohexylphosphino)-ethyl]aminepalladium(II))	

3.2 HIV-1 Inhibition

The metal complexes were tested for their inhibition of three of the major enzymes involved in viral replication; HIV reverse transcriptase, integrase and protease. All tested concentrations were within concentration-ranges that had previously been tested in enzyme inhibition assays for metal complexes [99, 122].

3.2.1 Reverse Transcriptase inhibition assay

Rationale: The HIV-RT assay is based on the ability of the enzyme (RT) to synthesize DNA starting from the poly(A) × oligo $(dT)_{15}$ template. The detection and quantification of the synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol, where Biotin-labelled DNA binds to Streptavidin micro plate modules (**Figure 20**). Next the digoxigenin that has been conjugated to peroxidase (anti-DIG-POD) binds to the labelled DNA. Addition of the peroxidase substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABST results in the cleavage of the substrate by peroxidase, which gives rise to a coloured reaction product. The absorbance of the samples can then be determined using a micro plate reader. Absorbance values are then correlated to the level of RT activity in the sample (www.roche-applied-science.com).

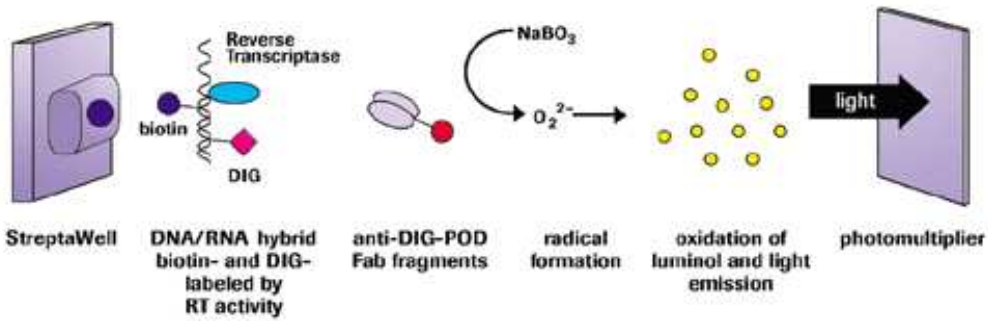


Figure 20: Principle of the HIV-1 reverse transcriptase inhibition assay. From: www.roche-applied-science.com

Method: A Reverse Transcriptase Assay Calorimetric Kit (Roche Applied Science, Germany) was used for the determination of RT inhibition, and the assay was performed according to the manufacturer's instructions and as previously described [100]. Briefly, 20 µL (0.2 U) of HIV-1 recombinant RT enzyme (Merck, Germany) and 20µL of reaction mixture were transferred into micro tubes containing 20 µL of compound sample diluted in lysis buffer and resulted in final compound concentrations of 25, 10 and 1 µM. The reaction mixture consisted of reconstituted template-template/primer hybrid poly(A), Oligo (dT)15 and diluted nucleotide (tris-HCl, 50 mM, pH7.8, with DIG-dUTP, biotin-dUTP and dTTP).The following controls were included in the analysis:

- **positive control**- enzyme+ lysis buffer+ reaction mixture, without any inhibitor
- **negative control**- lysis buffer and reaction mixture, no enzyme

- a **known inhibitor** of RT (BBR) developed in the HIV/AIDS research laboratory was also included [100].

The reactions were incubated at 37℃ for an hour. A reaction plate was prepared using micro plate modules that had been pre-coated with streptavidin. The samples were transferred to the wells (60 µL of sample per well) and the plate was covered with foil and incubated for an hour at 37℃.

The plate was then rinsed 5 times with washing buffer (250 µL) and was blotted on paper towel to completely remove buffer. Anti-DIG-POD working stock solution (200 mU/mL) was added to each well (200 µL). The plate was covered with foil and incubated for 1 hour at 37℃. Thereafter the solution was completely removed and the wells were rinsed with 250 µL washing buffer 5 times. The ABST was then prepared by dissolving 1 tablet in 5 mL substrate buffer and the solution was protected from light by covering with foil. ABTS substrate was added to each well (200 µL) and the plate was incubated at room temperature protected from light by covering with foil until sufficient colour development (green) for photometric detection was obtained (approximately 10 min). The absorbance was measured at 405 nm using a Multiskan Ascent plate reader (Thermo Labsystems). Enzyme activity was calculated using Microsoft Excel (Microsoft Corporation, USA). See **Appendix II, Formula 1** for the calculation of enzyme activity and percentage inhibition.

3.2.2 Protease inhibition assay

Rationale: The HIV-1 protease inhibition assay is based on the ability of HIV protease to cleave a flourogenic HIV protease substrate-1 (Sigma Aldrich, USA). The substrate has the structure: Arg-Glu-(**EDANS**)-Ser-Gln-Asn-**Tyr-Pro**-Ile-Val-Gln-Lys-(**DABCYL**)-Arg. **EDANS** stands for 5-(2-aminoethylamino)-1-naphtalene sulfonate, and **DABCLY** is 4'-dimethylaminoazobenzene-4-carboxylate. The Try-Pro bond is the cleavage site for HIV protease. The substrate also has 2 covalently modified amino acids that can be used to detect cleavage [125].

Method: The protease substrate was dissolved in DMSO to prepare a 1 mM stock. This substrate stock was then diluted to 20 µM with assay buffer (0.1 M sodium acetate, 1 M NaCl, 1mM EDTA, 1 mM dithiothreitol and 1mg/ml bovine serum albumin; pH 4.7). The protease enzyme (Bachem, Switzerland) was diluted to final concentration of 1 ug/mL in assay buffer. The compounds to be tested for HIV-protease inhibition were prepared to a final concentration of 200 µM (final assay concentration was 100 µM). Acetyl pepstatin (AP, Sigma, Germany), the positive control, was prepared in DMSO (1 mg/ml) and the reactions were performed in a black costar 96-well plate (plate layout provided in **Table 5**). The fluorescent substrate was added

last, and this was done in the dark. The plate was incubated at 37℃ for an hour covered with foil to protect the substrate from light. An endpoint fluorescence measurement on a Flouroskan plate reader was then performed at an excitation wavelength of 355 nm and emission of 460 nm (Flouroskan Ascent FL, Thermo Lab Systems, USA). Enzyme activity and inhibition was calculated similarly to reverse transcriptase.

Table 5: Reaction contents for the HIV-1 protease inhibition assay.

	Negative Control (Blank) (µL)	**Untreated Control (µL)**		**Positive treated control (AP-10 µg/ml)**	**Final Compound concentration (µM)**			
					100	**25**	**10**	**5**
DMSO			10					
Assay Buffer	51	50	40	40		37.5	45	47.5
Enzyme (1µg/mL)			1	1	1	1	1	1
Substrate (10µM)	49	49	49	49	49	49	49	49
Inhibitor (Compound)				10	50	12.5	5	2.5
Final Volume	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

3.2.3 Integrase inhibition assay

Rationale: The Xpressbio HIV-1 Integrase assay kit (Expressbio Life Science Products, USA) was used to detect the inhibition of HIV-1 integrase by the complexes. The assay is a non-radioactive assay that detects the inhibition of the strand transfer reaction. The assay uses two-types of oligonucleotide substrates. The first substrate (donor substrate, DS) has the sequence 5'-ACTGCTAGAGATTTTCCACACTGACTAAAAGGTC-3' and is labelled with biotin at the 3' end. This biotin binds to the plate which is coated with streptavidin. The second oligonucleotide substrate (target substrate, TS) is labelled with digoxigenin at the 5' end and has the sequence 5'-GACCCTTTTAGTCAGTCAGTGTGGCAAAATCTCTAGCAGT-3'. For the stand-transfer reaction to take place, HIV-1 integrase cleaves the terminal two bases at the 3' end of the donor substrate and catalyses a strand-transfer reaction to integrate the donor substrate to the target

substrate. An HRP-labelled antibody that is directed against the digoxigenin on the 5' end of the target substrate is then added for the detection of reaction products [123], [126]

Method: Reaction buffer and blocking buffer were warmed to 37℃ (water bath, 10 min) and all the other kit components were warmed to room temperature except for the integrase enzyme. The streptavidin-coated plate strips were then coated with DS and these were diluted 100-fold in reaction buffer. The diluted DS DNA was added to each well (100 µL) and the plate was incubated (30 min at 37℃). The liquid was aspirate d and the plate was washed with 300 µL of 1× wash buffer per well. Blocking buffer was added to each well (200 µL) and the plate was incubated (37℃ for 30 min). The HIV-1 integrase en zyme (2 µM) was thawed on ice for 5 min before use and diluted at a ratio of 1:300 in reaction buffer by adding 2 µL enzyme and 598 µL reaction buffer. After the incubation the liquid was aspirated and the plate was washed three times with 200 µL reaction buffer. Thereafter integrase enzyme (100 µL) or reaction buffer (negative control) was added per well and the plate incubated for 30 min at 37℃. The compounds to be tested were prepared by diluting to two times the final desired test concentration (for final concentration of 10 µM, 20 µM was prepared). Sodium azide (20%, v/v) was used as a known inhibitor. The liquid was aspirated from the wells and the plate was washed three times with 200 µL reaction buffer per well. The compounds were added to their respective wells (50 µL per well) and the plate was incubated at room temperature (5 min). Target substrate (TS) DNA was prepared by diluting 10 µL TS DNA 100× solution with 990 µL reaction buffer. Diluted TS DNA was added (50 µL) per well directly to the 50 µL buffer/compounds already present in the wells. The plate was mixed by tapping 3-5 times and then incubated (37℃, 30 min). After incubation the liquid was removed and the plate was washed five times with 300µL wash solution. The reaction products were detected by adding horseradish peroxidase (HRP) antibody to each well (100 µL) and incubating (37℃; 30 min). Thereafter the liquid was aspirated and the plate was washed five times with 300 µL wash solution per well. Thereafter 3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase was added to each well (100 µL) and the plate was incubated at room temperature (10 min). TMB stop solution (100 µL) was then added to each well and the absorbance was read at 450nm (Multiskan Ascent, Thermo Labsystems, USA). Enzyme activity was calculated using Microsoft Excel (Microsoft Corporation, USA). See **Appendix II, Formula 1** for the calculation of enzyme activity and percentage inhibition.

3.3 Cell Viability and Proliferation

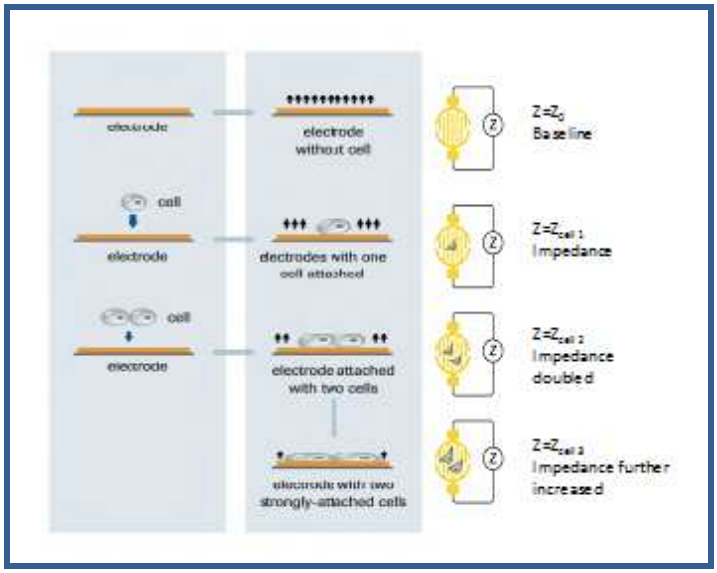
The effect of the compounds on cell viability was determined using two cell types; TZM-bl cells (a HeLa cell line derivative expressing high levels of CD4 and CCR5 as well as endogenous expressed CXCR4) and peripheral blood mononuclear cells (PBMCs). These cells are isolated from whole blood and basically encompass any nucleated cells found in the blood that play a role in immune responses. A major fraction of the PBMCs are $CD4^+$ T-lymphocytes which are targeT-cells for HIV infection. Also, $CD4^+$ T-lymphocytes and macrophages are able to produce both CCR5 and CXCR4 co-receptors, enabling infection by both R5 and X4 viruses. This makes these cells models for HIV infection and relevant to this study [126, 127].

Cell viability assays are usually carried out to assess the effects of a particular drug on a selected cell population or cell-line. There are a variety of viability dyes available for this purpose, but two dyes were used in this study. The 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay was developed by Mosmann in 1983 and is based on the transformation of the tetrazolium salt into purple formazan crystals by the enzyme succinic dehydrogenase in viable cells [129]. The formazan product accumulates in healthy cells as it is impermeable to the cell membrane and the amount of viable cells can be determined by absorbance readings using an ELISA reader post solubilisation of the produced formazan crystals. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenylo-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) dye works in a similar manner as MTT and the major difference between these two is that MTS produces a soluble formazan product, eliminating the solubilisation step required in the MTT assay [130]. There have been reports of significant variations in MTT-formazan production among different cell types as well as the degree of saturability of the assay [131]. This was confirmed in this lab where it was shown that MTT is not properly metabolized in PBMCs, and thus MTS was utilized for PBMC viability assessments. MTT was used for TZM-bl cells [132]. An important factor that affects cell viability is the concentration at which the compounds are being administered. The main aim is to improve the drug efficacy while minimizing undesirable side effects. In this study, serial dilutions of the compounds were analysed, and the best compound concentration would be one that does minimal damage to normal cells.

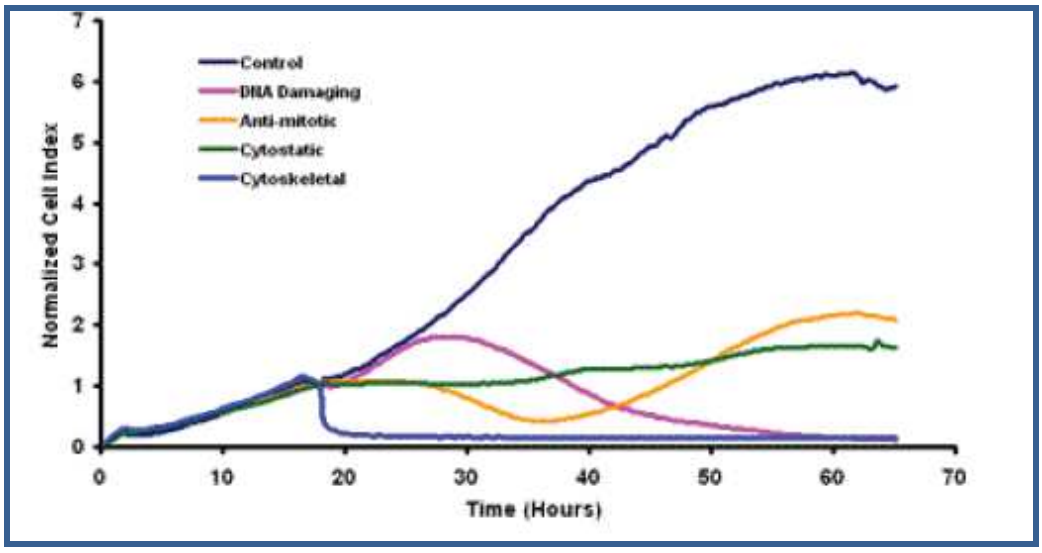
In addition to viability studies, the compounds were analysed for their effects on the proliferation of the cells. Carboxylflourescein diacetate succinimidyl ester (CFSE) staining and flow cytometry was used in PBMCs as flow cytometry works on cells in suspension. The assay is based on the lipophilic molecule (CFDA-SE) that is transported inside the cell where its acetyl groups are cleaved by esterases resulting in a markedly fluorescent molecule (CFSE) that covalently binds to proteins and is well retained in the cell. Flow cytometric analysis is based on

the serial halving of the fluorescence intensity as the cells divide. Halving of the cellular fluorescence intensity marks each successive generation in a population of proliferating cells, which can be readily followed by flow cytometry [133].

Since TZMbl cells are an adherent cell line and the re-suspension of these cells post treatment for flow cytometric analysis could be invasive and introduce variables, effects of the metal compounds on proliferation of these cells was determined using a real-time cell analyser (RTCA). The system offers label-free detection and is based on electronic impedance measurement. In the XCelligence platform (Roche Applied Science, Basel, Switzerland) impedance is generated from adherent T-cells attached to the gold electrodes at the bottom of the plate wells. Presence of cells on the wells has an effect on the local environment resulting in the increase in the impedance as the cells attach (**Figure 21A**). The more the cells attach, the greater the impedance and this is interpreted as the cell index (CI) [127, 131]. In addition to the real-time monitoring of cell proliferation, the system has an added advantage of detecting unique growth profiles that could be induced by compound treatment and are dependent on the biological mechanism of action of the compound (**Figure 21B**). These profiles include cytostatic, antimitotic, and cytoskeletal behaviour [134].



(A)



(B)

Figure 21: Diagram of the micro-electrodes on the bottom of the wells of an E-plate (A) and the advantage of compound-specific profiling offered by the XCelligence platform (A). (**A**) The cell index is zero when there are no cells present in the well. Addition of the cells results in the increase of the CI, which continues to increase as the cells propagate. (**A**) The system can also predict the potential mechanism of action of a particular compound, and these are based on the profiles of compounds with known mechanism. From: www.roche-applied-science.com.

Overall; viability dyes were used to perform initial viability screens as well as to obtain cell cytotoxicity (CC_{50}) values for each compound. These were then followed by more sensitive techniques (flow cytometry and RTCA) which offer a detailed look at the proliferation of these cells in the presence of the metal compounds. CFSE in flow cytometry showed whether the cells progressed to the daughter generation or remained in the parent generation, while RTCA investigated the type of compound mediated toxicity based on known profiles (**Figure 21B**).

3.3.1 Cell Culture

TZM-bl cells were first thawed in Dulbecco Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) supplemented with 5% HEPES buffer (v/v) (Gibco, Life Technologies, USA), 1.1% sodium pyruvate (v/v) (Hyclone, Thermo Scientific, USA), 1% Gentamicin (v/v) (Sigma-Aldrich, USA) and 20% foetal bovine serum (v/v) (FBS, Gibco, Life Technologies, USA) and incubated over night (37℃; 5% CO_2, 95% humidity) in a $75cm_3$ culture flask. The next day, 5ml spent media was removed and DMEM media supplemented with 10% FBS (v/v) was added (15ml). The cells were propagated for 2-3 days and upon reaching confluence, the cells were detached from the bottom of the flask through trypsinization. Briefly, all the media was removed and the layer of cells was washed with pre-warmed 1% PBS (v/v) in order to remove all residual media. Three millilitres of Trypsin-EDTA (Sigma-Aldrich, UK) was then added to the flask and left for 30 sec at room temperature. Thereafter the trypsin was removed and the plate was incubated for 3 min (37℃, 5% CO_2, 95% humidity). The cells were counted using a hemacytometer, and re-suspended at 1×10^5 cell/ml [135].

3.3.2 PBMC isolation

Ethics clearance for this research was obtained from both the Faculty of Natural and Agricultural Sciences and the Health Science Ethics Committees (University of Pretoria) with approval numbers EC080506-019 and 163/2008. Blood was collected from consenting HIV negative donors (Student Health Services, University of Pretoria) in EDTA anti-coagulant Vacuette® tubes (Greiner Bio-one, Austria). The blood was diluted 1:1 with RPMI media (Sigma Aldrich, USA) with 2 g/L $NaHCO_2$, 0.05% (v/v) Gentamycin and antibiotic/antimycotic. The diluted blood was layered onto Ficoll-Histopaque®-10771 (Sigma Aldrich, UK) at a 2:1 ratio. The tube was centrifuged at 1500 ×g for 30 min (Allegra 25R Centrifuge, Beckman Coulter, USA) and thereafter the buffy coat (white cloudy layer) was transferred to a new 50 mL falcon tube. RPMI media (30ml) was added and the tube was centrifuged (850 ×g for 10 min) to wash off platelets. The supernatant was discarded and Ammonium Chloride Potassium (ACK; 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was added to the cells (5 mL) in order to lyse any remaining erythrocytes and incubated for 5 min at room temperature. RPMI media was then added to wash off the lysis buffer and the tube was centrifuged (250 ×g for 10 min). The supernatant was discarded and the pellet was re-suspended in 5 mL RPMI media supplemented with 10% FBS (v/v). Cell counting was performed with a 10x dilution of the cells with tryphan blue and a hemacytometer. See **Appendix II, Formula 3** for the formula for calculating cell viability. A cell viability of greater than 90% was acceptable for further analysis. The cells were then diluted to a final concentration of 1×10^6 cells/ml. Phytohemagglutinin-protein (PHA, 4 µg/ml; Sigma Aldrich, Germany) was then added in order to stimulate cell

growth and the cells were incubated for 2 hours (37℃; 5% CO_2, 95% humidity) before treatment with the different compounds.

3.3.3 MTT Assay

DMEM media was added to a 96-well plate and a dilution series of the compounds was performed resulting in concentrations of 1.56- 100 µM or 0.78- 50 µM. TZM-bl cells were treated at a concentration of 1×10^5cells/ml per well. The plates were then incubated for 3 days (37℃, 5% CO_2, 95% humidity). After the 3 day incubation, the plates were centrifuged (250 ×g for 10min) and 160µl of the spent media was removed. MTT (Sigma Aldrich, Germany) working solution (5.0 mg/ml MTT in DMEM, 1:5 ratio, 100 µL) was added to each well and the plate was incubated for 2 h (37° C, 5% CO_2, 95% humidity). Formazan crystals produced after incubation were solubilized with acidified isopropanol (1 ml of 1 M HCl: 9 ml propanol) [136]. The absorbance of the samples at 550 nm was measured against a background control (media only) as well as untreated controls (cells only) using a microtiter plate reader (Multiskan Ascent, Thermo Labsystems, USA). The percentage viability was calculated (see **Appendix II, Formula 3**) and GraphPad Prism 5 (GraphPad Software, Inc., USA) was used to obtain the compound concentration resulting in death of 50% of the cells (CC_{50}).

3.3.4 MTS Assay

The CellTiter 96® AQ_{ueous} One Solution (Promega, USA) was used in the assay and PBMCs were treated in a similar way as for the MTT assay. After 3 days of incubation (37℃, 5% CO_2, 95% humidity) the plate was centrifuged (250 ×g, 10 min) and 160 µL of spent media was removed from each well. A working solution of MTS solution (1:9 dilution of the MTS solution with RPMI media supplemented with 10%FBS) was then added to each well (100 µL) and the plates were incubated for a further 24 hours (37℃, 5% CO_2, 95% humidity). The absorbance of the samples was then read at a wavelength of 492 nm, with a reference wavelength of 690 nm using a microtiter plate reader. The percentage viability was calculated in a similar manner as above and GraphPad Prism 5 was used to obtain CC_{50} values [119].

3.3.5 Carboxyfluorescein diacetate succinimidyl ester staining and Flow Cytometry

The proliferation of PBMCs was monitored using the CellTrace™ CFSE kit (Molecular Probes, Oregon, USA). The assay was performed as described by Wang *et al.* (2005). Briefly, PBMCs were isolated as described above and harvested (800 ×g, 10 minutes) from RPMI media with pre-warmed 5% FBS/PBS (v/v). The cells were then re-suspended in the same solution at a concentration of 1×10^7 cells/ml and an aliquot of that sample was stored and used as an unstained sample. In order to obtain uniform staining, half of the required volume of 5% FBS/PBS was used to dilute the CFSE stain before adding it to the cells so that uniform

staining can be obtained. For each 1×10^7 cells/ml, 1 µL of 5 mM CFSE was added and the cells were gently pipetted with the stain to obtain uniform labelling. The cell-dye mixture was then incubated at 37℃ for 15 minutes with gentle shaking in between. The staining was then quenched by the addition of 5 volumes of ice-cold RPMI media supplemented with 10% FBS (v/v). The cells were then incubated on ice for 5 minutes and then pelleted by centrifugation (800×g for 10 minutes). Unincorporated CFSE was removed through washing by re-suspending the pellet in cold media again and this was done for a total of three washes (X.Q. Wang *et al.* 2005). PHA was added for the stimulation of cell growth and the cells were incubated for 2 hours (37℃, 5% CO_2, 95% humidity). The cells were then treated with the compound at various concentrations and the plates were incubated for 3 days (37℃, 5% CO_2, 95% humidity). After incubation the contents of each well were then transferred into polystyrene round-bottom tubes (BD, USA) for FACS analysis and centrifuged (250 ×g, 10 min). The supernatant was removed and wash steps were performed by re-suspending the cells in ice-cold 1% PBS (1 mL). These wash steps were repeated twice. After the third wash, 600 µL of the supernatant was removed [137]. The cells were stained with Propidium Iodide (PI) (2 µl/tube, 15 min). Flow cytometry analysis for each sample was then performed using the BD FACSAria II (BD, USA).

3.3.6 Real Time Cell Analysis

The assay was only carried out for compounds that had showed toxicity in the MTT assay (CC_{50} below 20 µM). The main reason for this was to determine the mode of compound mediated cytotoxicity and attempt to profile these toxic metal complexes. The XCelligence (Roche Diagnostics, Germany) platform was used for the real time analysis of TZM-bl cells treated with different concentrations of the metal compounds. The assay was performed according to manufacturer's instructions. Briefly, the cells were plated (25 000 cells/well) and allowed to attach for at least 24 hours (37° C, 5% CO_2, 95% humidity). The cells were then treated with four different complex concentrations (40, 20, 10 and 5 µM). Auranofin at 10 µM (Sigma Aldrich, Germany) was used as a positive control for toxicity. Proliferation was automatically monitored every minute for 1 hour and then every 30 min for 3 days. Changes in the cell index values indicated the different effects of complexes on cells; increasing CI for proliferating cells and decreasing CI suggestive of cytotoxicity [132].

3.4 Anti-cancer activity

3.4.1 Cell Culture

HeLa cells were thawed in Minimum Essential Media (Separations, South Africa) supplemented with 20% FBS and 5% antibiotic/antimitotic and incubated over night (37℃; 5% CO_2, 95% humidity) in a large flask. The next day all the media was removed and fresh MEM media (10% FBS; 5% antibiotic/antimytotic; 15 mL) was added to the cells. The cells were left over night to reach confluence and the next day the cells were detached from the bottom of the flask through trypsinization. Briefly, all the media was removed and the layer of cells was washed with pre-warmed 1% PBS (v/v) in order to remove all residual media. Trypsin-EDTA was then added to the flask which was then left for 30 sec at room temperature. Thereafter the trypsin was removed and the plate was incubated for 3 min (37℃, 5%CO_2, 95% humidity). The cells were re-suspended in MEM media (5% FBS; 5% antibiotic/antimytotic) and counted using a hemacytometer.

3.4.2 Effects of metal compounds on HeLa cell growth; MTT assay

MTT has been extensively used in cancer studies for the determination of potential toxicity of drugs on various cancer cell lines [138]. The assay was performed as described by Marzano *et al.* (2007).Briefly, HeLa cells were re-suspended to 1×10^5cells/ml and were plated in a 96 well plate (100 µL of cells per well). The plate was incubated (37℃; 5% CO_2, 95% humidity) over night to allow for the cells to attach. Media was added to a separate 96-well plate and a dilution series of the compounds was performed. The compounds were then transferred to the plate with cells resulting in final compound concentrations of 0.78- 50 µM. The plate was then incubated for 3 days (37℃; 5% CO_2, 95% humidity). The plates were then treated as described in **Section 3.3.3** above and GraphPad Prism 5 was used to obtain CC_{50} values [139].

3.5 Effects of metal complexes on Tuberculosis

All anti- *Mycobacterium tuberculosis* assays were performed at the Plant Science Department at the University of Pretoria under the supervision of Prof. N Lall.

3.5.1 *M. tuberculosis* inhibition assay

Rationale: The ability of the metal compounds to inhibit *M. tuberculosis* was evaluated using the alamar blue fluorescent dye. Alamar blue has been extensively used in the determination of bacterial growth and these include bacteria such as *Staphylococcus aureus* [140]. The dye is an oxidation-reduction dye and it is generally used for the determination of cellular growth and/or viability. The assay is based on the oxidation of a blue non-fluorescent resazurin salt into a red/pink fluorescent resofurin salt by viable bacteria [141]. Bacterial growth can then be measured using a fluorometer, spectrophotometer or determined by a visual colour change [142]

Method: Briefly, bacteria was cultured by inoculating fresh 7H9 medium (Middlebrook broth, 0.1% Casitone and 0.5% glycerol supplemented with oleic acid, albumin, dextrose and catalase) with H37R *mycobacteria* and incubated until the day of the experiment (35℃, 5% CO_2, 95% humidity). The compound samples were diluted in 7H9 media (200 µM; 100 µL). Media was added to each well (100 µL) and a dilution series was performed resulting in final concentrations of 0.78-50 µM. Isonicotinic acid hydrazide (INH) also known as isoniazid was used as a positive control for mycobacterial inhibition. The prepared bacterial inoculum (100 µL) was added to each well. The plate was sealed with parafilm and incubated (35℃, 5% CO2, 95% humidity for 10 days). After 9 days of incubation an alamar blue and 10% tween solution mixture (1:1 dilution) was added to each well (40 µL) and the plate was incubated for a further 24 hours in order to monitor colour changes. The minimum inhibitory concentration was determined as the lowest concentration that prevented colour change [142].

3.5.2 Glutathione Reductase inhibition assay

Rationale: Mycobacteria do not utilize glutathione (GSH), but have the analogue to glutathione, mycothiol (MSH). The peptide exists in the bacteria in millimolar concentrations and similarly to glutathione, the protein plays a role in oxidative stress management. Mycothiol is essential for the growth of *M. tuberculosis* and bacteria with deficiencies in the substance have been reported to possess increased sensitivity to oxidative stress. This therefore makes this redox pathway a potential biological target for novel anti-TB chemotherapies. MSH is oxidized to a symmetrical disulphide MSSM and mycothiol reductase (Mtr) is essential for the conversion of the disulfide MSSM to MSH. Inhibition of Mtr will result in the deficiency of MSH in the bacterium [143]. Mtr is an analogue of glutathione reductase (Gtr; responsible for the reduction

of GSSG to GSH) and inhibition of Gtr could influence GSH levels. GSH has been shown to be toxic to mycobacteria and plays an important role in innate immunity. Therefore, in the search for potential Mtr inhibitors, it is important to eliminate all Gtr inhibitors [144]. Here in, selected compounds were screened for their effects of the enzyme. These were compounds that had shown good MICs against *M. tuberculosis* and this was done in order to eliminate these compounds as Gtr inhibitors.

Method: The standard micro plate assay mixture (200 uL) contained Gtr (27 mU), Hepes (50 mM, pH 7.5), EDTA (0.1 mM), NADPH (140 uM), DTNB (100 uM), and varying concentrations of inhibitor. Inhibitor stock solutions were made up in dimethyl sulfoxide (DMSO) and all final assay mixtures contained 5% DMSO. Inhibitor often precipitated out of solution on dilution from the 100 mM DMSO stock solution into assay buffer. To prevent this from occurring, sample was diluted into the assay buffer accompanied by rapid mixing. At very high assay concentrations, a milky emulsion was seen to form, but subsequent addition and mixing of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) produced a clear solution. Reactions were initiated by the addition of Gtr. Enzyme activity was monitored over 40 min by the increase in absorbance at 412 nm due to the formation of 2-nitro-5-thiobenzoate (TNB).

3.6 Drug-likeness Predictions

Rationale: Before a particular drug can be administered to humans as a therapeutic agent, the product requires sequential rounds of optimization and screening. These include assays that test the Absorption, Distribution, Metabolism, Elimination and Toxicity (ADMET) of the particular drug [145].

In this study *in silico* predictions were employed using Discovery Studio 3.1 (Accelyrs Software Inc., USA, 2012). The software employs a model with four ADMET descriptors and these are briefly described below:

1. **Human Intestinal Absorption**: Based on the model, a well-absorbed compound is one that is absorbed at least 90% into the blood stream. The model has 4 prediction levels: 0 (good), 1 (moderate), 2 (Poor) and 3 (very poor). This model was validated against several data sets and also includes 95% and 99% confidence ellipses. This is seen on the ADMET_AlogP98, ADMET_PSA_2D plane. The confidence ellipses function to define regions where well absorbed compounds are predicted to lie. The 95% ellipse is expected to show 95% of well-absorbed compounds. Similarly 99% of well absorbed compounds are expected to fall within the 99% ellipse. Positioning of the compound on the plane does not however mean that the compound will be well, moderately, or poorly absorbed [146].

2. **Aqueous Solubility**: This model mainly predicts the solubility of the particular compound in water at 25℃ using linear regression a nd was developed from a data set consisting of 775 compounds with molecular weights ranging from 50 to 800 g/mol. The software has five aqueous solubility levels which predict the drug-likeness of a particular compound. These include level 0 (extremely low), level 1(No drug-likeness, low, but possible), 2 (Yes there is drug-likeness, but low), 3 (Yes there is drug-likeness and its good), 4 (Yes there is drug-likeness and its optimal), 5 (No drug-likeness, the compound is too soluble) and 6 (Molecule with one or more unknown AlogP98 types- the base 10 logarithm of molar solubility as predicted by the regression) [146].

3. **Blood Brain Barrier**: This model predicts penetration of the blood brain barrier by the compound following oral administration. The model has four prediction levels which include 0 (very high penetrant), 1 (high penetrant), 2 (medium penetrant), 3 (low penetrant) and 4 (Undefined). In addition to the linear regression model, 95% and 99% confidence ellipses are also included in the model, and these are shown on the

ADMET_PSA_2D, ADMET_AlogP98 plane. These ellipses differ from those associated with human intestinal absorption.

4. **Cytochrome P450 2D6 (CYP2D6):** Cytochrome P-450 enzymes function in the addition of electrophilic centres such as OH groups to non-polar foreign chemicals. These enzymes are located in the endoplasmic reticulum of most T-cells and are especially found in high amounts in the liver. This is done in order to solubilize these foreign compounds, allowing excretion from the body. The model predicts the inhibition of the enzyme CYP2D6 using 2D chemical structure input. The enzyme plays a role in the metabolism of a wide range of substrates in the liver and a lot of drug-drug interaction cases involve the inhibition of this enzyme.

5. **Hepatotoxicity:** The hepatotoxicity model predicts the potential organ toxicity of the compound. The liver is one of the portals of entry to the tissue of the body and due to this it is exposed to many potentially toxic substances [147]. The software uses a model developed from available literature data of 436 compounds which have been shown to exhibit liver toxicity.

6. **Plasma Protein Binding:** This model predicts the binding capability of the compound to the carrier proteins found in the blood. This is important as it can affect the efficiency of the drug since the bound fraction is temporarily shielded from metabolism. The physiochemical character of the compound will determine the nature and strength of binding to a particular plasma protein. A training set of 854 compounds from two data sets with plasma protein binding level data was used to generate this model [148].

7. **AlogP:** The AlogP is the measure of the octanol-water partitioning coefficient and it reflects the hydrophobicity of the molecule. The AlogP is greatly associated with Lipinski's rule of five which states that a compound is more likely to possess poor absorption and permeation when it has:
    - More than 5 hydrogen-bond donors
    - More than 10 hydrogen-bond acceptors
    - A molecular weight greater than 500g/mol
    - An AlogP value greater than 5
    - The compound can only break one of these rules

Method: As stated above, Discovery Studio 3.1 (Accelyrs Software Inc., USA, 2012) was employed for the ADMET prediction studies. The compound structures were drawn using Chemsketch Freeware Version 11.01 (Advanced Chemistry Development Inc., Canada, 2012) and were saved in Molecule format (mol).

The following flow diagram (**Figure 22**) briefly shows the different processes that were run in order to obtain ADMET predictions. Minimization mainly performs energy minimization on the molecule by updating the coordinates of the molecule and adding energy properties. Before this can be done, the molecule must be typed with supported force field. Force field is the functional form and parameter sets used to describe the potential energy of a system of particles. Upon completion of minimization, ADMET predictions can them be made.

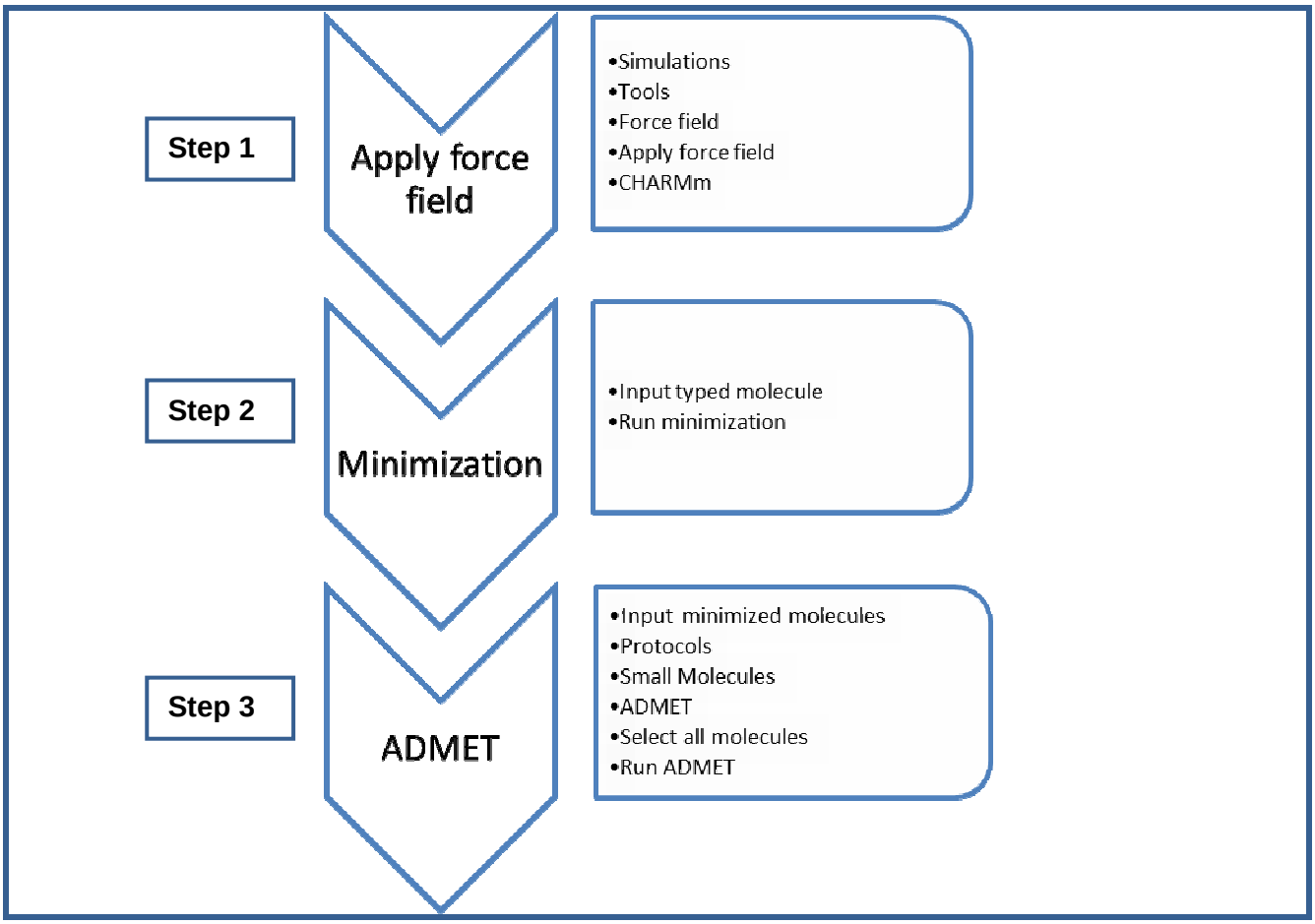


Figure 22: Step-by-step prediction of ADMET using Discovery studio 3.1. There are mainly three protocols that are run to obtain the ADMET predictions for a compound that has been saved in mol. format.

In order to compare ADMET predictions of the analysed compounds to those of currently available HAART, structures of the drugs were obtained from PubChem

(www.pubchem.ncbi.nlm.nih.gov) and the procedure described above was used to obtain the ADMET prediction values for these known inhibitors.

3.7 Statistical Analysis

Statistical analysis of data was carried out using Microsoft Excel 2010 (Microsoft Corporation, USA) and the statistical significance was determined using the paired t-test. Differences were considered significant at a level of $P < 0.05$.

4. RESULTS

There is currently no cure for HIV and the infection can be controlled by the use of HAART. These drugs are however associated with toxicity resulting in non-compliance by patient. This in turn causes the emergence of resistant viral strains. In addition to the challenges associated with HAART, there a number of problems associated with the treatment of OIs associated with HIV. These include emergence of MDR and XDR TB strains as well as drug-resistant tumours of cervical cancer. It is therefore crucial to develop new forms of treatment that are effective against HIV as well as its associated OIs.

This study investigated the activity of metal complexes against HIV and two IOs; TB and cervical cancer.

4.1 HIV-1 Enzyme inhibition

4.1.1 HIV-1 Reverse Transcriptase inhibition

HIV-1 reverse transcriptase is one of the most important enzymes required for the replication of the virus. Twelve metal complexes where tested for their inhibition of the enzyme at 3 different concentrations (25 µM, 10 µM and 1 µM). **Figure 23** represents the obtained percentage inhibition values for the compounds.

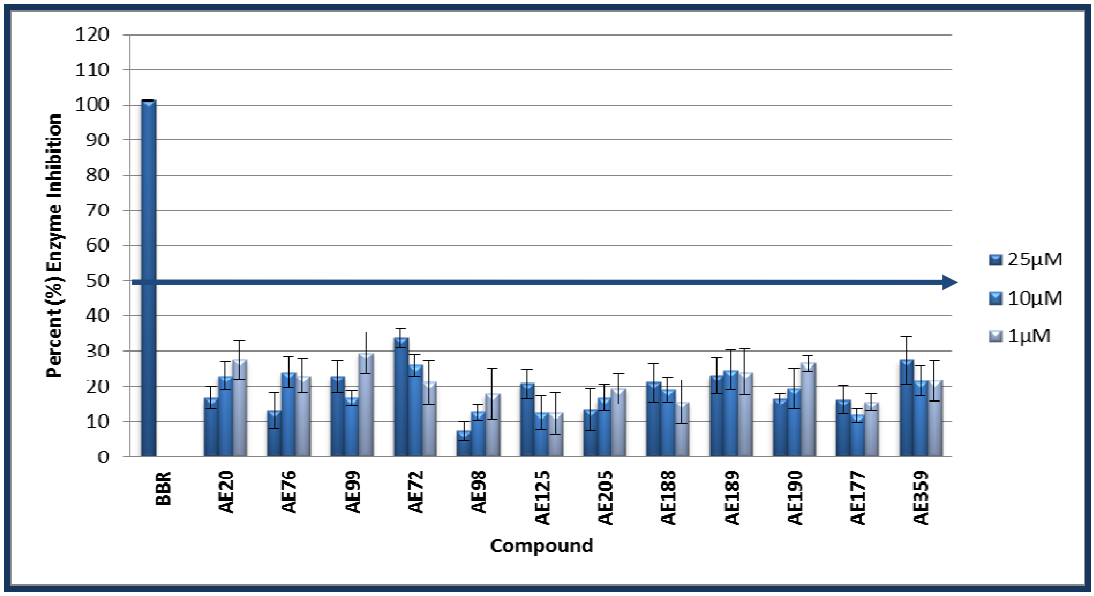


Figure 23: Inhibition of HIV-1 Reverse Transcriptase Inhibition. All the compounds showed percentage inhibition values of below 40%. The results are representative of 3 biological replicates, each performed in duplicate, %SE.

In general HIV studies, enzyme inhibition of ≥ 50% is considered as significant compound activity. None of the compounds showed percentage inhibition values above 50% and the platinum compound **AE72** had the highest enzyme inhibition of 33%.

4.1.2 HIV-1 Protease inhibition

The compounds were screened for the inhibition of HIV-1 protease and this was performed at three concentrations; 100, 25 and 10 µM. All negative percentage inhibition values were zeroed and **Figure 24** represents the obtained inhibition profiles.

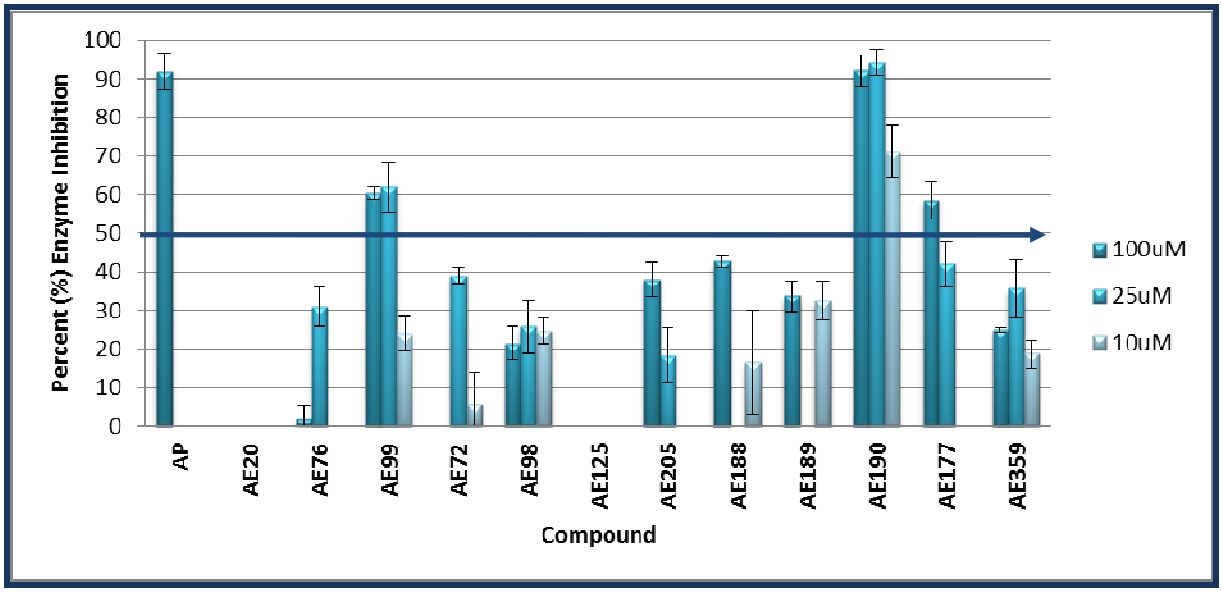


Figure 24: The inhibition of HIV-1 Protease by metal complexes. Four of the 12 analysed compounds showed inhibition of the enzyme with percentage inhibition values above 50%. The results are representative of 3 biological replicates, each performed in triplicate, %SE.

Three compounds; **AE99**, **AE190** and **AE99** showed percentage enzyme inhibition of above 50%. The table below (**Table 6**) shows calculated p-values at the lowest active concentration for each active compound.

Table 6: *P*-values for compounds active against HIV PR as calculated by the paired T-test.

Compound	Concentration (µM)	*P*-value
AE99	100	0.0009
AE190	10	0.0009
AE177	100	0.0013

The ligands used to prepare these complexes are **AEL1 (AE177)**, **AEL2 (AE99)** and **AEL6 (AE190)**. **Figure 25** shows percentage enzyme inhibition obtained when the ligands were analysed for their inhibition of the enzyme. None of the ligands was able to inhibit enzyme activity. This suggests that the metal ions of the active compounds play an important role in the activity of these complexes.

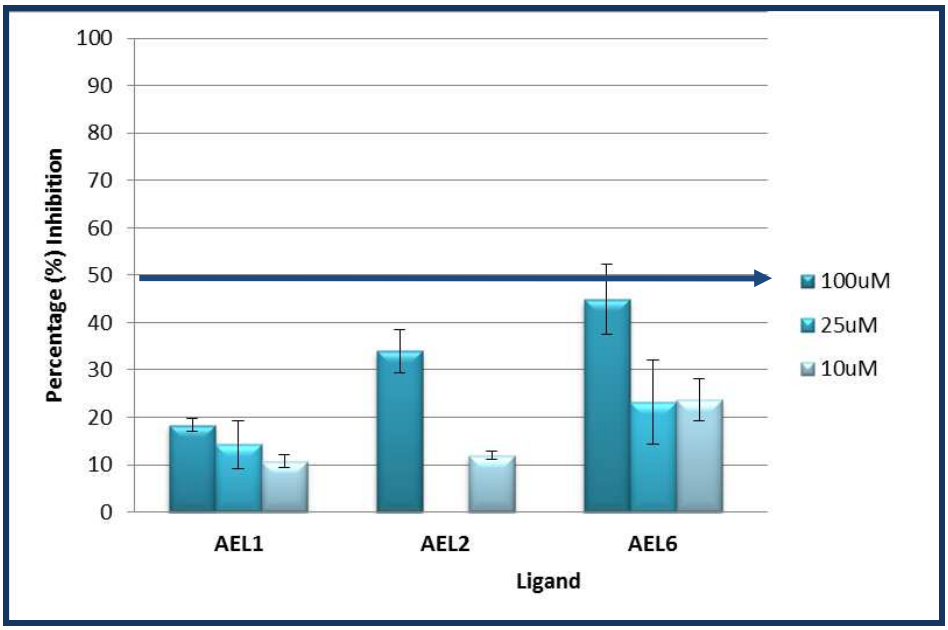


Figure 25: Inhibition of HIV-1 Protease by selected ligands. All the ligands of the active compounds showed no inhibition of the enzyme, with percent enzyme inhibition values well below 50%. The results are representative of 3 biological replicates, each performed in duplicate, %SE.

4.1.3 HIV-1 Integrase inhibition

The compounds were screened for their inhibition of the activity of HIV-1 integrase based on the inhibition of the strand transfer reaction using a kit (Xpressbio, USA). Initial screens were performed for all the compounds and thereafter only active compounds were subjected to repeats. All negative percentage inhibition values were zeroed **Figure 26** represents the inhibition profiles of these compounds.

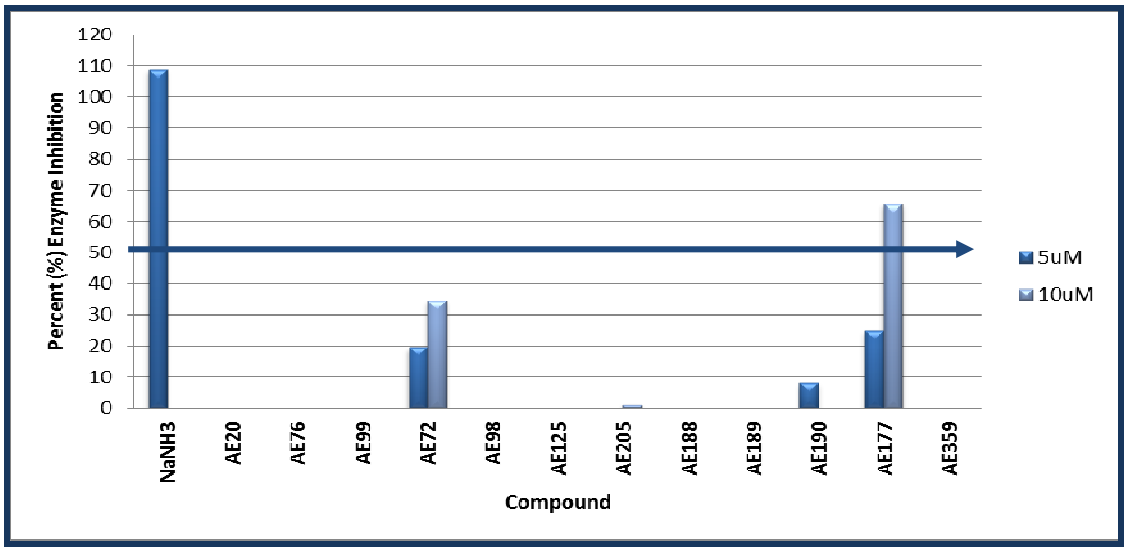
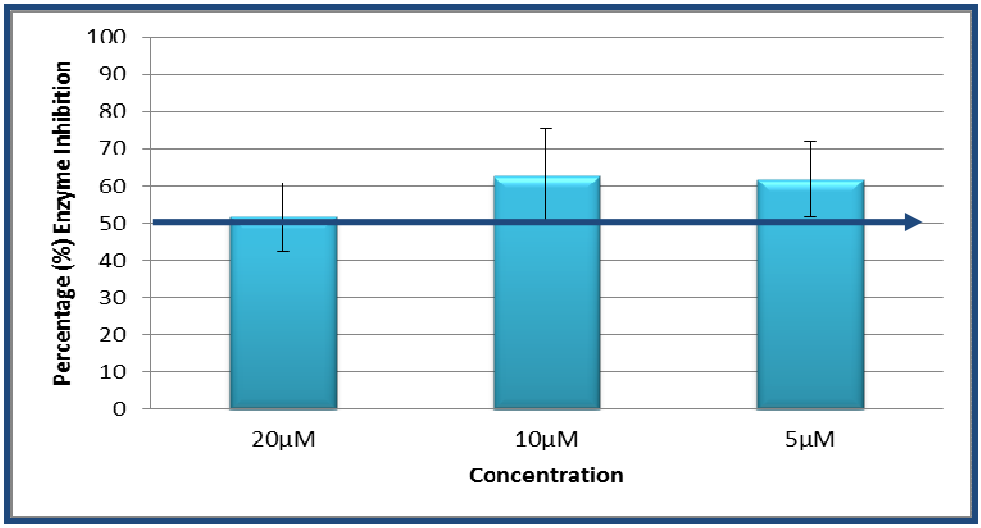


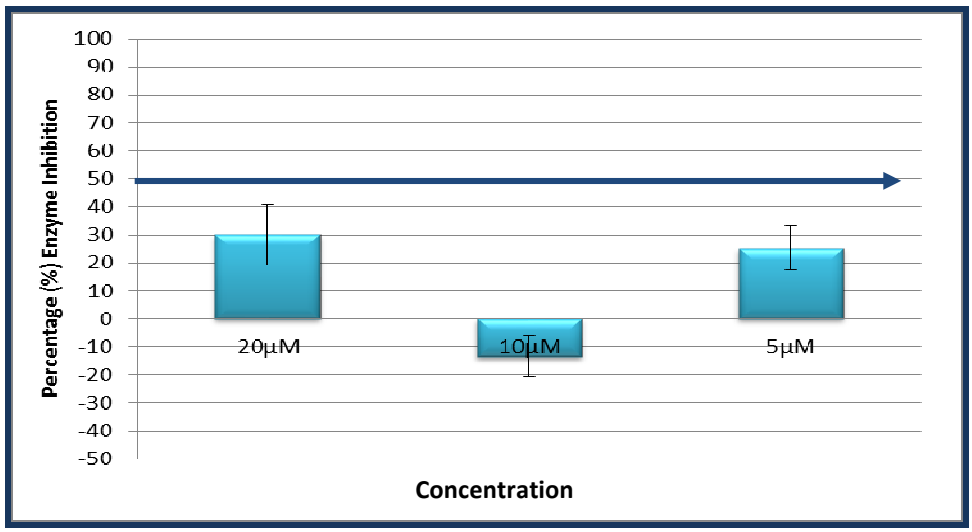
Figure 26: The inhibition of HIV-1 integrase by metal compounds. The compounds were screen at two concentrations 5µM and 10µM. Only one complex demonstrated potential inhibitory activity.

One compound, **AE177** showed potential activity with an initial percentage enzyme inhibition value of 65% at 10µM. When re-tested, the complex retained its activity (**Figure 27A**) with an average activity above 60% at 10 µM (p= 0.01). The compound was also tested at additional concentrations; 20µM, which also exhibited an average activity above 50%. Notably, repeats at 5µM showed an average activity that is similar to inhibition at 10µM and was not as inactive as shown in the initial screen (**Figure 26**). This could potentially suggest that concentrations ≤ 10µM could be optimal concentrations for enzyme inhibition, while concentrations greater than 20µM could be losing inhibitory activity. Differences in the percentage inhibition between 20µM and 10µM were shown to be insignificant, with a calculated *P*-value of 0.199.

The ligand used to synthesize the complex **AE177**, **AEL1** was also tested for IN enzyme inhibition (**Figure 27B**). The percentage inhibition was well below 50% for all three concentrations.



(A)



(B)

Figure 27: The inhibition of HIV-1 integrase by the bimetallic compound AE177. (**A**) The compound maintained the inhibition of the enzyme at 10µM with a slight decrease in the activity at 20µM. (**B**) The ligand of AE177, AEL1 did not show any enzyme inhibition, with percentage enzyme inhibition values well below 50%, n=3, %SE.

4.2 Metal complex effects on cell viability and proliferation

4.2.1 Compound CC50 values

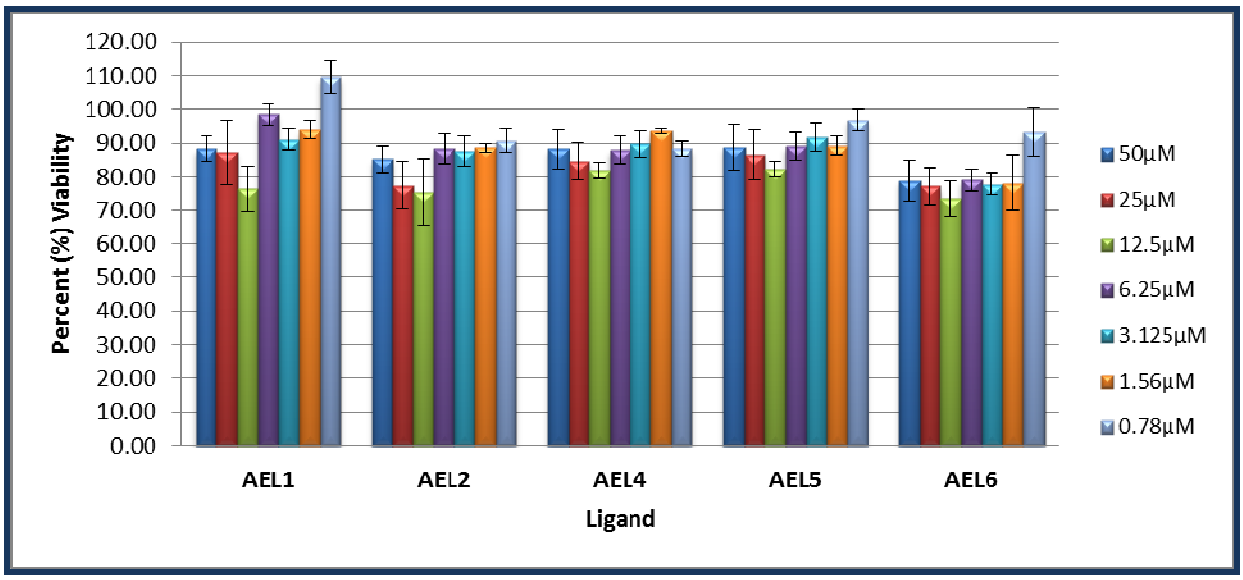
The effects of the metal compounds on the viability of cells were determined using two cell types, TZM-bl cells and PBMCs. The obtained viability graphs are shown in **Appendix III** (**Figure A1**) and **Table 7** shows the obtained CC_{50} values.

Table 7: The Cell Cytotoxicity (CC50) concentrations of each compound with their SD.

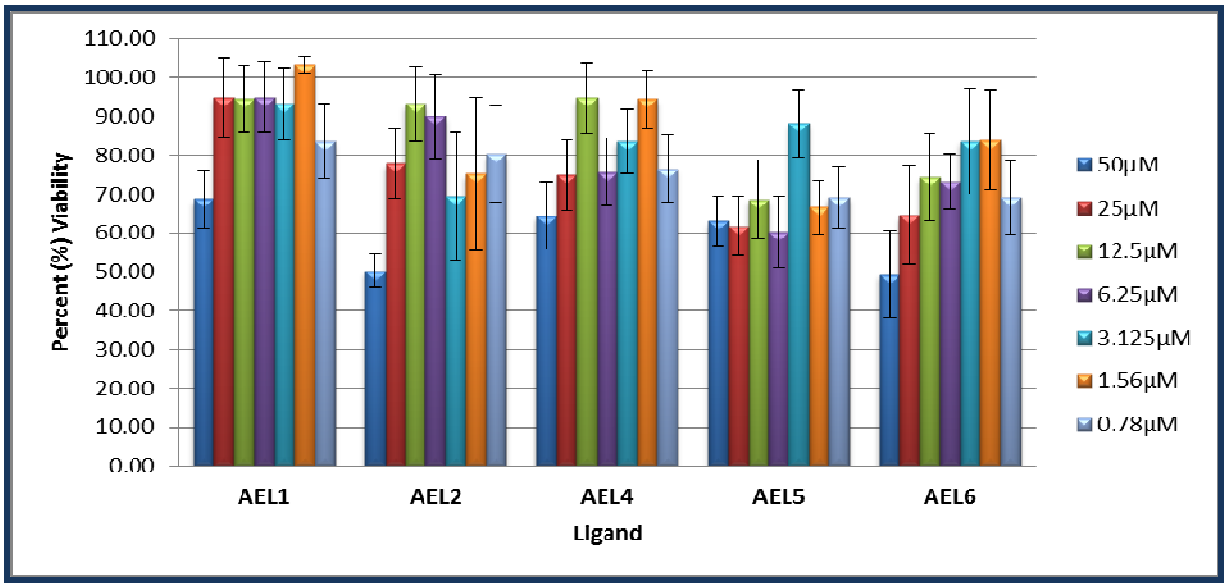
No.	Compound Name	CC_{50} (µM) PBMCs	CC_{50} (µM) TZM-bl Cells
1	AE20	1.081 ±0.005	15.76 ±1.52
2	AE76	0.576 ±0.043	18.06 ±2.29
3	AE99	>100	>100
4	AE98	>100	>50
5	AE72	>100	>50
6	AE125	1.740 ±0.998	9.737 ±0.675
7	AE205	<0.78	1.272 ±0.065
8	AE188	3.064 ±0.032	4.355 ±0.204
9	AE189	3.438 ±0.079	8.105 ±0.153
10	AE190	2.091 ±0.002	2.322 ±0.015
11	AE359	2.338 ±0.006	3.763 ±0.028
12	AE177	1.348 ±0.007	7.413 ±0.005

In general, the compounds showed greater toxicity to PBMCs than to the cultured TZM-bl cell line. The gold based compound **AE205** caused the lowest cell viabilities, with an estimated CC_{50} value of below 0.78µM for PBMCs and a slightly higher 1.272µM for TZM-bl cells. The palladium based compounds **AE99** and **AE187**, as well as the platinum based compounds **AE98** and **AE72** were the least toxic with CC_{50} values above 100µM for both PBMCs and TZM-bl cells.

Figure 28 below shows percentage viabilities of cells in the presence of the ligands. All analysed concentrations resulted in viability values of ≥ 65%. This therefore means that any observed toxicity for the metal complexes is due to the presence of the metal ion.



(A)

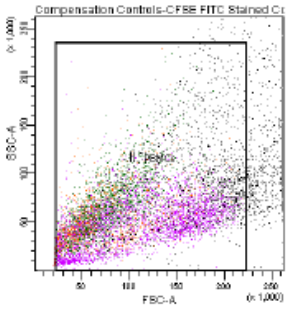


(B)

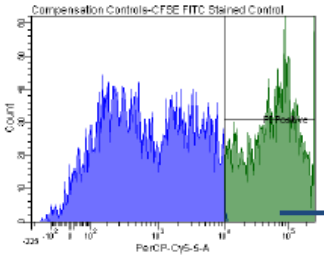
Figure 28: The effects of the binding ligands on cell viability. The ligands did not cause significant cell death. Percentage viability values were generally above 65% in the treatment of both TZM-bl (A) cells and PBMCs (B) except in two cases (50µM of **AEL2** and **AEL6**.The results are representative of 3 biological replicates, each performed in triplicate, %SE.

4.2.2 Flow Cytometry

Flow cytometry was used to determine the effects of the metal compounds on the proliferation of PBMCs based on the reduction of the CFSE stain. The following gating strategy was used to obtain histograms showing the parent and the daughter populations:

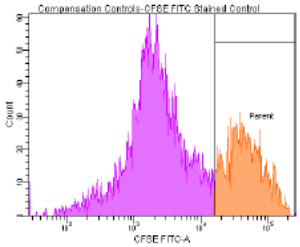


Gating on all the events (analysis of effects on the whole PBMC population)

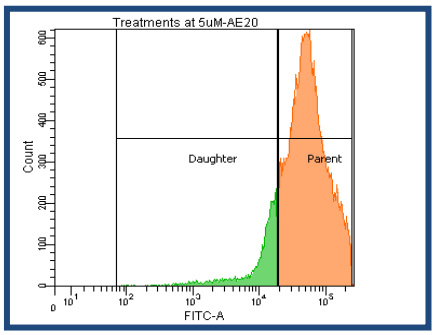


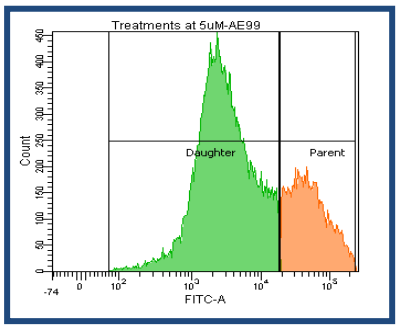
Distinguish live cells from dead cells based on the uptake of Propidium Iodide by dead cells, shown by high intensity on PerCP-Cy5-5A

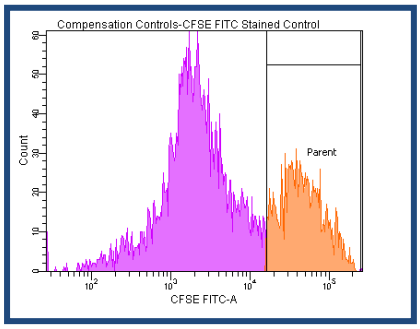
From the live gate, daughter and parent populations were obtained.



The representative histograms for the compounds are shown below. As predicted with the MTS assay, all the compounds with appreciable toxicity to PBMCs did not show much proliferation (**Figure 29A**) while compounds with no observed toxicity to PBMCs showed proliferation of the cells into the daughter population (**Figure 29B**). The proliferation histograms of the rest of the complexes are shown in **Figure 30**.







(A) **(B)** **(C)**

Figure 29: Effects of the compounds at 5µM on the proliferation of PBMCs. The compound **AE20** (**A**) did not show presence of a daughter peak and most of the cells remained in the parent population. **AE99** (**B**) showed proliferation of the majority of the cells into the daughter population and this was similar to the proliferation of untreated cells (**C**) meaning that the compound did not have an effect on cell proliferation.

Only two populations were observed meaning that the cells underwent one division, and this could be due to the fact that a heterogeneous population of cells (PBMCs) was analysed. Homogeneous populations have been reported to give better resolutions than non-homogeneous populations [149].

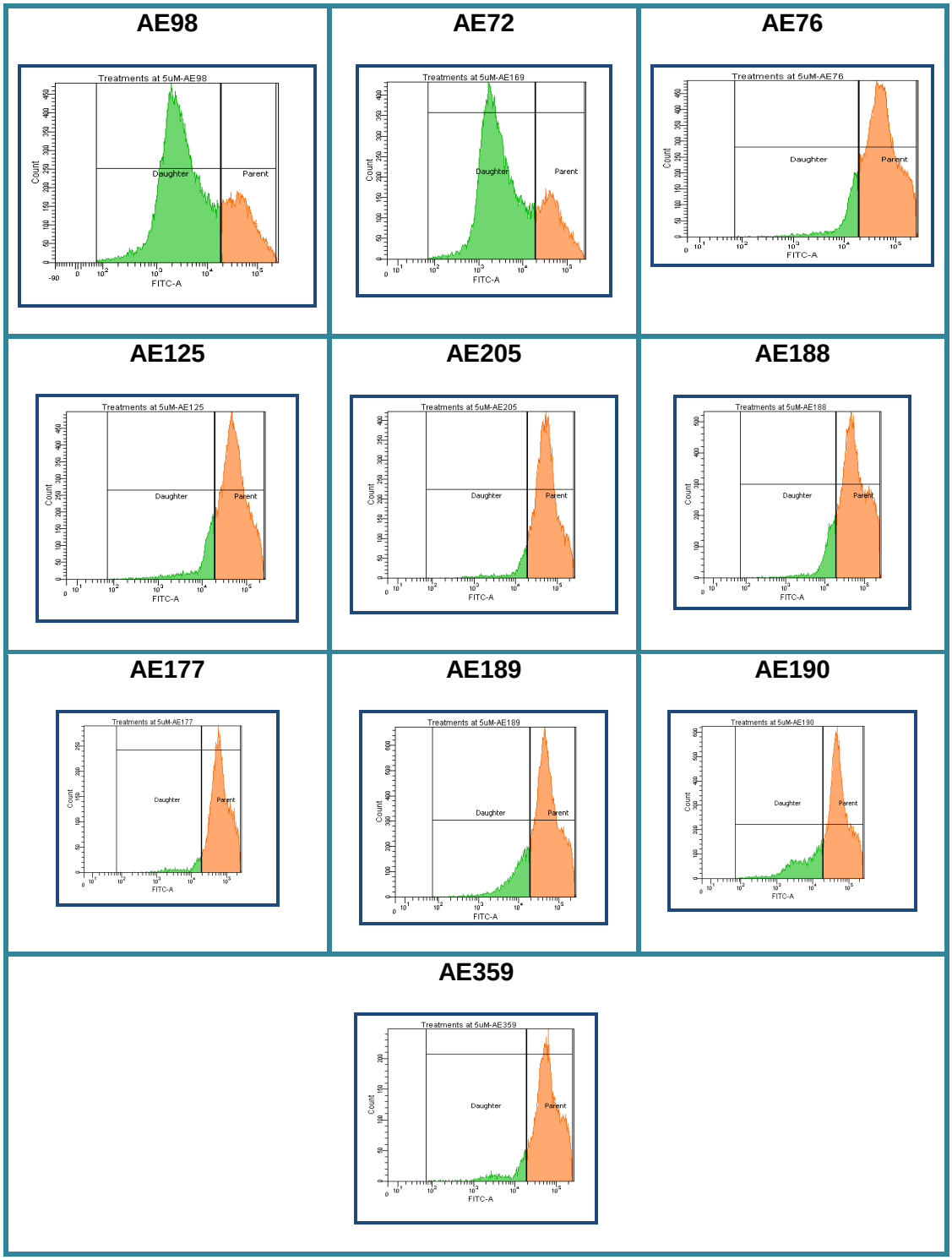


Figure 30: The effects of the various metal complexes at 5μM on the proliferation of PBMCs. Complexes that were shown to be toxic in the MTS assay had only one peak, while complexes which were not toxic showed proliferation into the daughter population. The results are representative of 3 biological replicates, each performed in duplicate.

As expected, all the ligands showed proliferation into the daughter population (**Figure 31**), and this was confirmations of their non-toxicity to cells.

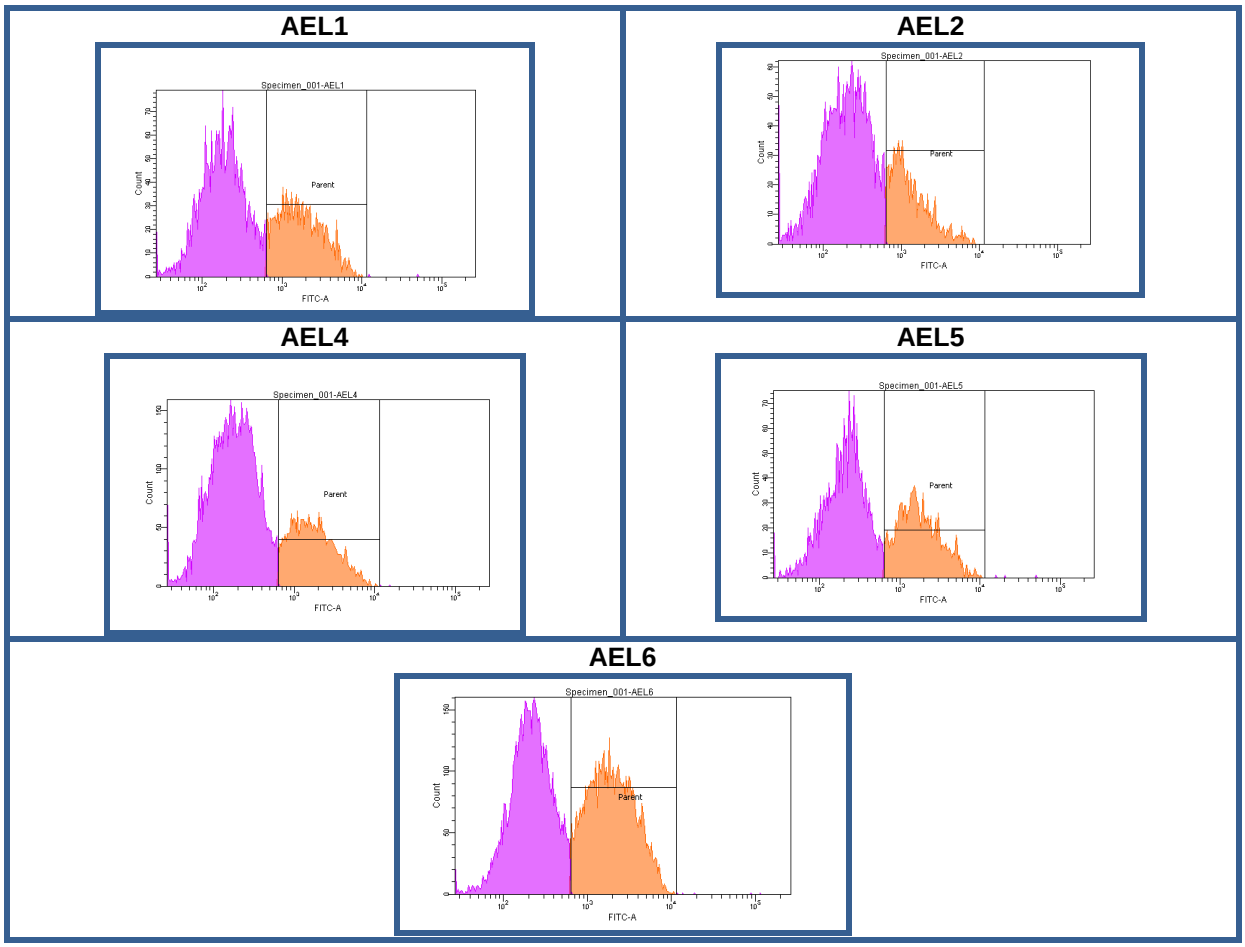


Figure 31: The effects of the ligands at 5 µM on the proliferation of PBMCs. All the ligands showed presence of daughter population.

4.2.3 Real Time Cell Analysis

Cell proliferation was monitored on TZM-bl cells using an RT-CES device. The device offers real time monitoring of cell proliferation and allows for the detection of cellular responses after the addition of compounds. A cell titration experiment was performed (**Figure 32A**) and the cell concentration (25 000 cells/well) which resulted in cell index values of between 1 and 1.5 was chosen as the ideal cell concentration for further experiments [128]. Compound treatments were done along side a vehicle control (cells only), medium only, as well as Auranofin (10µM, cytotoxicity control) and these are indicated in **Figure 32 B**.

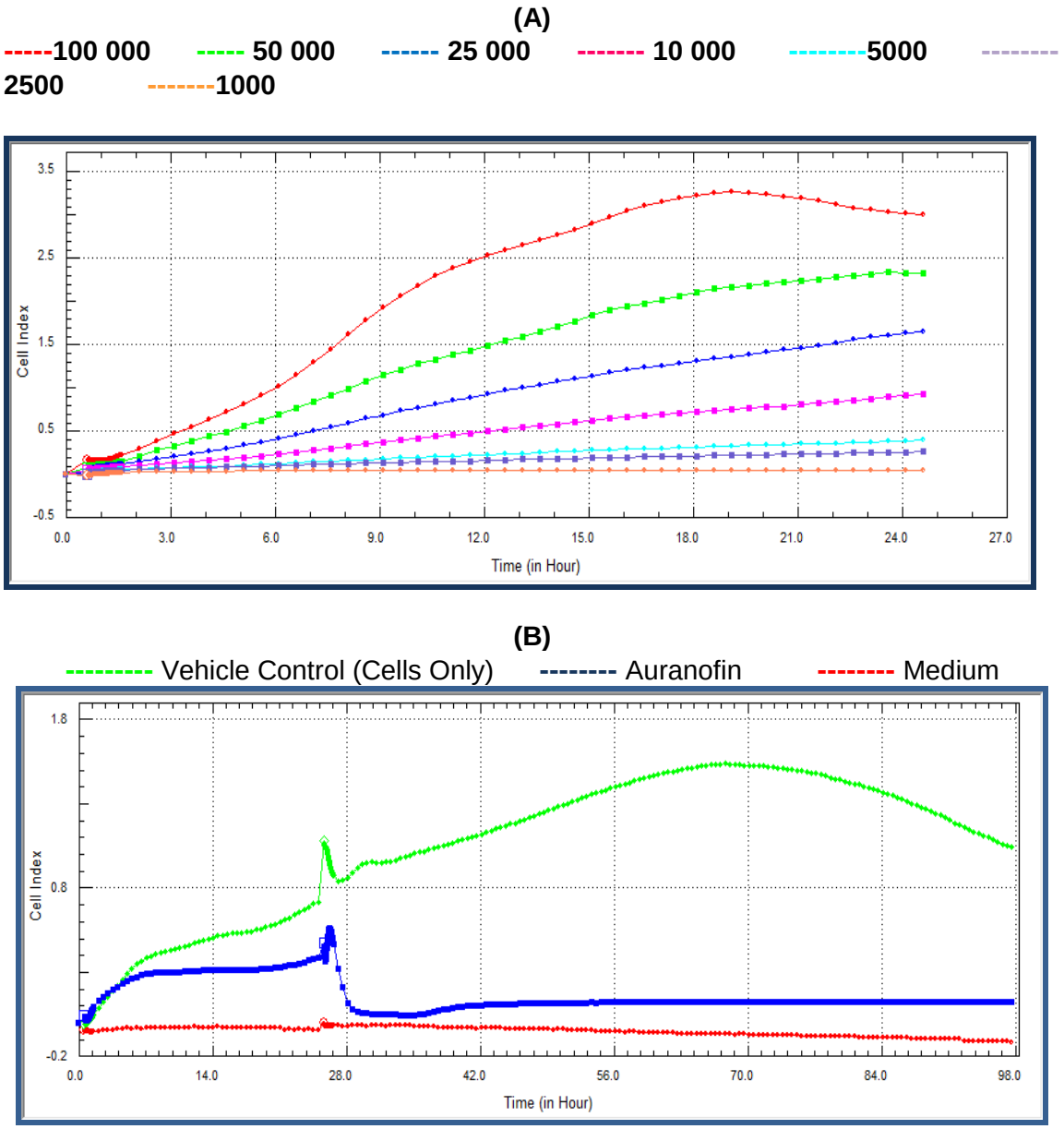


Figure 32: Cell Titration for real time cell analysis (A) and controls for compound treatments (B). The cell concentration of 25000 cells per well was used for all compound treatments. The experiment was stopped for the addition of the compound, resulting in the observed peaks (Increase in CI) upon resuming the experiment. Every experiment was performed in the presence of wells containing cells only, media only and Auranofin at 10µM. The results are representative of 3 biological replicates, each performed in duplicate.

The representative proliferation curves for the selected compounds are shown in **Figure 33**. These compounds were selected based on CC_{50} values (<20µM, refer to **Table 8**) on TZM-bl cells and the main objective was to confirm observed compound toxicity in MTT and to determine if the compounds resulted in unique proliferation profiles such as cytostasis (arrest of cellular growth and division). Higher concentrations (40µM and 20µM) of the metal compounds exhibited profiles similar to Auranofin for most of the compounds confirming toxicity (**Figure 33**). Lower concentrations had profiles that were similar to the vehicle control, indicating non-toxicity and confirming obtained MTT data. The growth profiles for the rest of the compounds are shown on **Figure 34.**

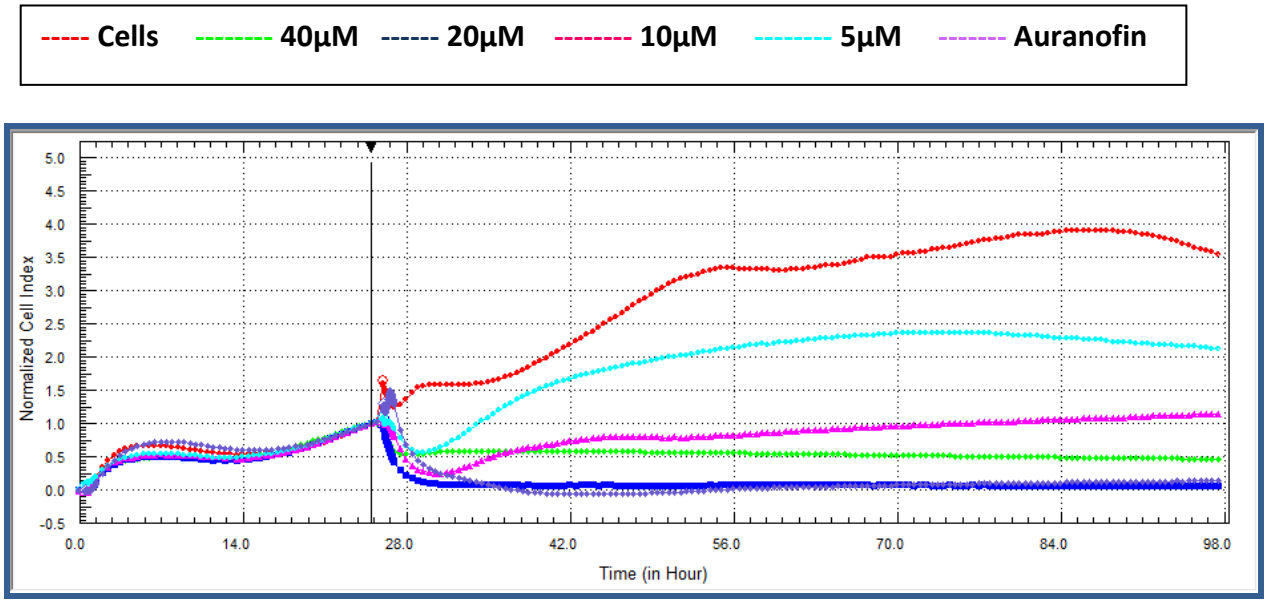


Figure 33: Representative growth profiles as based on real-time cell analysis. The compound **AE20** had growth profiles that were similar to Auranofin for the concentrations 40-10µM indicating toxicity, while the lower concentration (5µM) had a proliferation profile similar to untreated cells. The compound seemed to behave slightly better than Auranofin at 10µM, showing stabilized cell index values from 42hrs to 98hrs. The results are representative of 3 biological replicates, each performed in duplicate.

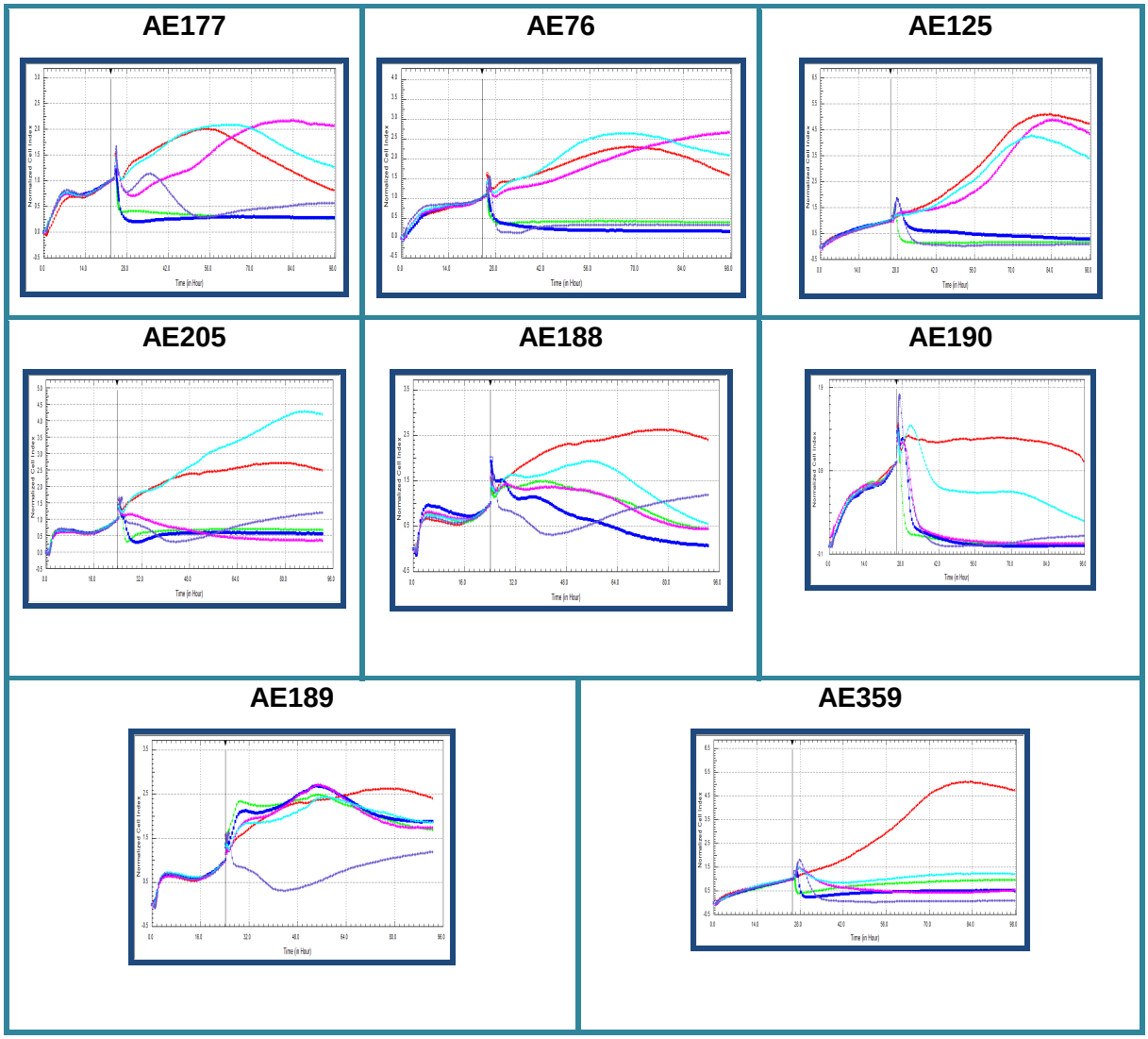


Figure 34: Effects of the compounds on TZM-bl cells as analysed by a real time cell analyser. **--- Cells** **-------- 40µM** **------ 20µM** **-------- 10µM** **-------- 5µM** **------- Auranofin.** The higher concentrations (40 and 20µM) behaved in a similar manner as Auranofin. Exceptions were AE189, which showed profiles similar to untreated cells even at high concentrations. The results are representative of 3 biological replicates, each performed in duplicate.

In addition to the obtained profiles, the xCelligence software has an added advantage of calculating the estimated CC_{50} value for each compound and these are shown in **Table 8**. When these were compared to the MTT data, they were higher for most of the compounds (**AE125, AE205, AE188, AE189, AE190** and **AE177**). This may be because of the initial step of allowing cells to first adhere to the plate before addition of compound (recommended by instrument manufacturer); therefore allowing the cells to be more stable and to metabolise the compound slightly better. Variations associated with formazan production in the MTT assay have been observed, were the duration of the exposure of treated cells to MTT results in the changes in the IC_{50} of the analysed compound [150].

Table 8: Cell Cytotoxicity values obtained from RTCA. The highlighted compounds had CC50 values that were higher than those obtained in the MTT assay.

Compound	CC_{50} (µM)	CC_{50} (µM) MTT
AE20	9.68	15.76
AE76	10.70	18.06
AE125	20.20	9.737
AE205	6.96	1.272
AE188	8.22	4.355
AE189	23.25	8.105
AE190	11.90	2.322
AE359	3.29	3.076
AE177	14.70	7.413

The compounds **AE20, AE76** and **AE359** exhibited CC_{50} values lower than those predicted by the MTT assay indicating appreciable toxicity for these compounds.

4.3 Anti-Cancer activity

Cervical cancer is one of the pathologies that are associated with HIV infection. Development of treatments that could be effective against both HIV and cervical cancer could be beneficial in the holistic treatment of HIV. Metal complexes were screened for inhibition of the growth of HeLa cells, a cervical cancer cell line (**Figure 35**).Three complexes; **AE99**, **AE98** and **AE72** did not show appreciable cell growth inhibition. All analysed concentrations of **AE99**, **AE98**, and **AE72** exhibited percentage viability values above 70% (**Figure 35**). Nine complexes showed potential toxicity, with at least two concentrations resulting in the death of more than 50% of the cells.

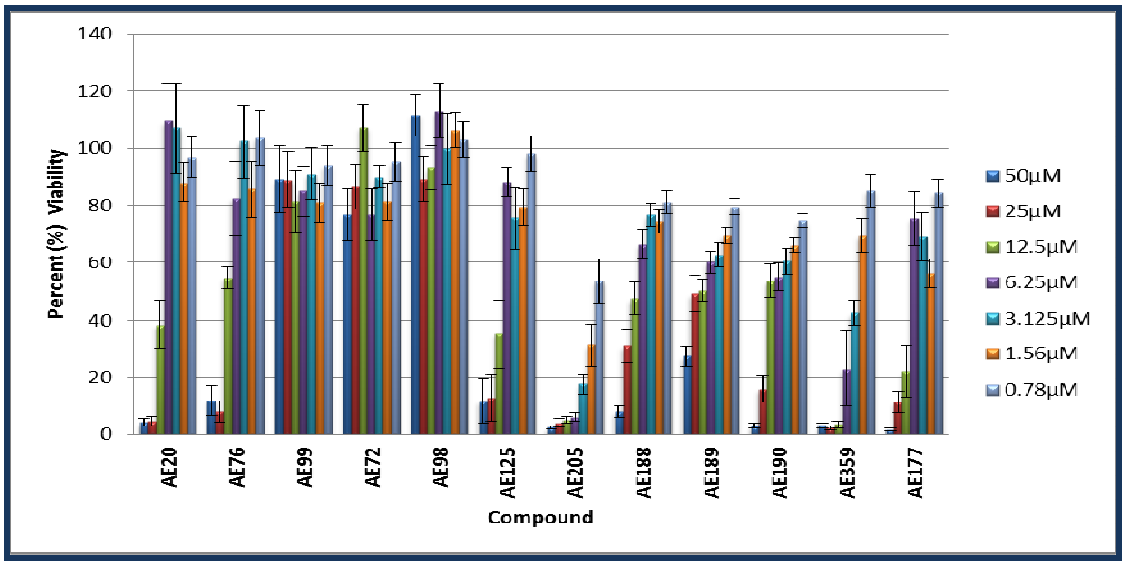


Figure 35: The effects of the metal complexes on the growth of HeLa cells. Nine complexes showed potential toxicity with percentage viability values of below 50% for some of the concentrations. The results are representative of 3 biological replicates, each performed in triplicate, %SE.

When the ligands that are associated with the active complexes were tested on HeLa cells, none of the ligands had inhibitory effects on cell growth (**Figure 36**) which indicates that all observed potential chemotherapeutic activity is due to the presence of the metal ions not the ligands.

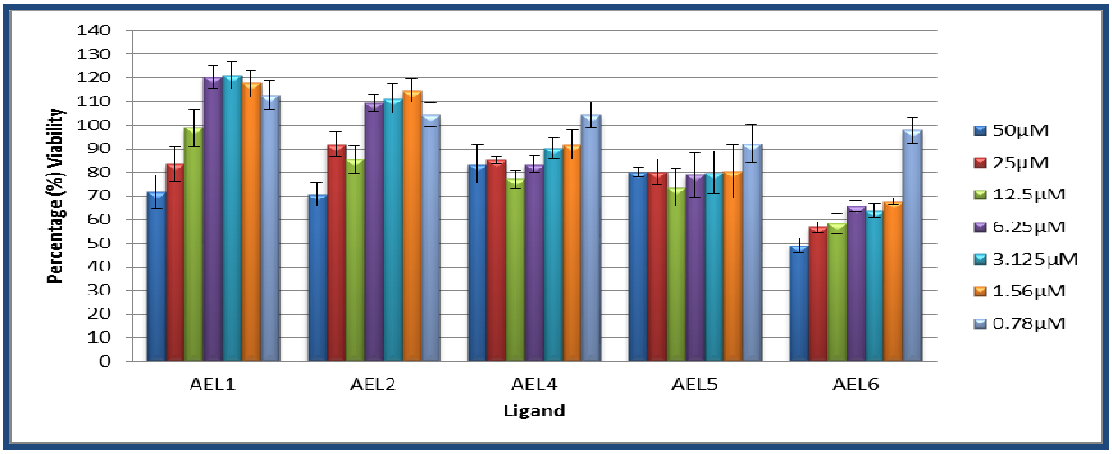


Figure 36: Effects of the ligands on the growth of HeLa Cells. Most of the ligands caused percentage viabilities of ≥ 60% when added to HeLa Cell. The results are representative of 3 biological replicates, each performed in triplicate, %SE.

Furthermore, complexes with potential activity were analysed for their effects on Vero Cells, a monkey kidney cell-line (**Figure 37**). This was done in order to determine the selectivity index (SI) value which is calculated as the ratio of cytotoxicity on Vero cells to HeLa cells. If the SI value is ≥ 10, the biological efficacy of a compound is considered to not be due to general cytotoxicity [151].

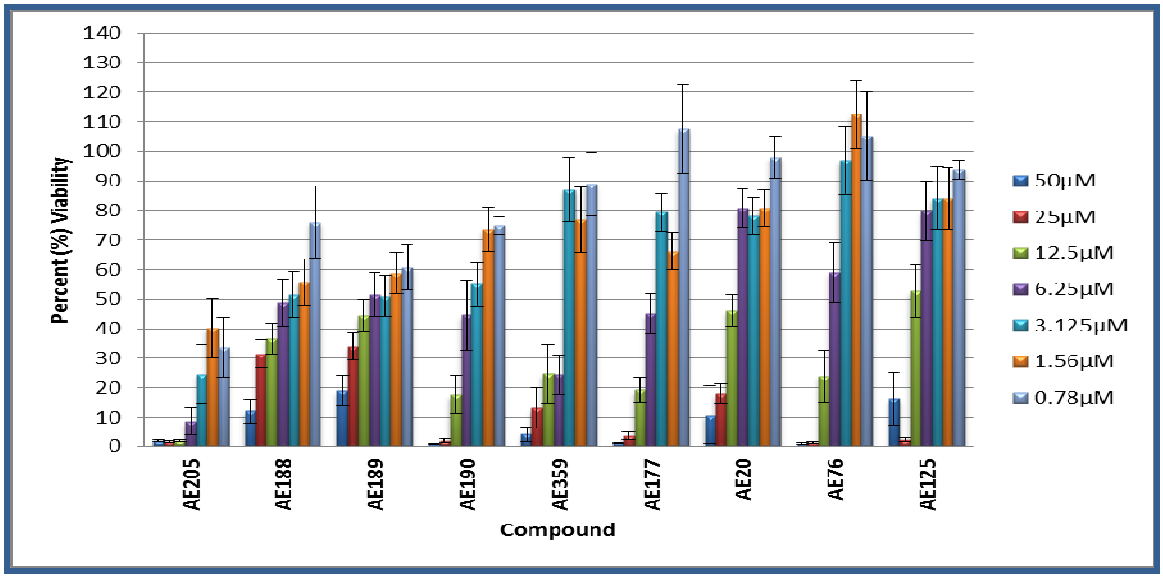


Figure 37: Effects of selected complexes on the growth of Vero cells. The complex **AE205** seemed to be more toxic on Vero cells than on HeLa cells, while the complexes **AE20** seemed to be slightly less toxic on these cells. The results are representative of 3 biological replicates, each performed in triplicate, %SE.

Table 9 shows the obtained 50% cell growth inhibitory concentrations (IC_{50}) values for complexes with potential activity. These are complexes which resulted in IC_{50} values below

20µg/ml. Based on the US National Cancer institute guidelines, a crude extract is generally considered to have *in vitro* cytotoxicity activity if the IC_{50} value in cancer cells is ≤ 20µg/ml, while for pure compounds the IC_{50} value is ≤ 4µg/ml [152].

Table 9: The effects of the compounds on the growth of the cervical cancer cell-line (HeLa) and the monkey kidney cell-line (Vero).

Compound	IC_{50} (µg/ml) HeLa cells	IC_{50} (µg/ml) Vero cells	Selectivity index (SI)
AE20	4.89± 0.44	6.24± 0.45	1.27
AE76	5.311± 0.183	4.72± 0.41	0.88
AE125	6.18± 0.26	8.17± 0.21	1.32
AE205	0.81± 0.02	0.16± 0.04	0.19
AE188	8.47± 0.22	7.08± 0.60	0.8
AE189	9.93±0.94	4.69± 0.55	0.5
AE190	4.82± 0.40	2.09± 0.16	0.4
AE359	1.36± 0.03	2.09± 0.11	1.5
AE177	6.16± 1.31	6.01± 0.40	1.0
Cisplatin	8.91± 2.42	4.50± 0.77	0.5

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Based on the above recommendation, two complexes are considered to be potential potent chemotherapeutic agents; **AE205** and **AE359**, while **AE20**, **AE76**, **AE125**, **AE190** and **AE177** could be considered to possess moderate chemotherapeutic activity.

4.4 *M. tuberculosis* inhibition assays

TB is one of the major opportunistic infections associated with HIV infection and the bacterial infection continues to affect a large number of patients worldwide. The metal compounds were assessed for their ability to inhibit the growth of *M. tuberculosis*. **Table 10** shows the obtained MIC values for each of the complex.

Table 10: Minimum Inhibitor Concentrations of the compounds on the growth of *Mycobacterium tuberculosis*.

Compound	MIC (µM)
AE20	50
AE76	50
AE99	100
AE98	200
AE72	200
AE125	25
AE205	50
AE188	1.56
AE189	1.56
AE190	1.56
AE359	25
AE177	50
INH	0.25

The compounds **AE98** and **AE72** had the highest MIC values, indicating non-activity, while **AE188, AE189** and **AE190** exhibited the lowest MIC values and potential activity towards the bacterium. These active complexes were further tested for their inhibition of the enzyme glutathione reductase (**Table 11**). Only **AE190** did not show inhibition of the enzyme. In the case of **AE188** and **AE189**, inhibition of the enzyme was shown to be due to the presence of the metal ions as the ligands that synthesize these complexes did not show any inhibition.

Table 11: The inhibition of Glutathione reductase (Gtr) by selected metal complexes.

Compound	Percentage (%) Enzyme Inhibition
AE188	73.6
AE189	95.5
AE190	12.16
AEL4 (AE188)	10.47
AEL5 (AE189)	10.54
AEL6 (AE190)	8.25

The inability of **AE190** to inhibit the activity of the glutathione reductase could be an indication of the potential selectivity of this compound to the bacterial analogue, mycothiol reductase.

4.5 Drug-likeness Predictions

One of the major steps in drug development is the prediction of the drug-likeness of a particular lead compound and this is done using different techniques which ultimately seek to predict the potential absorption, distribution, metabolism, excretion and toxicity (ADMET) of the compound. A simple and quick way of doing this is through the use of *in silico* methods, and in this study Discovery studio 3.1 (Accelerys Sofware Inc., USA) was utilized to perform ADMET predictions for each compound. **Table 12** represents the predictions obtained for each compound as well as for the ligands.

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Table 12: ADMET prediction scores for the compounds. The shaded regions indicate compounds with good properties for the specific predictor.

Name	Solubility	BBB	CYP2D6	Hepatotoxicity	HIA	PPB	AlogP98
AE20	2	0	FALSE	TRUE	0	TRUE	4.91
AE76	2	0	FALSE	TRUE	0	TRUE	4.841
AE99	0	4	FALSE	TRUE	3	TRUE	9.683
AE98	1	0	FALSE	TRUE	1	TRUE	5.33
AE72	0	4	FALSE	TRUE	3	TRUE	9.82
AE125	0	4	TRUE	TRUE	3	TRUE	7.202
AE205	1	4	FALSE	TRUE	3	TRUE	7.516
AE188	0	4	FALSE	TRUE	3	TRUE	14.405
AE189	1	4	FALSE	TRUE	3	TRUE	7.516
AE190	0	4	FALSE	TRUE	3	TRUE	7.312
AE359	1	0	FALSE	TRUE	1	TRUE	6.083
AE177	1	0	FALSE	TRUE	2	TRUE	6.547
AEL1	2	0	FALSE	TRUE	0	TRUE	5.095
AEL2	2	0	FALSE	TRUE	0	TRUE	5.026
AEL4	0	4	TRUE	TRUE	3	TRUE	7.387
AEL5	1	4	FALSE	TRUE	3	TRUE	7.886
AEL6	0	4	TRUE	TRUE	3	TRUE	8.809

Key= Solubility level: 0 (extremely low), level 1(possible), 2 (low), 3 (good), 4 (optimal); **Blood brain barrier penetration level:** 0 (very high penetrant), 1 (high penetrant), 2 (medium penetrant), 3 (low penetrant),4 (undefined); **CYP2D6:** true (inhibitor), false (non-inhibitor); **Hepatotoxicity:** true (toxic), false (non-toxic); **Human intestinal absorption:** 0 (good), 1 (moderate), 2 (Poor) ,3 (very poor). **Plasma protein binding:** true (binder), false (non-binder). According to Lipinski's rule of five, **ALogP** must be below five for a drug-like compound.

The compounds **AE20** and **AE76** were predicted to be the most drug-like with good properties for five of the seven ADMET predictors. All the compounds were predicted to be hepatotoxic as well as possible binders to plasma proteins.

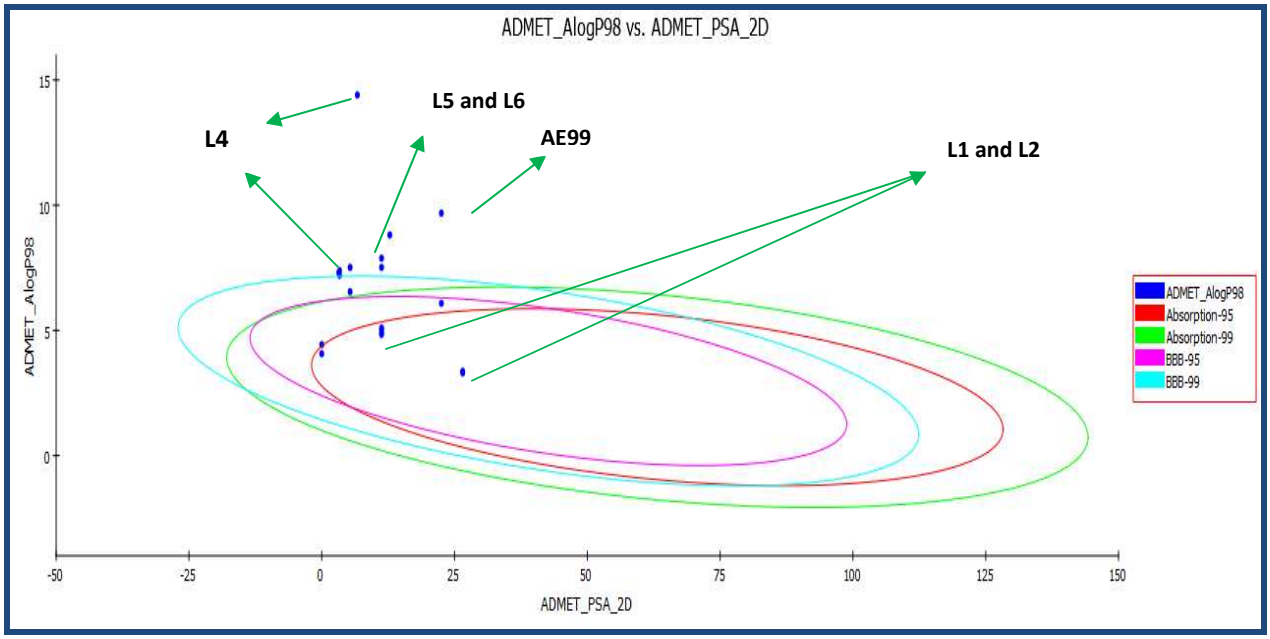
ADMET predictions were also performed for drugs currently used in HAART and these are shown in **Table 13**.

Table 13: ADMET Prediction scores for known drugs currently used for the treatment of HIV.

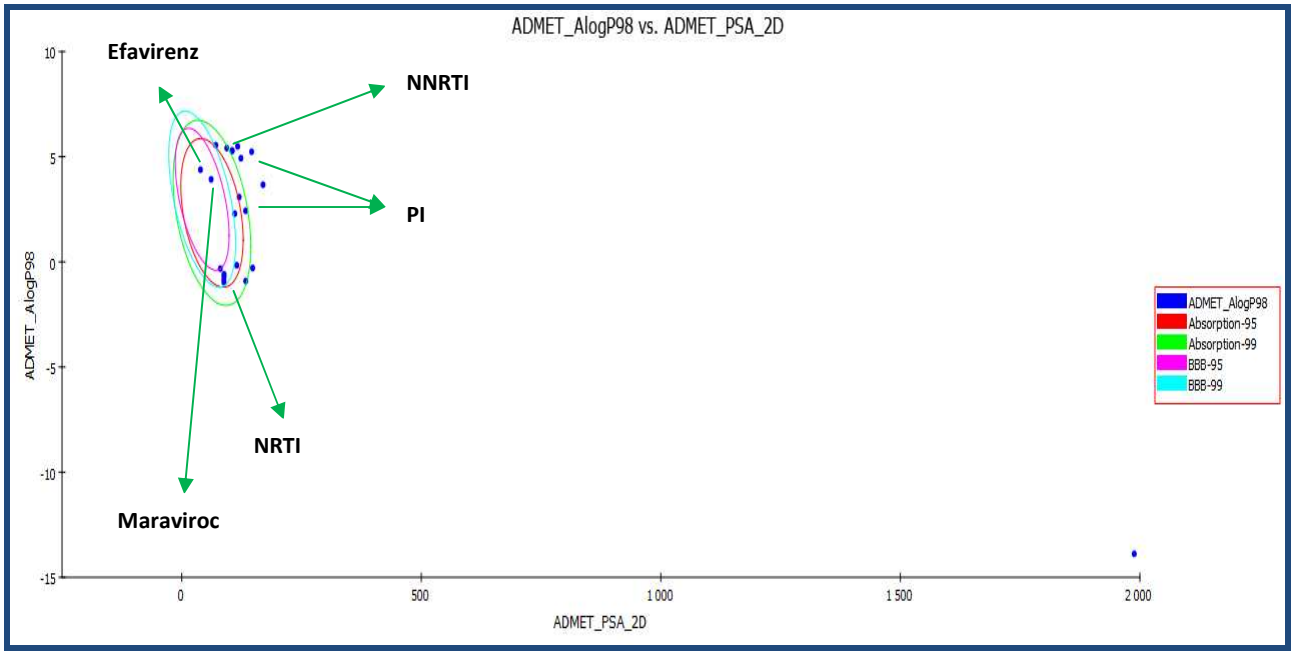
Drug Type	Drug Name	Solubility	BBB	CYP2D6	Hepatotoxicity	HIA	PPB	AlogP 98
PI	Amprenavir	3	4	FALSE	TRUE	1	TRUE	2.429
PI	Indinavir	3	4	FALSE	FALSE	1	FALSE	3.082
PI	Lopinavir	3	4	FALSE	TRUE	2	TRUE	4.926
PI	Ritonavir	2	4	TRUE	TRUE	2	FALSE	5.237
PI	Saquinavir	2	4	FALSE	TRUE	3	TRUE	3.667
PI	Stavudine	4	3	FALSE	TRUE	0	FALSE	-0.321
PI	Tenofovir	4	4	FALSE	TRUE	1	FALSE	-0.911
NNRTI	Dapivirine	1	4	FALSE	TRUE	1	TRUE	5.549
NNRTI	Delavirdine	2	4	FALSE	TRUE	0	TRUE	2.292
NNRTI	Efavirenz	1	1	FALSE	TRUE	0	TRUE	4.381
NNRTI	Nelfinavir	2	4	FALSE	TRUE	2	TRUE	5.284
NNRTI	Rilpivirine	1	4	FALSE	TRUE	1	TRUE	5.409
NNRTI	Etravirine	1	4	FALSE	TRUE	2	TRUE	5.492
NRTI	Emtricitabine	4	3	FALSE	TRUE	0	FALSE	-0.68
NRTI	Lamivudine	4	3	FALSE	TRUE	0	FALSE	-0.59
NRTI	Didanosine	4	3	FALSE	TRUE	0	FALSE	-0.844
NRTI	Zidovudine	4	4	FALSE	TRUE	0	FALSE	-0.163
NRTI	Zalcitabine	4	3	FALSE	FALSE	0	FALSE	-0.991
EI	Enfuvirtide	5	4	FALSE	FALSE	3	FALSE	-13.88
EI	Maraviroc	2	1	FALSE	FALSE	0	TRUE	3.921
INI	Raltegravir	3	4	FALSE	TRUE	2	FALSE	-0.291

Indinavir was the only protease inhibitor with no predicted hepatotoxicity while all the reverse transcriptase inhibitors (NRTIs and NNRTIs) were predicted to be hepatotoxic except **Zalcitabine**. None of the entry inhibitors were predicted to be hepatotoxic. Only two drugs; **Efavirenz** and **Maraviroc** had blood-brain barrier penetration. In fact, Maraviroc was shown to have good predictions for all the ADMET descriptors except for plasma protein binding. This means that the bioavailability of the drug can be lowered requiring administration of higher concentrations in order to obtain drug efficacy.

The hydrogen bonding ability of the metal compounds is indicated by the PSA plot and when used together with the AlogP98, human intestinal absorption predictions can be made. The ADMET_AlogP98, ADMET_PSA_2D plane includes 95% and 99% confidence ellipses. The confidence ellipses function to define regions where well absorbed compounds are predicted to lie. The 95% ellipse is expected to show 95% of well-absorbed compounds, both in the intestine and through the blood brain barrier. Similarly 99% of well absorbed compounds are expected to fall within the 99% ellipse (Accelerys Sofware Inc., 2011). Complexes of the ligands **AEL1** and **AEL2** as well as the ligands themselves showed good absorption falling within the 95% ellipse. **AE99** was the only **AEL1** compound with both poor blood brain barrier penetration and human intestinal absorption falling out of the 99% ellipses for both parameters (**Figure 38A**).



(A)



(B)

Figure 38: ADMET dot plot for the compounds (A) and current HIV drugs (B). The AlogP989 is plotted against the PSA. **L4, L5** and **L6** compounds were predicted to have poor BBB penetration with most of the compounds falling out of the 95 and 99% ellipses **(A)**. The entry inhibitor Maraviroc was predicted to have good BBB penetration and HIA falling in the 95 and 99% ellipses for both ADMET descriptors **(B).**

As expected from **Table 13**, **Maraviroc** and **Efiverenz** were predicted to have good BBB penetration as well as good HIA falling within the 95% ellipses for both these ADMET

descriptors. All other analysed HIV drugs were predicted to fall out of the 95% ellipse for BBB penetration and most NNRTIs were predicted to have moderate HIA occupying the 99% ellipse (**Figure 38B**). Four of the six analysed NNRTIs also showed AlogP values above 5, violating one of Lipinski's rule of five.

5. DISCUSSION

Nearly three decades after its discovery, HIV is still a major health threat especially in Sub-Saharan Africa. There is currently no cure or vaccine against the virus and there is therefore a need for new forms of treatment that offer more effective management of the infection. The main objective of this research was to develop new metal based inhibitors of the viral enzymes (HIV-1 RT, PR and IN) and also to evaluate these potential metallodrugs for their effect on selected opportunistic infections (cervical cancer and M. Tb) characteristic of AIDS in South Africa.

5.1 HIV-1 Enzyme inhibition

In a review of metal complexes as enzyme inhibitors, Louie and Meade (1999) stated that metal complexes can coordinate to enzyme active site residues to block substrate interactions (competitive inhibition) or to residues outside the active site to affect structural integrity (non-competitive inhibition). The authors also suggest that the metal complexes could be interacting with targets through weak linkages of hydrogen bonding and van der Waals interactions [95]. Herein metal complexes were investigated for their inhibition of HIV-1 RT, IN and PR.

5.1.1 HIV-1 Reverse Transcriptase inhibition

Reverse transcriptase functions during the conversion of viral RNA to pro-viral DNA and the inhibition of this step could therefore halt the whole viral replication process. Twelve metal based compounds were analysed for their inhibition of this enzyme and none of the compounds exhibited activity.

Generally the enzyme is inhibited through two mechanisms; (1) binding of NRTIs which are analogues of nucleotides but act as chain terminators during DNA synthesis and/or (2) the binding of NNRTIs into an allosteric pocket of the enzyme inhibiting DNA synthesis non-competitively [25, 26]. A closer look at both these types of inhibitors could provide an explanation for the lack of activity of the tested metal complexes.

As stated before, NRTIs are analogues of the natural nucleotides and nucleotides are composed of a base (purine or pyrimidine), a phosphate and a sugar [153]. This means that for any compound to act as an NRTI, it must resemble a nucleotide. None of the metal compounds are structurally similar to nucleotides. The only possible similarities are the nitrogen ions on the pyridine rings of the ligands. In addition a phosphate group is required in order for these inhibitors to be incorporated into the DNA strand. Current NRTIs are phosphorylated by cellular enzymes after being taken up by the cells [26]. This could be the required step in order to activate the metal complexes studied here; meaning inhibition of

enzyme activity may be possible in whole virus assays. An example of such an assay is the luciferase reporter gene assay in TZM-bl cells that measures the production of neutralizing antibodies against HIV-1 pseudoviruses. The assay was first described by Montefiore (2005) and instead of looking at specific areas of the replication life cycle; the assay looks at whole virus infectivity [154]. It is based on the inhibition of the infection of cells by a molecularly cloned HIV-1 pseudovirus. A single round of infection takes place and produced particles are automatically unable to infect new cells. Some of the complexes in this study were tested in this assay at the National Institute for Communicable Disease of South Africa (NICD). Higher concentrations showed 100% virus neutralization for the complexes **AE76** and **AE20**; however these were discontinued due to the observed toxicity (see **Appendix IV**).

Non-nucleotide reverse transcriptase inhibitors consists of two linked aromatic rings and the linker itself could be the third ring [45]. Metal compounds analysed in this study all contain at least one aromatic ring; therefore one would expect them to be more like NNRTIs instead of NRTIs. The presence of one aromatic ring disqualifies the compound from the “two-hinged-ring” model, but compounds such as tetrahydroimidazo-(4;5;1-1 jk)(1,4)-benzodiazepin-2(1*H*)-one (TIBO) analogues (containing one ring) have been shown to be potent inhibitors of HIV-1 RT, binding in the same position as NNRTIs [45]. Oxovanadium–thiourea complexes consisting of two thiourea compounds as ligands and a central vanadium have also been reported to have an effect against HIV-1 replication in PBMCs at submicromolecular IC_{50} values of between 0.008-0.128µM. These compounds were also able to directly inhibit HIV-1 RT at IC_{50} values of between 0.187-2.1µM [12]. This is evidence that metal complexes can possess activity against HIV-1 RT, even if they do not fit into the “two-hinged-ring” rule, and modifications such as phosphorylation and increased lipophilicity to the metal complexes in this study could potentially result in the inhibition of the enzyme.

5.1.2 HIV-1 Protease inhibition

HIV-1 Protease is responsible for the production of mature virus particles and the enzyme has been one of the most targeted components of the viral life cycle. Of the twelve metal compounds, three showed activity against this enzyme.

The active compounds were **AE99**, **AE190** and **AE177**. The ligands of all these metal compounds had no enzyme inhibition suggesting the necessity of the metal ions in the activity of the compounds. **AE99** and **AE190** are both palladium based compounds. Palladium ions had been previously reported for anti-HIV activity; however this was not against HIV-1 PR. Polysulfonates of Pd(II) showed activity against both HIV-1 and HIV-2 during whole virus infectivity studies [155].

AE177 is a Pd-Au bimetal and Au(III) compounds have been shown to inhibit HIV-1 PR and their activity was speculated to be through ligand exchange reactions (reaction where one ligand in a complex ion is replaced by a different one) [100]. Lebon and colleagues (2002) have reported Cu(II) complexes of N1-(4-methyl-2-pyridyl)-2,3,6-trimethoxybenzenamide which interact with the active site of the enzyme leading to competitive inhibition. The ligands of the these complexes were said to be poor chelators of the cupric ion under enzymatic assay conditions, and the released cupric ions interacted with cysteine residues on the surface of the enzyme [103]. The phosphine ligands could be doing the same; releasing Pd^{2+} and Au^{+} ions that are able to interact with this cysteine residue.

Metallocarboranes have also been shown to inhibit HIV PR showing potent, specific and selective competitive inhibition [156]. Cigler *et al.* (2005) were able to determine the crystal structure of the parent cobalt bis(1,2-dicarbollide) in complex with the enzyme, and the structure revealed that two molecules of the parent compound bind to the hydrophobic pockets in the flap-proximal regions of the S3 and S3 sub-sites of PR. Therefore, in addition to filling the binding pockets in the same manner as PIs, the compound blocked flap closure [156]. Indeed the presence of a thiol on Cys95 at the enzyme dimerization interface could be a contributing factor to the observed activity of the compounds in this study [157]. Cysteine residues are very common in enzyme active sites and their thiolate anions are one of the most powerful nucleophillic ions available from amino acids [158]. Complexes of Pd(II) have previously been shown to hydrolyse internal amide bonds of peptides by attaching to the sulphur atom of S-methylcystein [159]. Bonding of positively charged Pd(II) ions and Au(I) ions to the Cys95 residue can therefore result in the covalent modification of the protease, subsequently rendering it inactive.

5.1.3 HIV-1 Integrase inhibition

HIV-1 integrase plays a role in the integration of the produced viral DNA into the host chromosome and the inhibition of this step could mean no production of viral proteins needed for the assembly of new virus particles [27]. The bimetallic compound **AE177** was the only compound with activity against the enzyme.

The study of potential inhibitors of this enzyme is fairly new, with the first and only IN inhibitor, Raltegravir, approved as recent as 2007 [32]. This inhibitor binds the active site of the enzyme and docking studies of this compound into the crystal structure of the IN active site core domain showed that it docked near the Mg^{2+} ions. The keto-enol moieties were coordinated by the Mg^{2+} ions, allowing chelation of the ions [123]. This could be how **AE177**

achieves its activity, were the metal ions on the compound (Au and Pd) undergo ionization releasing ligands that have a number of electronegative atoms (N, P, S) (**Figure 39**). These in turn interact with the Mg^{2+} ions, inhibiting enzyme activity. Diketoacids, which are the first compounds to exhibit potent HIV IN inhibition, are also believed to function in a similar manner, with an ability to sequester one or both of the metal ions in the active site. If this is true for **AE177,** a complex could be forming between the compound, metal ions (Au and Pd) and integrase, resulting in blockage of the transition state enzyme-DNA complex and competition with the target DNA substrate [126]. Lack of activity from other mono metals (Au or Pd) could mean that both metal ions are required for the observed activity.

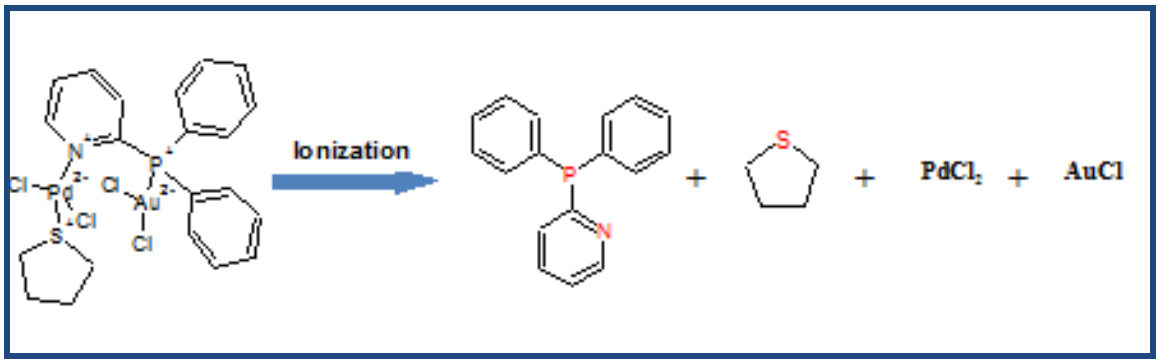


Figure 39: Ionization of AE177. The release of the ligands binding to the metal ions results in the exposure of electronegative ions (red) capable of forming interactions with active site Mg^{2+} ions.

In addition to the metal ions, the enzyme active site also includes residues such as Lys159 which is able to establish cationic and hydrogen bonding interactions with inhibitors. Other residues that could form hydrogen bond interactions include Thr66, His67 and Gln148. Hydrophobic interactions with active site residues such as Asn155 are also possible, and this might also be how **AE177** exhibits enzyme inhibition [123].

5.2 Effects of Metal compounds on cell viability

One of the many steps in the multifaceted process of drug discovery is the determination of whether the compound of interest is cytotoxic or not. Here the tetrazolium dyes MTT and MTS were used and CC_{50} values for each compound was determined (**Table 7**). Ten compounds showed very low CC_{50} values, which is indicative of the potential toxicity of these compounds to PBMCs and TZM-bl cells. **AE205** exhibited the highest toxicity with an estimated CC_{50} value of below 0.78µM on PBMCs. The compound is gold based and toxicity at such low concentrations suggests that the compound is better suited as an anti-cancer drug [105]. Three compounds had CC_{50} values above 50µM and two of these were platinum based compounds (**AE98** and **AE72**). This is surprising as one would expect platinum compounds to be more toxic as compounds such as cisplatin and carboplatin have been

very potent against cancerous cells. With that said, it is not unusual for metal based compounds to be non-toxic and a gold(III)chloride compound has been reported to show over 80% cell viability at concentrations as high as 100µM [100].

In general, the compounds seem to show greater toxicity to PBMCs than TZM-bl cells with as high as 36-fold difference in the CC_{50} for some of the compounds. These differences may be due to the differences in the properties and mechanism of the dyes. The dyes vary in cell permeability characteristics, formazan product solubility, redox potentials and proposed sites of interaction with the mitochondrial respiratory chain. MTS is a tetrazolium analogue of MTT possessing phenyl moieties that have negatively charged sulfonate groups. This slight modification results in the production of soluble formazans and the solubilisation reaction is no longer required. However, the positive charge of the inner core of MTS can be counter balanced by the negative charge of the sulfonate group on one phenyl ring, generating an inner salt. This therefore means that MTS would not be expected to readily enter the cell via membrane potential (lipophilic properties may counter negative charge resulting in limited ability to cross the plasma membrane). Therefore, although both dyes can enter the cell and be reduced intracellularly, MTS does so to a lesser extent and some live cells could be missing the dye, giving off a signal of increased toxicity [131].

5.3 Effects of metal compounds on cell proliferation

Apart from cytotoxicity, it is important to know if the compound of interest promotes cell proliferation and if not, to what extent or does it halt the cell cycle at a particular point inhibiting proliferation. HIV infection is associated with chronic immune activation and proliferation. This has been said to result in observed immune dysfunction and damage. It would therefore be ideal for potential anti-HIV compounds to inhibit proliferation [160]. Metal based compounds have been shown to possess effects on the proliferation of a number of cells and gold compounds have been suspected to work at cellular level by inhibiting T-cell proliferation and therefore modulating the immune systems [161]. Here two methods were used to determine the effects of the metal ions on the two c

]\ll types.

5.3.1 Effects on cell proliferation using Flow Cytometry

Flow cytometry was used to determine the effects of the compounds on the proliferation of PBMCs at 5µM. Gating was done on all events and CFSE intensity was used to determine proliferation into the daughter cell population. As expected from the MTS assay, compounds with no toxicity initiated proliferation into daughter cells. Propidium iodide staining showed the presence of some viable cells for compounds that were toxic in the MTS assay; however, CFSE staining did not show proliferation of the cells into the daughter population. This could

mean that the cells are being arrested in one of the stages of the cell cycle. There are mainly two molecular processes that take place during the cell cycle; DNA synthesis (S phase) and mitosis (M phase) where resulting daughter chromosomes are distributed to each daughter cell. These steps have resting intervals in between and master controllers of these events are heterodimeric protein kinases which regulate the activities of various proteins involved in DNA replication and mitosis [162]. Metal ions have been shown to interact with proteins, and it is possible that the metal compounds in this study are interacting with these regulatory proteins [162, 163].

The observed effect of these compounds on the proliferation of PBMCs could mean that the compounds possess immunomodulatory effects. Metal ions have been shown to have effects on the immune system, resulting in conditions such as chronic inflammatory reactions, hypersensitivity as well as allergic and autoimmune disease [159, 164]. Although such effects could be counter productive to the aims of this study, it is also possible that some compounds exhibit positive immune modulation where compounds such as **AE20** could inhibit the proliferation of infected cells and non-toxic compound such as **AE99** could promote proliferation of uninfected CD4 cells, possibly increasing CD4 cell counts, as well as of cytotoxic cells.

5.3.2 Real-time cell analysis of the compounds on TZM-bl cells

Impedance-based label free technology is becoming more and more popular in drug discovery and this is because it is non-invasive and interferes minimally with cell morphology. As stated before, the system can provide a number of cell growth profiles that gives a glimpse into the mode of action of the compound. One of those is cytostatic behaviour. If the compounds have any effect on proliferation, it should ideally be anti-proliferative and inhibit the proliferation of infected CD4 cells. HIV replicates in actively dividing cells and decreasing these cells could result in the decrease in HIV production (Lori *et al.* 1997). The compounds were analysed at concentrations varying from 40-5µM.

Higher concentrations (40 and 20 µM) of the compounds that were shown to be toxic in the MTT assay (e.g. **AE76**) had profiles similar to Auranofin, while their lower concentrations had growth profiles similar to untreated cells. This could suggest that toxicity observed from these compounds is through a similar mode of action as Auranofin. Auranofin has been reported to inhibit thioredoxin reductase [139]. Cytosolic and mitochondrial isoforms of the enzyme play a role in the regulation of cellular events including cell signalling, cell proliferation and apoptosis. In addition, the redox system formed by the enzyme and its

substrate thioredoxin has a large variety of roles including the removal of hydrogen peroxide. Therefore, by inhibiting thioredoxin reductase, Auranofin alters the redox state of the cell leading to increased production of hydrogen peroxide. This leads to oxidation of components of the thioredoxin system, subsequently leading to augmented apoptosis [139], [166].

Comparison of the obtained metal complex profiles to known profiles (**Figure 21B**) showed the potential of **AE188** (5µM) of being a DNA-damaging compound. This is proven by the observed anti-cancer activity of this complex (**Table 10**). With regards to the other complexes, it is important to remember that the physiology of each cell-line is complex, unique and subject to changes *in vitro*; and that could be the reason why some of the known profiles were not observed for these complexes [128].

5.4 Anti-Cancer activity of metal compounds

One of the opportunistic infections associated with HIV infection is cancer and cervical cancer is becoming more prevalent in HIV-1 infected women in Sub-Saharan Africa. The metal compounds were tested for their inhibition of the growth of HeLa cells and nine of the twelve metal complexes showed growth inhibition of the cells , with IC_{50} values as low as 0.81ug/ml for **AE205** (**Table 10**).

Platinum based compounds such as cisplatin were the first metal based compounds to show activity against cancerous cells and are some of the most widely used chemotherapeutic agents. None of the platinum based compounds in this study (**AE98** and **AE72**) showed activity against HeLa cells. In fact the most potent compounds were **AE205** and **AE359** which are both gold based compounds. These compounds exhibited IC_{50} values lower than cisplatin indicating a higher potency and a potential ability of these compounds to overcome intrinsic cisplatin resistance [166]. There have been studies of the anti-cancer mechanism of both gold(I) and gold(III) compounds. Gold(I) compounds have been reported to exhibit weak interactions with DNA, suggesting that their mechanism of action is different from that of cisplatin [167]. Gold(III) compounds on the other hand have been reported to show stronger interactions with DNA and this may be due to the ability of gold(III) to form square planar complexes similar to cisplatin. However, interactions could also be weak and reversible, depending on the ligands binding to the Au(III) [105].

There have been efforts to develop tumour-inhibiting profiles of new metal complexes and these include palladium based compounds. These are anticipated to have superior efficacy, reduced toxicity, a wider spectrum of activity, lack of tumour cell resistance, increased selectivity and improved pharmacological characteristics [167]. Another compound that

showed an IC_{50} lower than that of cisplatin is **AE190**. The compound is a palladium based compound and it had an $IC_{50}$of 4.82µM. In a study on the activity of tris(dibenzylideneacetone)dipalladium against human and murine melonoma cells, Bhandarkar *et al.* (2008) found that the compound was a potent inhibitor of N-myristoltransfarase-1. In addition, the compound inhibited a number of pathways required for melanoma tumouregenisis and this could be one of the ways **AE190** inhibits HeLa cell growth [168]. Ray *et al.* (2007) also showed a palladium (II) complex with a similar IC_{50} to **AE190** against HeLa cells. Mechanistic studies of this complex revealed that it caused over expression of cyclin B1 antibody which is associated with the arrest of the cell cycle in the G2/M phase and induction of apoptosis [11].

5.5 Effects of complexes on the growth of *Mycobacterium tuberculosis*

All HIV infected patients, especially in Sub-Saharan Africa, are at risk of co-infection with TB. Due to the non-compliance of patience to the treatment, there has been emergence of MDR and XDR strains of the bacterium making treatment of co-infected individuals very difficult. Therefore, development of compounds with activity against HIV and TB could potentially decrease the number of drugs to be taken, increasing the chances of mycobacterial clearance for co-infected patients.

The metal based compounds were tested for their inhibition of the H37Rv strain of *Mycobacterium tuberculosis.* Three of the twelve compounds (**AE188**, **AE189**, and **AE190**) showed potent bacterial inhibition with MIC values of 1.56µM. All three compounds were palladium based. Ferreira *et al.* (2012) recently synthesized a 1,3-bis(diphenylphosphino)propane Pd(II) complex which inhibited the same strain of *Mycobacterium tuberculosis* with an MIC value of 5.15µM [169]. This therefore suggests that the palladium complexes in this study are the most potent Pd(II) complexes active against *M. tuberculosis* to be reported, amplifying the novelty of this research.

Mycobacteria do not contain glutathione (GSH) but have the analogue, mycothiol (MSH). The enzyme exists in millimolar concentrations in the bacterium and similarly to GSH, the enzyme plays a role in oxidative stress management and is oxidized to MSSM, a symmetrical disulfide [144], [170] . MSH is essential for the growth of *M. tuberculosis* and bacteria with deficiencies in the enzyme have been shown to have increased sensitivity to oxidative stress. This therefore makes this redox pathway a potential biological target for novel antitubercular chemotherapy. Mycothiol reductase (Mtr) is essential for the conversion of the disulfide MSSH to MSH and therefore inhibition of Mtr will result in the deficiency of

MSH in the bacterium [143]. In this study the three most potent compounds were tested for their inhibition of glutathione reductase (Gtr). This was done in order to eliminate any compounds that would show inhibition against this enzyme as this would not be specific for Mtr and would therefore interfere with normal physiological GSH concentrations. **AE190** did not show inhibition of the enzyme and further analysis on the inhibition of Mtr could then reveal if the compound has specificity for this enzyme or not.

5.6 Drug-likeness of compounds

In silico methodologies have become popular in the drug discovery process. These methods include ADMET predictions as well as molecular docking of compound structures into enzyme active sites [116]. ADMET provides a quick screen of compounds for essential properties related to drug-metabolism using known models. The complexes in this study were screened using Discovery Studio 3.1 (Accelerys Sofware Inc., USA) for their potential solubility, human intestinal absorption, blood-brain barrier penetration, plasma protein binding, cytochrome P450 enzyme inhibition and ALogP values [145].

The solubility of an administered drug determines whether it could potentially be administered orally or intravenously which basically determines the “drug-likeness” of that compound. None of the metal compounds showed good or optimal solubility with the gold based compounds **AE20** and **AE76** showing moderate solubility. This therefore means that in the case of the latter compounds moving forward as anti-cancer drugs (**Table 9**), there are possibilities that they could be administered orally. This would make these compounds advantageous over cisplatin which has poor solubility and is therefore administered intravenously [11].

Knowing the blood-brain barrier (BBB) penetration probability of a particular drug is not only important for the regulation of the distribution of the drug and its potential toxicity, but in cases such as HIV infection were the virus is able to attack the brain, high BBB penetrants could be beneficial. Six compounds were predicted to be high penetrants and one of those is the bimetal **AE177** which showed good PR and IN inhibition. This means that, following extensive clinical tests, the compound could exhibit the aforementioned ability if included as part of a treatment regimen. Metals have been shown to participate in many neurodegenerative conditions and examples include the use of lithium in the treatment of bipolar disorder [171]. Although the exact mechanism of action of this metal ion is not known, the metal compounds’ BBB penetration could further be investigated for their effects on neurodegenerative disorders like bipolar and Parkinson’s disease.

Eventually an administered drug will be metabolised and excreted. Cytochrome P-450 enzymes play a huge role in the proper excretion of drugs. One of the most important cytochrome-P450 enzymes is CYP 2D6 and the enzyme plays a role in conjugation reactions such as sulphation and conjugation to glutathione [145]. Most of the metal compounds were predicted to not have any inhibitory effects on the enzyme, except for the compound **AE125**.

The hepatotoxicity predictor looks at the possible occurrence of dose dependent human hepatotoxicity. All the compounds were predicted to have potential liver toxicity, and this means that these compounds can potentially interfere with a number of functions performed by the liver. These include production of plasma proteins and the storage of energy reserves [145].

The ability of a particular compound to be absorbed determines the form of possible administration for that particular drug. Ideally a drug should be well absorbed into the blood stream and based on the HIA predictions, the compounds **AE20** and **AE76** were predicted to have good absorption; with **AE98** and **AE359** showing moderate HIA. Most of these compounds are gold based compounds and it not surprising that they should be absorbed because a number of gold based compounds (e.g. Auranofin) used in the treatment of rheumatoid arthritis, are administered orally [98], [99].
As stated before, metallo-compounds have been reported to interact with proteins. This therefore means that these compounds could potentially bind to plasma proteins and this could affect the bioavailability, delivery and storage of the compound [145]. All the compounds were predicted to have binding abilities to plasma proteins. Human Serum Albumin (HSA) is the most abundant protein making up about 52% of all serum proteins. The protein is known to bind to a wide range of drugs resulting in restriction of free and active drug concentrations. Reduction in the binding ability of lead compounds to such proteins could lead to increased bioavailability, otherwise the administered drug concentrations can be increased in order to compensate for plasma protein binding. Binding of compound to HSA does not always have to be a bad thing as organometallic compounds such as ruthenium(II)-arene which is an anti-cancer drug has been targeted to tumour cells through the exploitation of affinity to HSA [172].

The lipophillicity of a drug is one of the key physiochemical parameters that are linked to membrane permeability, and therefore drug absorption and distribution. The partition coefficient P or ALogP is the gold standard of expressing lipophilicity, and according to one of Lipinski's rules for drug-like compounds the ALogP should not be greater than 5 [145].

Three of the twelve compounds, **AE20** and **AE76** exhibited compliance to that rule with ALogP values of 4.9 and 4.84 respectively.

With regards to the drug-likeness of current HIV inhibitors, Discovery Studio (Accelerys Sofware Inc., USA) obtained ADMET data was similar to literature [82]. Although most of the ARVs had some undesirable predictions e.g. hepatotoxicity, most had ALogP values way below 5 (which shows the importance of lipophilicity in drug distribution and efficacy). Overall, the compounds **AE20** and **AE76** were predicted to be the most drug-like of the twelve complexes. These are more likely to act as cancer drugs since they exhibited moderate anti-cancer activity (**Table 9**) and are predicted to be soluble and easily absorbed. Moderate solubility and good absorption makes these compounds candidates for oral administration. Although BBB penetration could be viewed as an adverse effect for cancer drugs, such high penetrants could be beneficial in the treatment of brain tumours. The inability of these compounds to inhibit CYP2D means that they can be excreted, while their ability to bind to plasma proteins could lower their effective concentration, requiring administration of high doses. This can however be exploited to target these drugs [40].

6. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, three compounds with an effect on the activity of HIV-1 PR were successfully synthesized. One of these compounds also showed inhibition of HIV-1 IN, indicating possibility of one compound to target different processes of HIV-1 replication (**Table 14**).

Further more, one of the compounds with PR inhibition showed affectivity against the growth of cervical cancer cells as well as *M. tuberculosis*, indicating the potential use of one compound to treat different pathologies that could be related. This could especially be beneficial in HIV-TB co-infection. Indeed, the observed broad spectrum activity of some of the complexes is an interesting finding, but should also be discussed in the light of potential non-specificity of action. Experimental procedures to test this possibility are, however, outside the scope of this work.

ANSWERS TO RESEARCH QUESTIONS

The hypothesis of this study was that “Metal complexes (containing palladium, platinum and gold) could serve as lead compounds against HIV and its associated opportunistic infections; in particular tuberculosis and cancer". In order to verify this hypothesis, a number of research questions were posed. Responses to these questions are provided below and are also summarized in **Table 14**.

1. **Can the metal complexes inhibit the activity of HIV-1 RT, PR and IN?**

   None of the complexes inhibited the activity of HIV RT, while three complexes inhibited HIV PR. The bimetallic complex was one of these complexes and was also the only complex with IN inhibition. All enzyme inhibitions were shown to be significant with *P*-values below 0.05.

2. **What are the CC_{50} values of these complexes on peripheral blood mononuclear cells (PBMCs) and TZM-bl cells?**

   Three of the twelve complexes had CC_{50} values that were above the tested compound concentrations, indicating the non-toxicity of these complexes on both PBMCs and TZM-bl cells. Nine complexes had CC_{50} values below 5 µM on PBMCs and were slightly less toxic to TZM-bl cells. Only one compound that had exhibited enzyme activity (**AE99**) was non toxic at the enzyme inhibition concentrations.

3. **What are the effects of these complexes on cell proliferation and can this be observed through flow cytometry and real time cell analysis?**

   Three complexes showed proliferation of PBMCs into daughter cells in flow cytometry. These were the complexes that were not toxic to PBMCs (CC_{50} value above the highest tested concentration). All the tested complexes showed cell proliferation profiles that were similar to Auranofin at high concentrations in real-time cell analysis, while the lowest concentration (5 µM) had a proliferation profile similar to untreated cells. This confirmed MTT data for these complexes. No unique profiles were obtained for any of the complexes, while **AE188** showed potential as a DNA-damaging agent.

4. **Can the complexes inhibit the growth of HeLa cells and if so, what is the selectivity index for each complex?**

   Nine complexes showed IC_{50} values below 20 µg/ml, while two of those complexes (**AE205** and **AE359**) had IC_{50} values below 4 µg/ml against the growth of HeLa cells, which is the recommended cut-off for pure compounds by the US National Cancer Institute of America. Unfortunately all calculated selectivity index values were below 10, which is an indication of general not targeted cytotoxicity.

5. **Do the compounds exhibit anti-MTB activity, and if so, what is the minimum inhibitory concentration for the active complexes?**

   Three complexes showed potent *M. tuberculosis* inhibition. These complexes all had an MIC of 1.56 µM and one of these complexes did not show inhibition of glutathione reductase. This is an indication of the potential selectivity of this compound to the bacterial analogue of this enzyme, mycothiol reductase.

6. **What is the overall drug-likeness of each complex based on *in silico* ADMET analysis, and how do these compare to the current HAART?**

   Two complexes showed good ADMET predictions, with good solubility, human intestinal absorption and ALogP values of below 5. Unfortunately all of the complexes were predicted to be hepatotoxic, which is one of the major challenges with current HAART regimens. A major distinction between current HAART drugs and the complexes tested in this study is that although HAART drugs may be toxic, they have relatively low AlogP values, making them compliant to one of Lipinski's major rules for drug-like compounds.

Table 14: Summary of positive response from analysed metal complexes.

ACTIVITY	COMPOUND(S)
HIV PR inhibition	AE99, AE190, AE177
HIV IN inhibition	AE177
Non-toxic to cells	AE99, AE98, AE72
Anti-proliferation	AE20, AE76, AE125, AE205, AE188, AE189, AE190, AE359, AE177
Anti-cancer activity	AE20, AE76, AE125, AE205, AE188, AE189, AE190, AE359, AE177 (Red= most potent).
Anti-*M. tuberculosis*	AE188, AE189, AE190
Drug-like	AE20, AE76

Future work includes modifications of the compounds in order to reduce potential toxicity. The compounds could be further tested for the ability to act as entry inhibitors (interaction with CCR5), and the potential immune modulatory effects of the compounds should be confirmed by measuring the secretion of cytokines.

The mechanism of action of all active compounds can be elucidated using *in silico* molecular modelling, where the compounds can be docked into the active sites of PR and IN. These complexes can also be tested for their synergistic effect in the presence of current anti-HIV drugs as well as the inhibition of viral infectivity through whole virus assays.

With regards to the anti-cancer activity, active complexes can be tested for their effect on other cancer cell line as well as drug resistant strains. Investigation of the mechanism of action of the complexes can be performed in order to determine the target for these complexes. Targeting the complexes could result in higher specificity, therefore increasing the selectivity index of the complexes.

The complex with potent anti-TB activity and no inhibition of glutathione reductase can further be analysed for activity against mycothiol reductase in order to determine if this is the target pathway for the complex.

Finally, the use of metal compounds in medicine reveals extensive potential for organometallic compounds. The significant identification of four HIV PR inhibitors, one IN inhibitor, two potent anti-cancer agents and three potent *Mycobacterium tuberculosis* inhibitors shows the possible ability of these compounds to inhibit HIV, cancer and tuberculosis, supporting the hypothesis of this study.

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8. APPENDIX

Appendix I: List of reagents and company information

The following reagents were used in this study:

Table A1: Information of reagents used in the study

Reagent	Catalogue Number	Company	Country
HIV Reverse Transcriptase Calorimetric Kit	11468120910	Roche Appplied Science	Germany
rec HIV-1 Reverse Transcriptase Enzyme (expressed in *E.coli*)	382129-500U	Merck	Germany
HIV-1 Protease Substrate	H6660-1MG	Sigma Aldrich	USA
rec HIV-1 Protease (expressed in *E.coli*)	H-9040	Bachem	Switzerland
Acetyl Pepstatin (AP)	A4815	Sigma Aldrich	Germany
Xpressbio HIV-1 Integrase ssay kit	EZ-1700	Expressbio Life Science Products	USA
DMEM Media	41966029	Sigma Aldrich	USA
Hepe Buffer	15630056	Gibco, Life Technologies	USA
Sodium Pyruvate	SH30239.01	Hyclone, Thermo Scientific	USA
Gentamicin	G1272-10ML	Sigma Aldrich	USA
Foetal Bovine Serum	10270106	Gibco, Life Technologies	USA
Trypsin-EDTA	T4049-500ML	Sigma Aldrich	UK
EDTA Anti-coagulant Vacuette Tubes	VGRV454021	Greiner Bio-one, Lasec	SA
RPMI Media	R6504-10X1L	Sigma Aldrich	USA
Antibiotic/Antimytotic	SV30079.01	Separations	SA
Ficoll-Histopaque®-10771	10771-6X100ML	Sigma Aldrich	UK
Phytohemagglutinin-protein (PHA)	L9017-5MG	Sigma Aldrich	Germany
Thiazolyl Blue Tetrazolium Bromide (MTT)	M5655-1G	Sigma Aldrich	Germany
CellTiter 96® AQueous One Solution (MTS)	G3580	Promega	USA
CellTrace™ CFSE Kit	C34554	Molecular Probes	USA
Auranofin	A6733	Sigma Aldrich	Germany
MEM Media	SH30024.01	Separations	SA
DMSO	D2650-100ML	Sigma Aldrich	Germany

The following instrumentation and software programmes (**Table 17** and **Table 18**) were used in this study.

Table A2: List of important instruments used in the study

Instrumentation	Company	Country
Multiskan Ascent plate reader	Thermo Lab Systems	USA
Flouroskan Ascent FL	Thermo Lab Systems	USA
Allegra 25R centrifuge	Beckman Coulter	USA
FACSAria II Flow Cytometer	BD	USA
XCelligence Real Time Cell Sensor	Roche Diagnostics	Germany

Table A3 Software programmes used to analyse data

Programme	Company	Country
Microsoft Excel 2012	Microsoft Corporation	USA
GraphPad Prism 5	GraphPad Software Inc.	USA
Discovery Studio 3.1	Accelerys Software Inc.	USA
Chemsketch Freeware Version 11.01	Advanced Chemistry Development Inc.	Canada

Appendix II: Formulae for Data Analysis

The following formulae were used for data processing:

Formula 1: Calculating percentage enzyme inhibition

$$\%Activity = \left(\frac{\left(Ave_{absorbance\ compound}\right) - \left(Ave_{absorbanceb\ blank}\right)}{\left(Ave_{absorbance\ positve}\right) - \left(Ave_{absorbance\ blank}\right)} \right) \times 100$$

$$\%\ Inhibition = 100 - \%Activity$$

Formula 2: Calculating cell concentration using a haemocytometer:

$$Cell\ concentration = \left(\frac{Total\ live\ cells}{Number\ of \quad squares\ counted} \right) \times Dilution \times 10^4$$

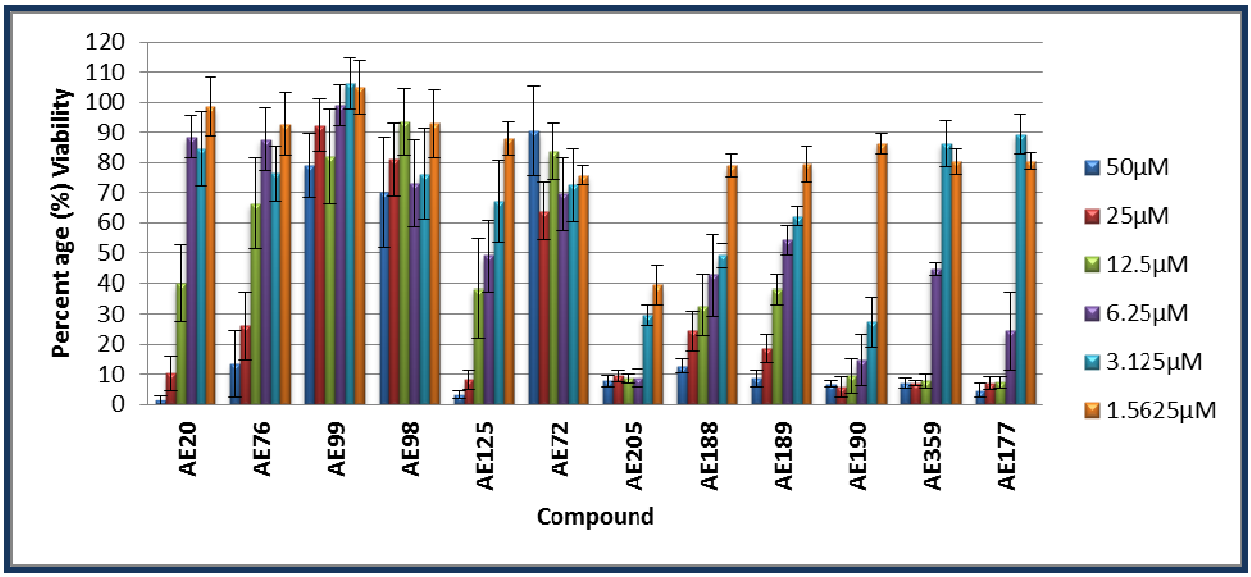
Formula 3: Calculating cell viability:

$$\%Viabillity = \left(\frac{\left(Ave_{absorbance\ compound}\right) - \left(Ave_{absorbanceb\ blank}\right)}{\left(Ave_{absorbance\ untreated\ cells}\right) - \left(Ave_{absorbance\ blank}\right)} \right) \times 100$$

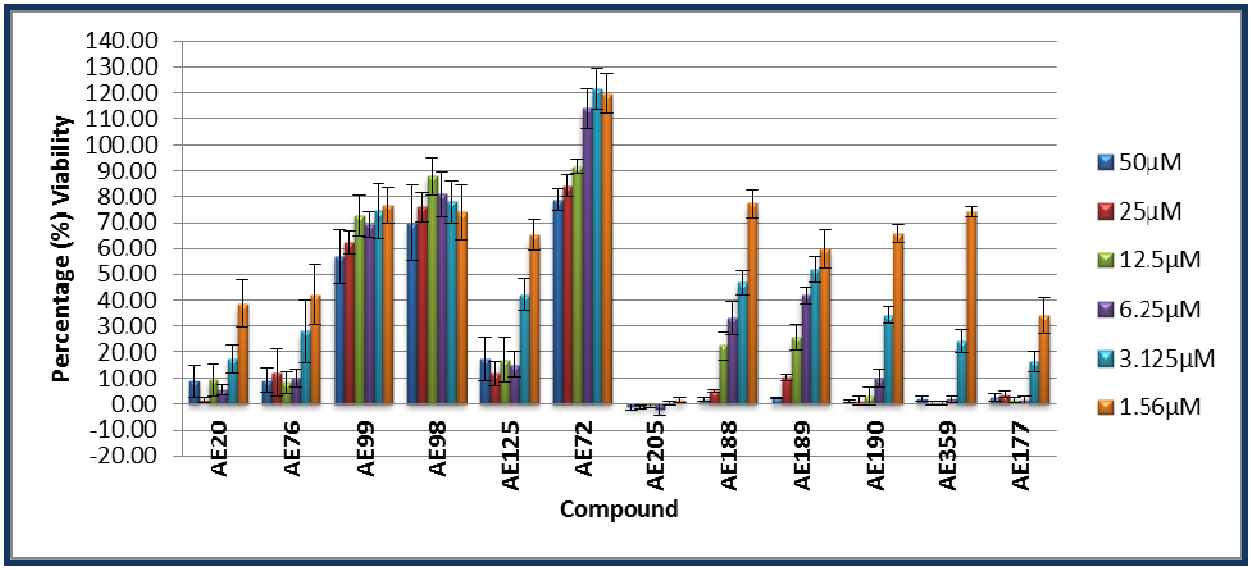
$$\%\ Cytotoxicity = 100 - \%Viability$$

Appendix III: Cell viability

The metal complexes exhibited the following effects on the growth of TZM-bl cells and PBMCs. These were used to calculate the CC_{50} values.



(A)

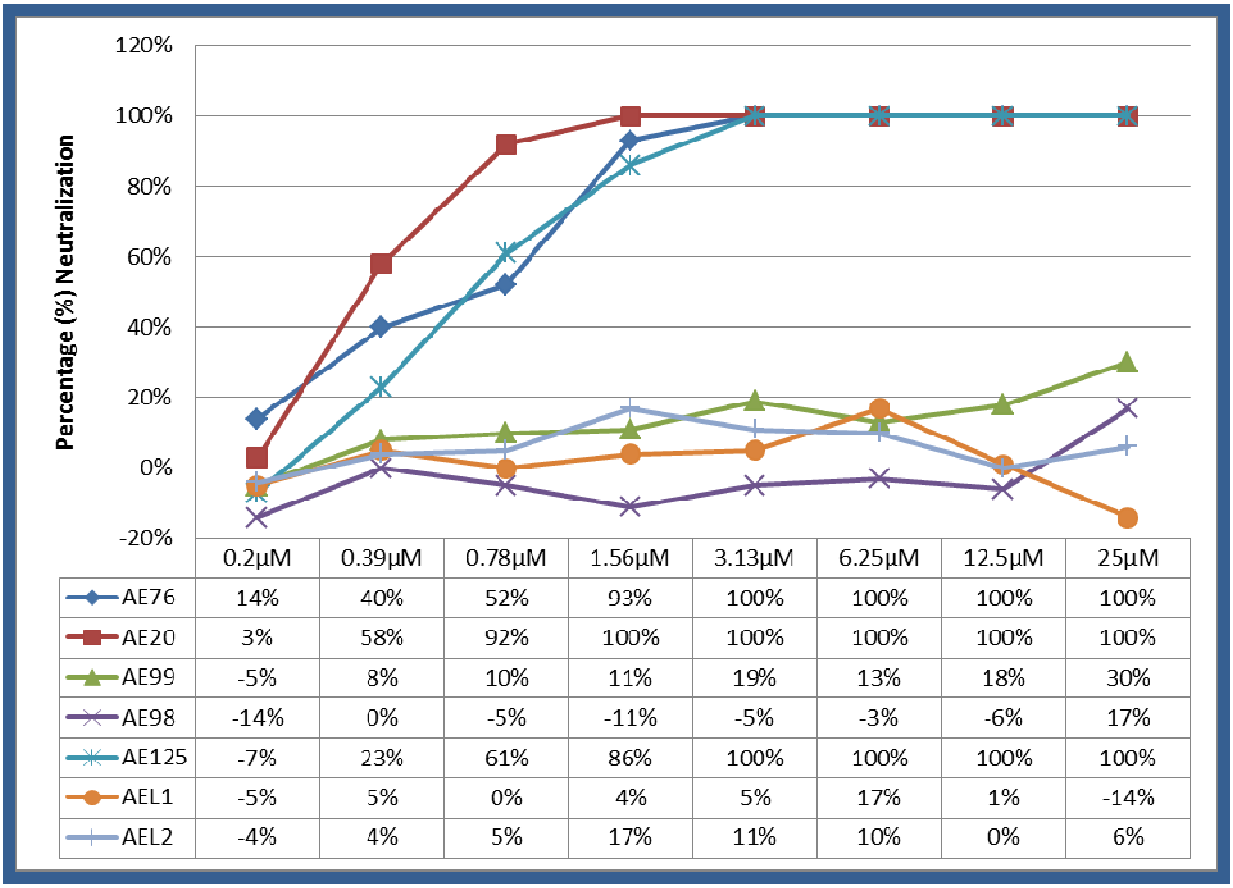


(B)

Figure A1: The effects of the cells on the viability of TZM-bl cells (A) and PBMCs (B) as determined using the MTT(A) and the MTS (B) assays.

Appendix IV: Whole virus neutralization assay.

The inhibition of viral infection by selected compounds. The gold based compounds, AE20,AE76 AND AE125 showed 100% inhibition at 25µM. However these compounds were shown to be toxic at this concentration and observed activity could be due to general toxicity.



	0.2µM	0.39µM	0.78µM	1.56µM	3.13µM	6.25µM	12.5µM	25µM
AE76	14%	40%	52%	93%	100%	100%	100%	100%
AE20	3%	58%	92%	100%	100%	100%	100%	100%
AE99	-5%	8%	10%	11%	19%	13%	18%	30%
AE98	-14%	0%	-5%	-11%	-5%	-3%	-6%	17%
AE125	-7%	23%	61%	86%	100%	100%	100%	100%
AEL1	-5%	5%	0%	4%	5%	17%	1%	-14%
AEL2	-4%	4%	5%	17%	11%	10%	0%	6%

Figure A2: The effects of selected complexes and their ligands on the infectivity of TZM-bl cells by a pseudovirus. Assays were performed at the NICD and analysis was discontinued due to compound toxicity.