CHRYSOTHERAPY: EVALUATING GOLD COMPOUNDS FOR ANTI-HIV ACTIVITY

by

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SUMMARY

Chrysotherapy: evaluating gold compounds for anti-HIV activity

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Background: The continuous emergence of drug resistant strains of HIV as a result of errors made by reverse transcriptase coupled with undesirable side effects of available drugs, latency problems, cost etc, warrants the continuous search for new drug candidates. Chrysotherapy which is the use of gold compounds for the treatment of various ailments has been practiced since 2500 BC. The use of gold compounds such as auranofin for the treatment of rheumatoid arthritis has lead to remission of this disease. Gold compounds such as auranofin not only prevented the progression of arthritis but also increased the CD4+ count of an HIV positive patient who was not on antiretrovirals. These compounds have been implicated in the treatment of cancers, autoimmune diseases and microorganism infections.

Objectives: In this work, novel gold compounds were evaluated with the aim of identifying lead compound(s) that can eventually serve as anti-HIV agents.

Materials and Methods: Eleven gold (I) phosphine complexes, four of their corresponding ligands (compound without gold atom), and a gold (III) complex were tested for the ability to inhibit reverse transcriptase (RT) and protease (PR) in direct enzyme assays. Uptake of the compounds by host cells was evaluated with inductively coupled plasma atomic emission spectrometry (ICP-AES). Potential toxicity of the gold compounds was screened for by viability dyes and flow cytometry assays. To determine inhibition of whole virus by other mechanisms in addition to RT or PR, p24 production by infected cells was evaluated. Prior to all these analysis, stability of compounds in solution was determined by ^{31}P nuclear magnetic resonance (NMR) and UV-visible spectroscopy.

Results: The compounds were shown to be stable in solution over a one week period and were taken up by both continuous cell lines and primary cells. Eight of the gold compounds significantly inhibited HIV-1 reverse transcriptase at concentrations of 25 and 250 µM while four compounds and the four ligands did not. In a fluorogenic assay against HIV-1 PR, four of the gold compounds demonstrated inhibitory activity. The gold compounds were toxic to cells lines but not to primary cells. One of the complexes (EK231) significantly reduced p24 ($p=0.0042$) production at a concentration of 25 µM.

Conclusion: Data provided here suggests that the therapeutic benefits of these gold containing compounds as potential HIV-1 reverse transcriptase and protease inhibitors should be considered.

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- Lindsay and Aurelie and all my other friends for your moral and spiritual support.

DEDICATION

I dedicate this work to my late father (Papa Lucas Che Fonteh – died 19th January 1981) who believed in the education of his 8 children but never had the opportunity to see them become the doctors and professors he had dreamt of.

Daddy, we miss you, May Your Soul Rest in Peace.

PREFACE

(I) Sections of this dissertation have been compiled in three manuscripts:

1) Fonteh P.N., Keter F., Meyer D., Spencer L., Darkwa J. (2008). Tetra-chloro-(3,5-dimethylpyrazolyl)methane)gold(III)chloride. A reverse transcriptase and protease inhibitor. *To be submitted to the Journal titled Metal Based Drugs.*

2) Fonteh P.N., Meyer D. (2008). Biological analysis of some novel gold(I) phosphine complexes as potential HIV inhibitors. *Still to be submitted.*

3) Williams A., Fonteh P.N., Phillipeos C., Meyer D. (2008). *In vitro* infection of CEM.NKR-CCR5 cells with cell free HIV-1 using spinoculation and polybrene, confirmed by Real-Time PCR. *To be submitted to the Journal of Molecular Immunolgy.*

(II) Portions of this work were presented at the 27th African Health Sciences Congress (3rd-7th December 2006, Durban South Africa) and won the 1st price in the poster presentation category.

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LIST OF IMPORTANT ABBREVIATIONS

ABTS	2, 2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium Salt Crystals
Anti-DIG-POD	antibody to digoxigenin conjugated to peroxidase
ARV	antiretroviral
CCR5	chemokine receptor 5
CTLs	cytotoxic T lymphocytes
DMSO	dimethyl sulphoxide
ELISA	enzyme-linked immunosorbent assay.
Env	envelope gene
FACS	fluorescence-activated cell sorter
FBS	foetal bovine serum
FITC	flourescein isothiocyanate
Fas-L	fas ligand
GS	gentamycine sulphate
HIV	human immunodeficiency virus
HNO_3	nitric acid
Hr	hour
ICP-AES	inductively coupled plasma atomic emission spectrometry
IL	interleukin
IN	integrase
K [Au $(CN)_2$]	potassium gold cyanide

LDH	lactate dehydrogenase
MTT	3-(4, 5-dimethlythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
Nef	negative effector
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PI	propidium iodide
Ppm	parts per million
PR	protease
PS	phosphatidyl serine
RA	rheumatoid arthritis
RP-HPLC	reverse phase high performance liquid chromatography.
RPMI	Rosewell Park Memorial Institute
RT	reverse transcriptase
ssRNA	single stranded ribonucleic acid
TCID50	50 % tissue culture infectious dose
TMB	tetramethly benzendine
U	units
UV	ultraviolet
UNAIDS	Joint United Nations Programme on AIDS
XTT	sodium 3'-[1-[(phenylamino)-carbonly]-3, 4 –tetrazolium]- bis (4-methoxy-6-nitro) benzene-sulfonic acid.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The prevalence of human immunodeficiency virus (HIV) and the impact it has on the socio-economic wellbeing of people continues to be a global problem. Infection with HIV typically results in continual loss of CD4+ T lymphocytes, which ultimately leads to immunodeficiency and enhanced susceptibility to opportunistic infections and malignancies, a syndrome called acquired immune deficiency syndrome (AIDS) (Lum *et al.,* 2003). Considering that there is no cure (Heagarty, 2003) or vaccine (Simon *et al*; 2006) to address the HIV/AIDS pandemic, coupled with the alarming statistics in the UNAIDS report of 2006, the need for measures to circumvent this problem continues. Therapeutic strategies such as the use of highly active antiretroviral therapy (HAART) have effectively led to decreases in morbidity and mortality rates in HIV infected individuals (Cressey and Lallemant. 2007, Porter *et al* 2003, Pallela *et al*., 1998, Powderly *et al*., 1998). The existence of latent forms of HIV (Chen *et al*., 2004) which integrates into the hosts' genome during therapy does not however allow for the complete eradication of the virus such that therapy has to be life-long. Apart from the problems encountered as a result of latency, HAART also presents with shortcomings such as development of drug resistant viral strains (Chen *et al* 2004, Skillmann *et al*; 2002), toxicities and lack of compliance (Ren *et al*; 2005, Chen *et al;* 2004) by patients because of intolerable side effects, cost etc. With no imminent cure or vaccine insight, coupled

with the shortcomings of HAART, the development of novel lead therapeutic agents with potential anti-HIV activity continues to be a priority.

The prevalence of HIV in Southern Africa and previous findings that some gold containing compounds inhibit this virus both *in vitro* (Traber *et al*., 1999, Tepperman *et al*., 1994) and *in vivo* (Yamaguchi, 2001, Shapiro and Masci, 1996), led to the establishment of Project AuTEK. AuTEK Biomed is a biomedical research project involved in anti-cancer, anti-malaria and anti-HIV screening. This project involves Mintek and the three main South African mining companies namely; Anglogold Ashanti, Gold Fields and Harmony Gold (Deane, 2006). The vision is that with the gold rich mines in South Africa, the synthesis, biological analysis and identification of novel gold compounds that have low toxicity against human cells and high efficacy against HIV subtype C (prevalent subtype in Southern Africa – Nkolola and Essex, 2006, Wouter *et al*., 1997) would go a long way to have significant socio-economic benefits to the southern African population and beyond. Potential new drug leads should supplement or perhaps even replace existing antiretroviral therapy.

Background information on HIV, AIDS, and the use of gold in medicine especially for the treatment of microorganisms as well as the rationale for this work follows next.

1.2 HIV AND AIDS

It is well established that HIV causes AIDS (Popovic *et al*., 1984). Two main forms of this virus based on genetic variability have been reported (Wouter *et al*., 1997). These are HIV-1 and HIV-2 with the former being the most widely distributed and also the most studied virus. These different strains differ in their transmissibility, pathogenesis and

pattern of spread with HIV-2 being less pathogenic with an enhanced immune control (Reeves and Doms, 2002).

Our study was based on HIV-1 and specifically HIV-1 subtype C as it is the most prevalent subtype in southern Africa (Nkolola and Essex, 2006, Wouter *et al.,* 1997). Unless otherwise stated, the use of the abbreviation HIV will refer to HIV-1.

AIDS resulting from HIV was first recognized in 1981 when a common pattern of symptoms was observed among a small number of homosexual men in the USA (Brennan and Durack., 1981, Gottlieb *et al*., 1981). It is characterized by severe wasting, breakdown in the immune system with decrease in CD4+ cells and a corresponding increase in viral load. An African HIV-1 sequence from 1959 has been implicated for the origin of the epidemic (Williams *et al*., 1983) though the authenticity of this case has still not been confirmed (Zhu and Ho, 1995). In 1999, Gao and his colleagues reported that simian immunodeficiency viruses (SIVs) from chimpanzees (cpz) cluster phylogenetically with HIV-1 hence the HIV-1 epidemic is likely to have originated from SIV_{cpz} while criteria identifying HIV-2 as a zoonosis (the process of viruses passing from animals to man) from the sooty mangabey were met i.e. similarity in genetic organization, phylogenetic relatedness and prevalence in natural host (Sharp *et al*. 1995).

1.2.1 Structure of HIV

HIV is a retrovirus (De Clercq, 1986) and therefore carries its genetic information in the form of RNA. It is also a lentivirus as it is slow in developing to disease (Girard *et al*., 2006). Each virion expresses 72 glycoprotein projections composed of gp120 and gp41. Gp41 is a transmembrane molecule that crosses the lipid bilayer of the envelope. Gp120 is noncovalently associated with gp41 and serves as the viral receptor for CD4 on host

cells. The viral envelope also contains some host-cell membrane proteins such as class I and class II major histocompartibility molecules (MHCs). Within the envelope is the viral core, or nucleocapsid, which includes a layer of a protein called p17 and an inner layer protein, called p24. The HIV genome consists of two copies of ssRNA, which are associated with two molecules of reverse transcriptase p64 and nucleoid proteins p10, a protease, and p32, an integrase (Goldsby *et al*., 2000). Figure 1.1 is a cross sectional schematic diagram of HIV.

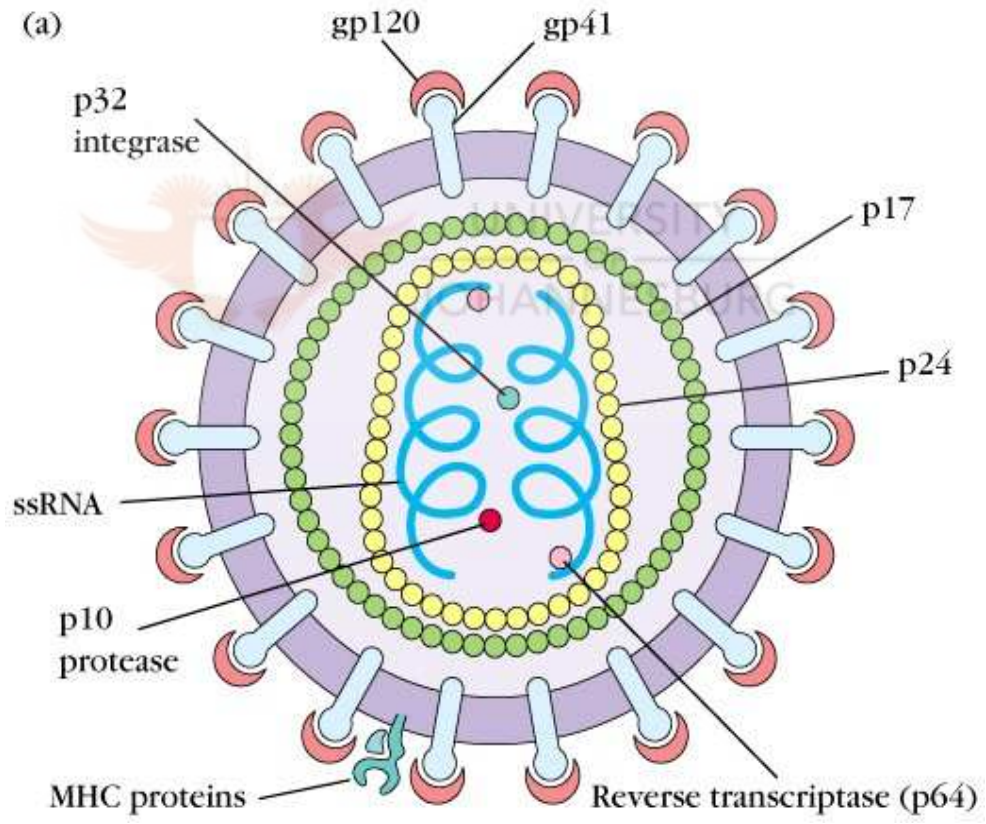


Figure 1.1: Cross-sectional schematic diagram of HIV. Taken from http://tutor.lscf.ucsb.edu/instdev/sears/immunology/images/figure19-08a.jpg (URL active on 19/11/2007)

1.2.2 Genome Structure

The HIV-1 genome is approximately 9.5 kb and displays significant sequence variations as a result of constant mutation and evolutionary pressure (Sanabani *et al.*, 2006). HIV as a lentivirus shares three common polypeptide genes (structural) known as *gag (*specific antigen*), pol* (polymerase) and *env* (envelope) (Wyatt and Sodroski, 1998). It also has several non structural genes unique to HIV. In total, HIV has nine genes (Figure 1.2, Table 1.1) compared to three or four genes encoded by most retroviruses (Semple, 2000). The *gag* gene provides the basic physical infrastructure of the virus and *pol* the mechanism by which reproduction occurs as found in all retroviruses. The other genes help the virus to enter the cell and enhance reproduction. The 5' long terminal repeat (LTR) contains sequences to which regulatory proteins bind. During the life cycle of the virus, the 3' end of the genome influences the integrated virus by acting as a promoter and transcriptional start site (Semple, 2000). Figure 1.2 is the genomic organization of HIV while Table 1.1 represents the genes and their protein products and functions.

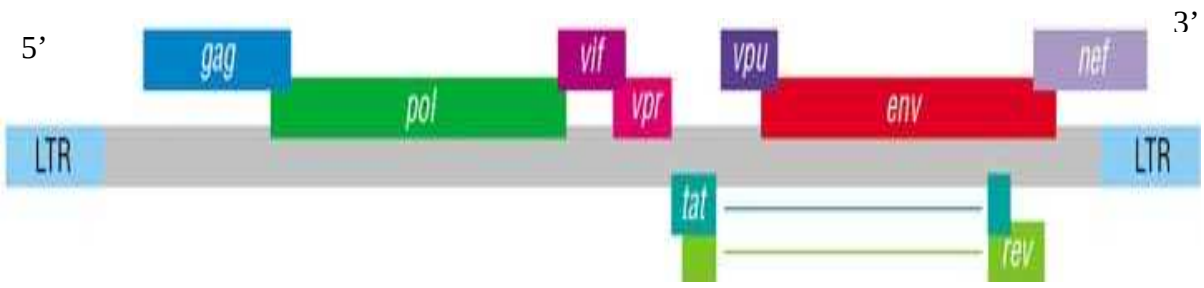


Figure 1.2: Diagram showing the genomic organization of HIV. Taken from http://www.new-science-press.com/info/illustration_files/nsp-immunity-10-4-10_12.jpg (Active 31/12/07).

Table 1.1: HIV genes and their protein products and functions. (Taken from Goldsby *et al.*, 2000).

Gene	Protein product	Function of encoded proteins
Gag		***Group specific antigen-Nucleocapsid proteins***
	p24	Forms inner core-protein layer
	p17	Forms outer core-protein layer
	p7	Binds directly to genomic DNA
	p9	Is a component of the nucleoid core
Pol		***Enzymes***
	p64	Has reverse transcriptase and RNase activity
	p51	Has reverse transcriptase activity
	p10	Is protease that cleaves gag precursor
	p32	Is integrase
Env		***Envelope glycoproteins***
	gp41	Is the transmembrane protein associated with gp120 and required for fusion
	gp120	Protrudes from the envelope and binds CD4
Tat	p14	***Trans-activator of transcription*** Strongly activates transcription of proviral DNA
Rev	p19	***Regulator of virion*** Allows export of unspliced and singly spliced mRNAs from nucleus.
Vpr	p15	***Viral protein R*** Weakly activates transcription of proviral DNA
Nef	p27	***Negative regulatory factor*** Increases viral replication; down-regulates host cell CD4
Vif	p23	***Viral infectivity factor*** Promotes infectivity of viral particle
Vpu	p16	***Viral protein U*** Is required for efficient viral assembly and budding

1.2.3 HIV Genetic Variability

HIV-1 has been classified into major (M), outlier (O), and non-M/O (N) groups (Sanabani *et al*., 2006, Charneau *et al.*, 1994). Within group M there are 9 genetically

distinct subtypes (A-J) and includes circulating recombinant forms (CRFs) which have been identified by phylogenetic analysis such as subtype G (Wouter *et al.,* 1997). Within the current HIV pandemic, geographic distribution of HIV subtypes has shown that HIV-1 subtype C (HIV-1C) is the most prevalent subtype causing more than half of all global infections and 94% of infections in Southern Africa (van Harmelen *et al*., 2001). HIV-1B has been the most widely studied and with the prevalence of HIV-1C a need to intensify research on it is necessary.

1.2.4 Life Cycle of HIV

HIV begins its infection of a susceptible host cell by the binding of gp120 to the CD4 receptor on the host cell (Singh *et al*., 2005). The CD4 antigen is present on the surface of many lymphocytes, which are a critical part of the body's immune system. For entry to be effective, coreceptors which are either M tropic (preferentially infect macrophages using CCR5 as coreceptor) or T tropic (preferentially infecting T cells using CXCR4 coreceptor) are utilized by the virus for fusion with the host cell membrane (Alfano and Poli, 2001). Following fusion, the virus enters the cell, releases it genetic material (RNA) which undergoes reverse transcription into cDNA catalysed by reverse transcriptase. This viral cDNA now known as a provirus enters the host cell nucleus where it integrates into the genetic material of the host cell catalysed by integrase. After integration, into the host genome, the viral DNA is treated as a normal cellular gene (Tarago-Litvak *et al*., 2002). Once integrated, the virus may persist in the host's DNA in the latent form (major barrier to the eradication of or cure of HIV) or is replicated alongside the host cell DNA to form immature virus particles through transcription and translation. The viral particle buds out of the cell taking the cell membrane with it. HIV protease enzyme cuts the protein chains

(*gag* precursor- Goldsby *et al*, 2000) in the new viral particle into individual proteins that combine to produce infectious mature virion. Various stages in the life cycle of this virus have been targeted by anti-viral drugs e.g. viral entry inhibition, reverse transcriptase inhibition, integrase inhibition and protease inhibition (Tarago-Litvak *et al*., 2002). The stages in the life cycle of HIV are represented in Figure 1.3.

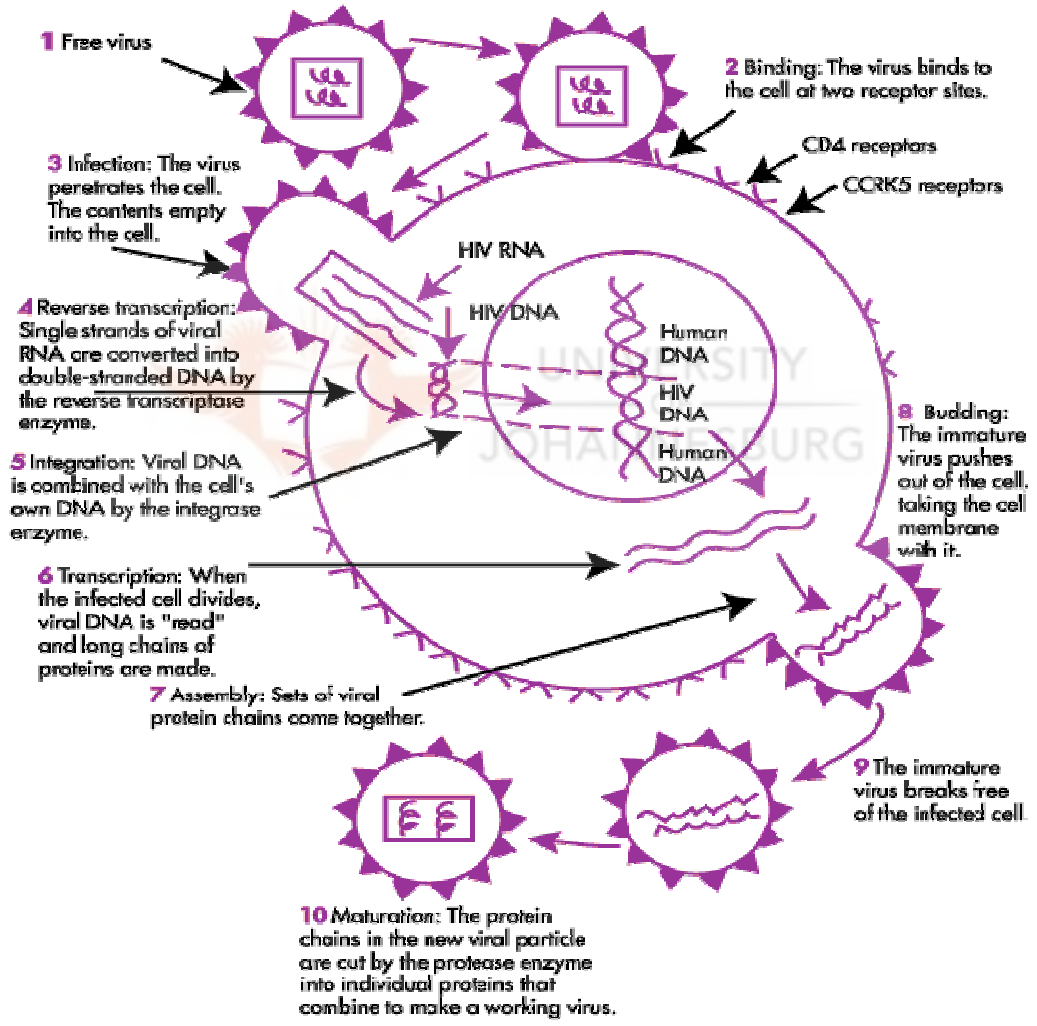


Figure 1.3: Life cycle of HIV. This figure represents the various stages of the life cycle and vital enzymes involved. The figure was taken from: http://www.engenderhealth.org/res/onc/hiv/understanding/images/hiv2d.gif. (URL active on 19/11/2007)

1.2.5 HIV/AIDS and the Immune System

HIV attacks both the humoral and cell mediated arms of the immune system (Fan *et al*., 2000). In early infection, both arms are functional with the humoral secreting neutralizing antibodies and the cell mediated producing both cytotoxic T lymphocytes (CTLs-CD8+) and helper T cells (T-helper cells-CD4+). In this early phase, i.e. prior to seroconversion and during primary infection HIV specific CTLs are activated and they bind to antigen presenting cells carrying HIV through T cell receptors (TCR). The CTL TCR binds to viral peptides associated with MHC class I molecules on the surface of infected cells causing the release of proteases and perforin which leads to destruction of the infected cells (Niiya *et al*., 2005). Alternatively, there is the interaction of Fas ligand (Fas-L - a cell surface molecule belonging to the tumor necrosis factor family) on the CTL surface with Fas molecules on the target cells resulting in the apoptotic lysis of the infected cells (Garcia *et al*., 1997). T-helper cells are vital as they interact with B lymphocytes or CTLs helping them to respond to antigens. The maturation and growth of B cells to produce antibodies in humoral immunity is stimulated by T-helper cells while the growth factor interleukin 2 (IL-2) required by both T-helper and CTLs is secreted by T-helper cells. In AIDS, the virus specifically targets and kills these crucial cells (T-helper cells) thus causing failure of both the humoral and cell-mediated immune system thus leading to impairment of immunological protection such that opportunistic infections and cancer develops (Lum *et al*., 2003, Fan *et al*; 2000). The virus finally escapes the immune system through its high mutation rate and development of escape variants (Price *et al*., 1997).

1.2.6 The Course of HIV Infection

The course of HIV infection is characterized by a gradual destruction of naïve and memory CD4+ T-lymphocytes populations with the hallmark being AIDS, the last disease stage (Figure 1.4). Primary infection is characterized by high plasma viraemia, low CD4+ cells and the absence of HIV-1 specific antibodies. Viraemia drops as CTLs develop and an individual viral load set point is reached during chronic infection. The risk of transmission is highest in the first week when viraemia peaks (Simon *et al*., 2006).

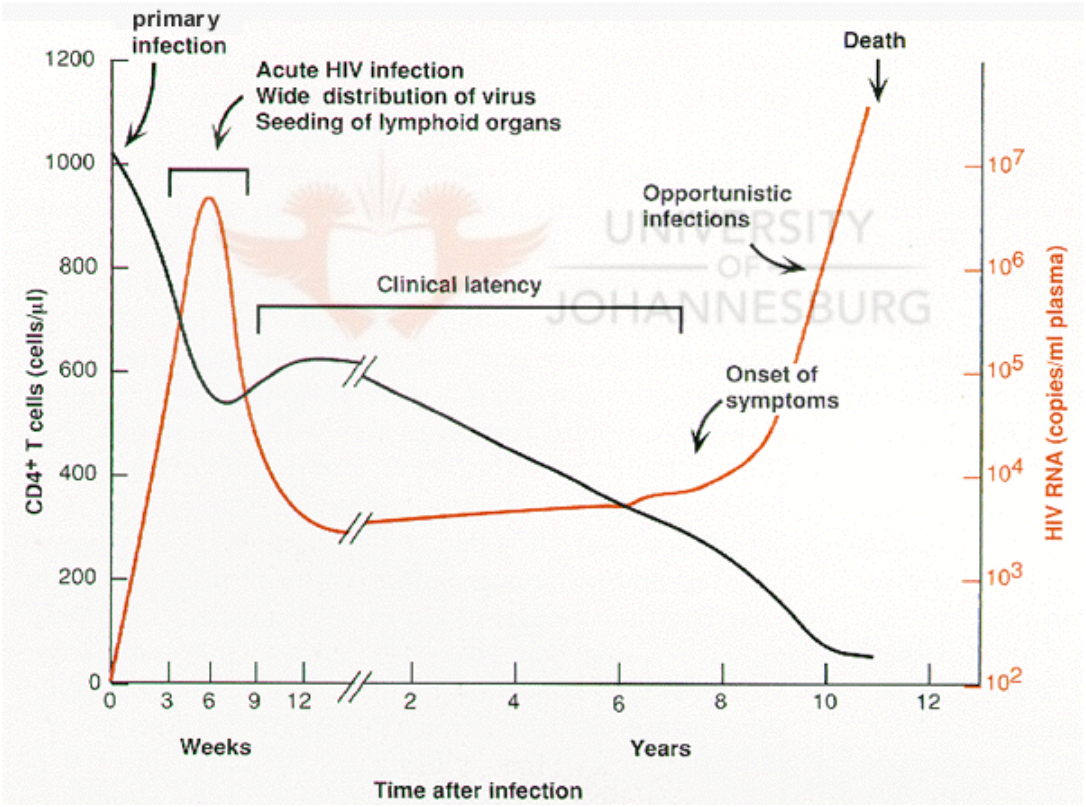


Figure 1.4: The time course of HIV disease.
Dynamic changes in the CD4+ T lymphocytes population over time is represented on the primary x-axis while plasma viraemia represented by HIV RNA copies/ml is shown on the secondary x-axis. Figure taken from http://msl.cs.uiuc.edu/~yershova/bcb495/bcbProject-3_files/image015.gif. (URL active on 05/11/2007)

1.2.7 Global Picture of AIDS

An estimated 39.5 (34.1-47.1) million people were living with HIV at the end of 2006. An estimated 4.3 (3.6-6.6) million people were newly infected and an estimated 2.9 (2.5-3.5) million deaths were recorded worldwide in the same year (UNAIDS, 2006). There is no region of the world that is untouched by this epidemic as represented in Figure 1.5 (UNAIDS, 2006). Heterosexual transmission remains the most dominant mode of transmission and accounts for about 85% of all HIV infections (Simon *et al*; 2006). Southern Africa remains the epicentre of the pandemic and continues to have high rates of new HIV-1 infections (Hayes and Weiss, 2006) with adult prevalence as shown in Figure 1.6 (UNAIDS, 2006). Growing epidemics of HIV in China and India are of concern as they are fast approaching those in Southern Africa (UNAIDS, 2006). Outside of sub-Saharan Africa, a third of all HIV infections are acquired through injecting drug use (an estimated 8.8 million) primarily in Eastern Europe and central and Southeast Asia (UNAIDS 2006).

Of concern in current trends is the increasing burden of HIV infections in women (Quinn *et al*; 2005). Women now make up about 42% of those infected worldwide; over 70% of whom live in sub-Saharan Africa. HIV-1 infection rates are three to six times higher in female adolescents than in their male counterparts and this difference has been attributed to sexual coupling patterns of young women with older men (Simon *et al*., 2006). It is however notable that among the new current trends, there are trends of recent decline in HIV prevalence in two sub-Saharan African countries (Kenya and Zimbabwe) and in urban areas in Burkina Faso (UNAIDS, 2006).

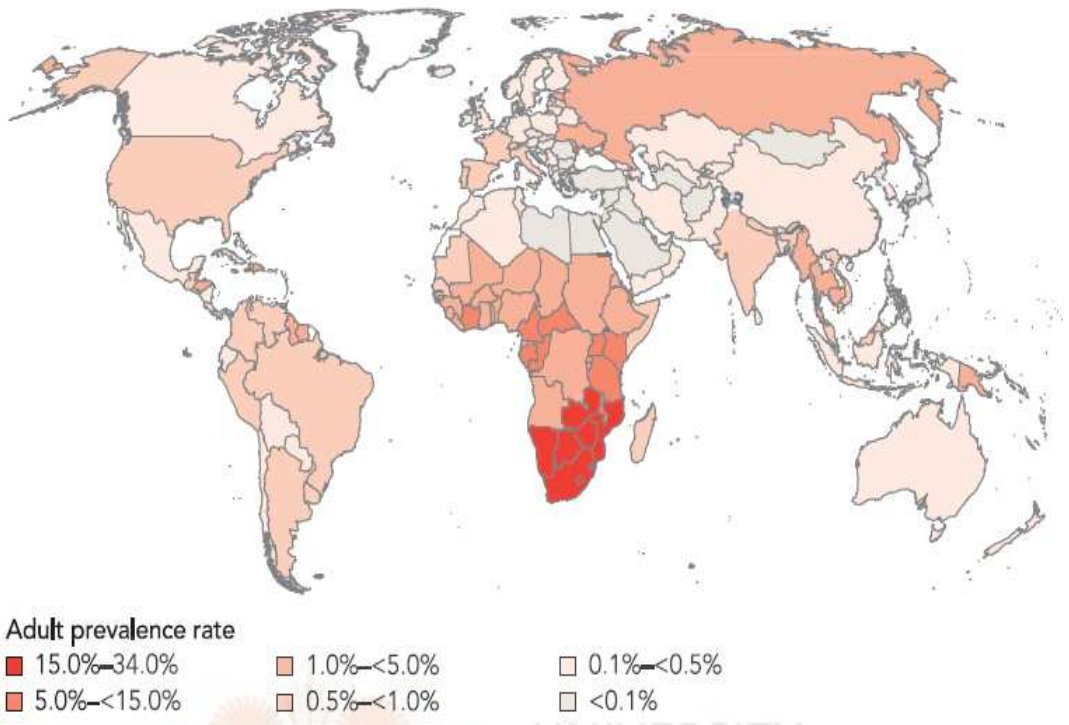


Figure 1.5: A global view of HIV infections showing adult prevalence rates in 2005 (UNAIDS 2006).

1.2.8 Current Treatment Measures

The prevalence of HIV and AIDS (as seen in Figures 1.5 and 1.6) and its existence for over 25 years now has seen some progress in antiretroviral treatment. Attempts at developing an effective vaccine have not yielded any fruits. Chemotherapeutic approaches towards AIDS could be based on treatment of the opportunistic infections, measures directed against the etiologic agent HIV or alternatively manipulating the immune system either by administration of cytokines e.g. IL-2 or by vaccines (Alfano and Poli, 2001).

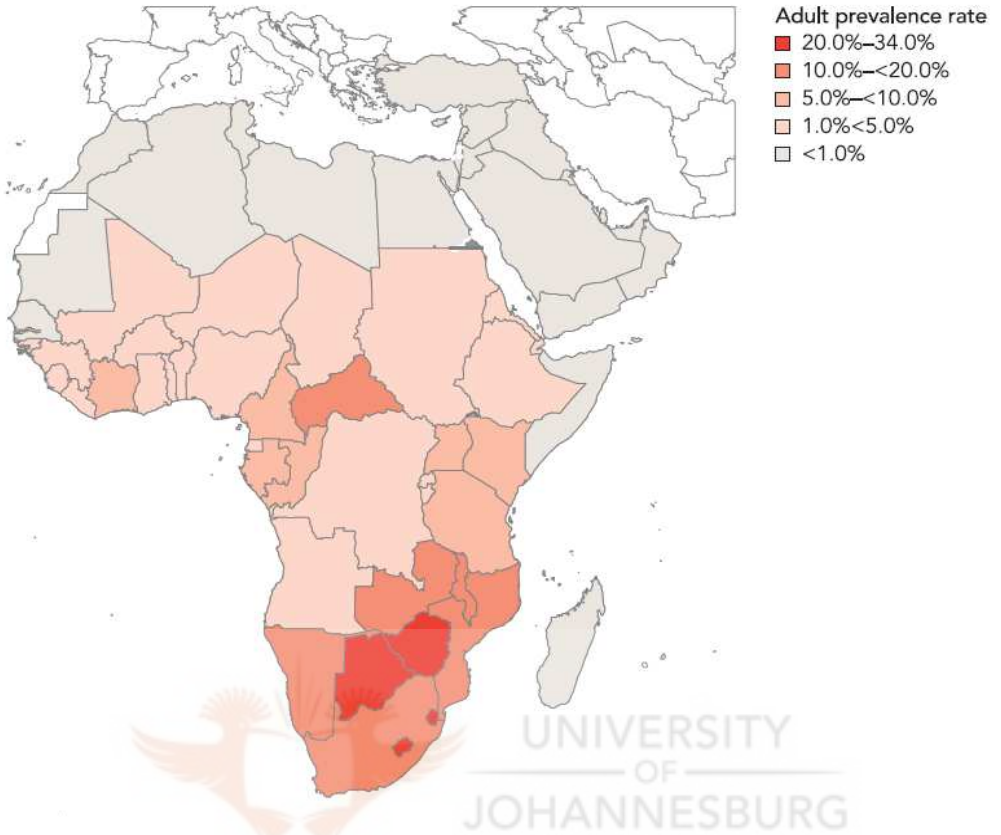


Figure 1.6: A global view of HIV infections showing adult prevalence rates in 2005 (UNAIDS epidemic update, 2006).

The use of HAART and attempts at vaccine development will be discussed in the following section.

1.2.8.1 Antiretroviral therapy and HAART

The US Food and drug Administration (FDA) has approved 21 antiretroviral drugs to date (Cressey and Lallemant, 2007). This includes 8 nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs), 3 non nucleoside reverse transcriptase inhibitors (NNRTIs), 9 protease inhibitors (PIs) and 1 fusion inhibitor. A daily combination of three of these antiretroviral drugs is recommended by International antiretroviral (ARV)

treatment guidelines for the treatment of immunocompromised patients. This triple combination forms what is known as highly active antiretroviral therapy or HAART. A standard HAART regimen consists of 2 NRTIs plus 1 NNRTI or 2 NRTIs plus a PI. This has led to profound decrease in mortality and morbidity in AIDS (Porter *et al*., 2003, Pallela *et al*., 1998) by suppressing the level of viral replication thus restoring the immune system. The more recent availability of an anti-HIV agent in the entry inhibitor class with several others in development indicates the continual efforts towards meeting the ongoing need for novel ARVs (Sension, 2007, Esté and Telenti, 2007). A recent development in this light was the approval of raltegravir (MK-0518) as the first integrase inhibitor drug (Cahn and Sued (2007), AIDSinfoNet (2007)).

HAART however comes with shortcomings such as toxicity on mitochondria (Lewis and Dalakas, 1995), lypodystrophy syndrome characterised by peripheral fat wasting, central adiposity, hyperlipidemia, insulin resistance and diabetes melitus (Carr *et al*., 1998). These together with the development of drug resistant HIV variants (Price *et al*., 1997) and other factors such as poor adherence, and other pharmacological issues such as protein binding and cellular resistance (Turriziani *et al*., 2000) and cost in resource limited areas have all rendered HAART ineffective.

1.2.8.2 Vaccine development

A safe, protective, inexpensive vaccine would be the most efficient and possibly the only way to curb the HIV pandemic (Ho and Huang, 2002). Despite numerous attempts, development of such a vaccine has not been realized. Only one HIV candidate vaccine has completed clinical trials of phases I, II and III in more than 20 years of the epidemic. The phase (III) trials were based on the use of recombinant envelope proteins, with the

aim of evoking virus neutralizing antibodies. However, results from this were disappointing (Bramwell and Perrie, 2005). There is divided opinion as to what type of immune response is required in the control of HIV. Neutralizing antibodies might be efficient in blocking virus particles but ineffective against cell associated virus whereas some CTLs are effective against virally infected cells and not against free virus. Latent infection further facilitates immune evasion and the rate of mutation inherent in HIV also aids in escape from responding T cells (Price *et al*; 1997). Promising vaccine strategies will thus depend upon highly conserved epitopes and upon vaccine strategies that induce potent immune responses capable of driving cytotoxicity and producing broadly effective neutralizing antibodies (Heeney, 2004). These vaccines must induce both humoral and cell mediated immune responses both systemically and at mucosal sites (Yuki *et al.*, 2007, Nkolola and Essex, 2006) as the majority of HIV infection is through mucosal sites by sexual contact.

With no cure (Heagarty. 2003) or vaccine (Simon *et al*; 2006) in sight the search for potential therapeutics to be used in combination treatment or replace current ARVs is a continuous quest.

1.3 METALLODRUGS

Drugs containing metals have a wide application in many areas of medicine for example for the treatment of asthma, inflammatory diseases (e.g. rheumatoid arthritis, ulcers, pemphigus) and more recently cancer, HIV and malaria (Shaw, 1999). Metallodrugs or metal based drugs are chemically synthesized agents containing a metal complexed to a suitable ligand. The metal could be gold (Au), platinum (Pt) etc. A ligand is a suitable molecule to which the metal is attached and different ligands can be used to alter

biological activity of a metal. It serves as a vehicle in transporting the metal to its active site (Shaw 1999). The active drug might not be the one administered as they tend to undergo ligand exchange or other reactions to form the active component and are therefore prodrugs (Shaw, 1999). These drugs are transformed by metal substitution or redox reactions before reaching their target site where the metal is required for proper functioning (Berners-Price and Sadler, 1996).

Mineral supplements such as Cr, Mn, Fe, Co, Ni, Cu Mo (Berners-Price and Sadler, 1996) are metallodrugs. Cisplatin is a platinum (II) complex that has improved the treatment of many types of cancer. The radioisotopes ^{99m}Tc and ^{111}In are used as radiodiagnostic agents (Mcfee *et al*., 1988) while bismuth has been shown to have anti-ulcer properties (O'Brien *et al*., 1990) to name but a few.

The metallodrugs of interest in this project are those containing gold. The gold and ligand together form what would be termed the gold complex or otherwise gold compound while the moiety in the absence of the metal would be termed the ligand. Compounds would refer to both the ligands and gold compounds (complexes).

Gold has long been used in medicine before the 1900 and the gold complex $K[Au(CN)_2]$ was introduced at the turn of the 20th century for the treatment of tuberculosis (Berners-Price and Sadler, 1996). In the following section, the unique properties of gold and some of its applications in medicine (with focus on its anti-HIV properties) and other arenas will be discussed.

1.3.1 Properties and Uses of Gold (Au)

Gold (periodic table symbol Au) is a soft, malleable, lustrous, yellow metal that resists corrosion (Merchant, 1998). It is found in group 1B of the periodic table and has an

atomic number of 79 and an atomic mass of 197 g/mol. It is normally present in human and animal tissues in only minute proportions and has no biological function in this state (Parish and Cottrill., 1987). Gold can exist in a number of oxidation states:-I, 0, I, II, III, IV, V but only gold 0, I, and III are stable in aqueous and therefore biological environments. Gold (I) and gold (III) are unstable with respect to gold (0) and are readily reduced by mild reducing agents. However, gold (I) is thermodynamically more stable than gold (III), with most gold (III) complexes being strong oxidizing agents and therefore reduced to gold (I). Biologically occurring reductants such as thiols reduce gold (III) to gold (I). This makes gold (III) compounds generally very toxic (Friker, 1996).

1.3.1.1 Gold protein interactions.

Gold compounds are capable of undergoing ligand exchange reactions with biological ligands such as the amino acid cysteine (Shaw, 1999, Sadler and Guo, 1998). This may contribute to their pharmacological activity and it has been proposed that gold drugs inhibit interaction of potentially degradative enzymes by reacting with thiol groups in metalloproteinases such as collagenase (Snyder *et al*., 1987).

Gold (I) also undergoes ligand exchange reactions with other biological proteins such as albumin and metallothioneins forming aurothioneins (Shaw, 1989). The pharmacological significance of these interactions is yet unclear (Friker, 1996).

1.3.1.2 Gold compounds and gene expression

Gold compounds exert a number of effects on the immune response, one of them being the regulation of cytokine levels. This has been attributed to the interaction of gold with thiol groups on the proteins responsible for regulating the transcription of the genes

encoding the cytokine expression i.e. transcription factors. Regulation of gene expression plays an important part in diseases like cancer, inflammatory diseases e.g. rheumatoid arthritis (RA), HIV/AIDS (Traber *et al*., 1999).

Because many transcriptions factors contain cysteine e.g. NF-κB which controls transcription of other inflammatory mediators e.g. TNF, it is possible that one molecular mechanism for gold anti-arthritic drugs is inhibition of transcription of crucial mediators of the inflammatory process. This also therefore applies to other diseases were regulation of gene expression is important.

1.3.2 Gold in Medicine

1.3.2.1 History

Chrysotherapy (from the Greek word for gold, chrysos) or aurotherapy are terms used to describe the treatment of ailments with gold compounds. Gold preparations have been used in medicine as early as 2500 BC when Chinese and Arabic physicians applied it in their practice of medicine (Huaizhi and Yuantao, 2001, Friker, 1996, Sutton 1986). Sutton (1986) further documents the history of the use of gold as mixtures and ointments of colloidal gold by medieval physicians for various ulcerative skin conditions including rheumatism. There is also a long history of application of gold in medicine as documented by Higby (1982) but these uses were limited by the fact that the metal was difficult to dissolve. It was however only in 1890 that Robert Koch (Friker, 1996, Sutton 1986) reported biological properties of gold compounds supported by experimental laboratory data. Koch showed that gold cyanide, K[Au(CN)2] was bacteriostatic towards the tubercle bacillus hence its use against tuberculosis. He however did not perform further studies on this as he was unable to demonstrate that gold salts exhibited

antitubercular activity in experimental animals (Sutton, 1986). The tubercle bacillus was however implicated to be the causative agent for RA hence the exploration of gold therapy for rheumatoid arthritis.

1.3.2.2 Chrysotherapy and Rheumatoid arthritis (RA)

Rheumatoid arthritis is an inflammatory disease characterized by progressive erosion of the joint resulting in deformities, immobility and a great deal of pain (Friker, 1996). It is an autoimmune disease with a progressive crippling effect on connective tissue and is not age related (Sutton, 1986). In 1935 Jacques Forrestier reported the beneficial effects of gold salts in slowing the evolution of RA (Sutton, 1986). This was later confirmed by John Singler (1974) who observed that gold salts slow down and often eliminate the progressive chronic joint changes associated with RA. This was however after Sutton *et al* (1972) had reported the potential of auranofin [2, 3, 4, 6-tetra-O-acetyl-1-thio-B-D-glucopyronosato-S (triethylphosphine gold] as an RA drug. Auranofin was approved for clinical management of RA in 1985 as it had an improved pharmacokinetic profile and reduced toxicity. It could also be administered orally unlike other available RA drugs (sodium aurothiomalate or myocrisin and aurothioglucose or solganol) that had hitherto been administered intravenously. Auranofin (radiura) is unique in that it produces therapeutic benefit when administered orally as it is quite lipophilic and thus eliminates the side effects associated with intravenously administered drugs. Furthermore, serum levels are reduced and maintained for longer with less retention of gold in tissues and hence renal toxicity is reduced. This is understandable as the oral drug acts systemically while the locally administered ones do not. However, its efficacy is reduced compared to

the polymeric gold (I) thiolates (Friker, 1996). Figure 1.7 represents structures of some important gold drugs.

Gold compounds that have been used effectively as RA agents are all gold (I) thiolates i.e. they contain AuSR units, where R is a suitable organic group in the case of aurothioglucose and sodium aurothiomalate. Auranofin however has a thiol and triethylphosphine group as ligands such that the coordination of gold is P-Au-S (Parish and Cottrill, 1987). All these are gold (I) complexes and the preference for gold (I) over gold (III) is for the following reasons:-

a) The gold should be univalent to avoid the toxicity associated with the oxidizing power of the trivalent state.
b) In order to stabilize gold (I) against disproportionation into inactive elemental gold and toxic gold (III), the gold must be bound to groups (ligands) with which it forms strong covalent bonds. These ligands must be 'soft' i.e. bound to gold through atoms of relatively low electronegativity such as sulphur, phosphorus or carbon. Hard atoms such as O_2, N_2 and halogens will not do.
c) The ligands must not be so tightly bound, and the compounds not so stable that metabolic reactions are difficult to occur.

The thiolates meet these criteria well and were therefore used as ligands of choice in the traditional anti-arthritic drugs. Moreover, they showed both immunosuppressive and anti-inflammatory characteristics.

Other forms of rheumatic diseases such as psoriasis, juvenile arthritis, palindrome rheumatism and discoid lupus erythematosus have been treated with gold drugs (Champion *et al.*, 1990).

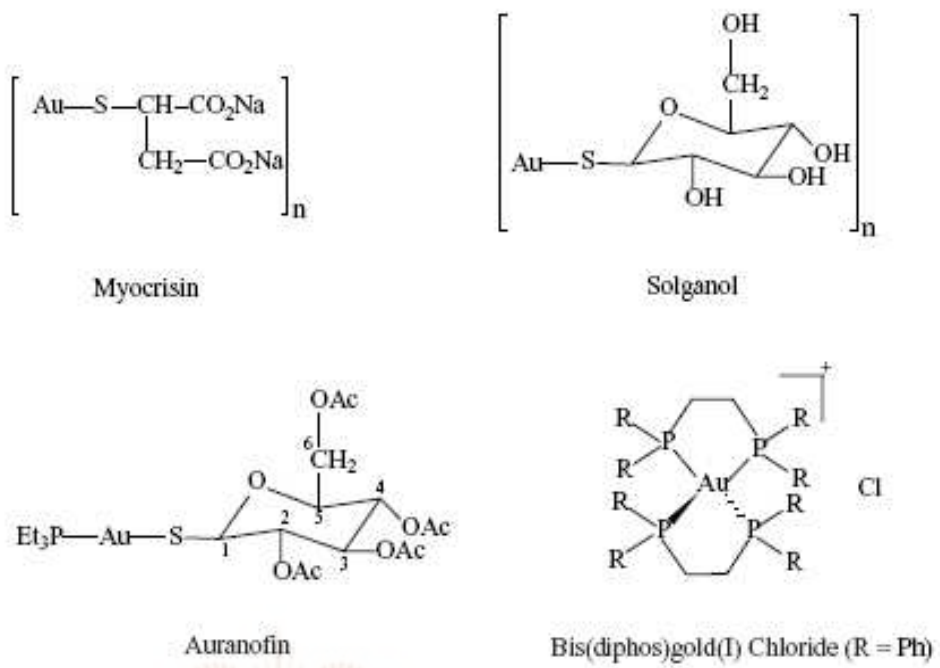


Figure 1.7: Structures of some important gold drugs. Myocrisin = aurothiomalate, solganol = aurothioglucose, auranofin is also known as radiura while bis(diphos)gold(I) chloride is also represented as Au(DPPE)chloride. (Ahmad, 2004).

1.3.2.3 Anti-tumour gold compounds

The discovery of cisplatin (cis-$[PtCl_2(NH_3)_2]$ as an anti-tumour agent in 1969 prompted the search for other metal containing anti-tumour drugs. Gold complexes were thus investigated specifically gold (III) as it gives rise to complexes that are isoelectric and isostructural with those of platinum (Bruni *et al*., 1999). Early reports indicated that the anti-arthritic drug auranofin was toxic to some tumour cells in culture and *in vivo* against P388 leukaemia (Lorber *et al*., 1979). Because of the inactivity *in vivo* on most cancer cell lines of gold (I), gold (III) which could form similar square planar complexes like

platinum was explored for anti-tumour activity. However because these gold (III) complexes are readily reduced to gold (I) (as the *in vivo* system is exclusively a reducing environment) it meant they would never reach their desired site. However, some gold (III) complexes have been evaluated against an *in vitro* panel of cells of different tissue types and different responses to cisplatin (Parish *et al.*, 1996).

A four coordinate gold complex, [1,2-bis(diphenylphosphino)ethane]gold (I) chloride ($Au(DPPE)_2Cl$ was reported in the mid 80s with promising anti-tumour activity (Friker, 1996, Berners-Price *et al.*, 1986, Mirabelli *et al.*, 1986). Unlike auranofin which was a thiolate, this complex incorporated a phosphine ligand and was more active against leukemia cells as well as in other tumour models (Berners-Price *et al.*, 1986, Mirabelli *et al.*, 1986). This compound was however not entered into clinical trials due to cardiotoxicity problems encountered during pre-clinical toxicology studies (Hoke *et al.*, 1989).

Research into the synthesis of gold complexes using ligands already demonstrating anti-tumour activity was however pursued and is currently one of the focus areas of Project AuTEK (Deane, 2006).

1.3.2.4 Antimicrobial activity of gold compounds

It was following the implication of a microbial infection as the causative agent of arthritis that gold complexes were investigated for treatment of rheumatoid arthritis. Early studies in the 1930-40s demonstrated that a variety of gold compounds were active against a broad spectrum of microorganisms (Liebarth *et al.*, 1981). There are indications that anti-arthritic gold complexes may suppress *Helicobacter pylori* (Girgis *et al.*, 1994) while

gold phosphine compounds are cytocidal *in vitro* to *Pseudomonas putida* (Rhodes *et al.*, 1992).

1.3.2.5 Anti-malarial activity of gold complexes

Some anti-malaria drugs which are useful for the treatment of arthritis exhibit similar pharmacological profiles to gold drugs hence the potential of gold as anti-malaria enhancers has been investigated (Navarro *et al*., 1997). One of the current focuses of Project AuTEK is to tailor compounds that had previously been used as anti-malaria agents e.g. chloroquine (CQ), but have become resistant into those that can inhibit both the CQ sensitive and CQ resistant strains. Research into this has been promising with the identification of one such compound (Deane, 2006).

1.3.2.6 Anti-HIV activity of gold complexes

The anti-HIV activity of gold complexes is not well documented as compared to the anti-arthritic and ant-tumour activity. The findings made by Okada *et al.* (1993) when they evaluated the anti-HIV activity of aurothioglucose and aurothiomalate (both RA agents) is relevant here. They found these compounds to completely protect MT-4 and CEM cells against HIV-1_{NL4-3} (a strain of HIV which has a unique cysteine residue close to the amino terminus of its gp41 evelope glycoprotein) induced cytopathogenicity. This inhibition was reportedly by gold (I) ligand exchange of the reactive species bis(thiolato) gold (I) and acidic thiol groups exposed on the surface of proteins as well as by reverse transcriptase inhibition in cell free assays. However, this inhibition was shown to be strain specific as other strains of HIV such as MN, RF and SF-2 were less sensitive to

inhibition compared to the NL4-3 strain possibly because of the differences in their envelop glycoproteins (Okada *et al.*, 1993).

In 1994, Tepperman *et al*., evaluated the anti-HIV activity of diacyanogold (I), $Au(CN)_2$. They showed that $Au(CN)_2$ was a common metabolite in patients treated with gold based drugs as it was found in both blood and urine samples. Gold was found to be of high levels in red blood cells suggesting that other cells such as T-lymphocytes which serve as the site for replication for HIV would also accumulate gold. Using the H9 continuous cell line, they were able to prove the uptake of gold and further evaluated inhibition of HIV by monitoring the activity of reverse transcriptase. Non cytotoxic concentrations of $Au(CN)_2$ showed a 50% decrease in RT activity (Tepperman *et al*., 1994). $Au(CN)_2$ thus has a high cellular uptake and is relatively non cytotoxic compared to other compounds that have been examined (such as solganol) and therefore capable of reaching intracellular levels of gold to provide useful therapeutic treatment for AIDS (Zhang *et al*., 1995).

In 1996 observations made from clinical data of an HIV+ patient treated for psoriatic arthritis with auranofin showed a significant increase in CD4+ cells. These cells normally decline in HIV and because the patient was not on anti-HIV drugs, auranofin must have caused remission of HIV (Shapiro and Masci, 1996). Treatment of LP-BM5 murine leukemia virus-infected mice with gold sodium thiomalate led to immunomodulatory effects (Yamaguchi *et al*., 2001). LP-BM5 murine leukemia virus causes a disease in mice that shows abnormal immunosuppression and lymphoproliferation and has similar features to AIDS. Recent findings on the anti-RT activity of gold (III) compounds containing a porphyrin ligand have been reported (Wai-Yin *et al.,* 2004). Preliminary

findings in the Project AuTek Biomed group in 2005 identified 2 of 10 novel AuTek gold compounds inhibiting HIV *in vitro* in a direct enzyme assay and through prevention of p24 secretion (Traore *et al*., 2005). The potential role of gold containing compounds as anti-HIV agents can thus not be overemphasized.

1.3.3 Other Uses of Gold

Being a metal, gold has properties such as being malleable and lustrous, and therefore has extensive use in the making of jewellery. Other applications include the use of gold and its alloys in dental application, implants, as electrodes for physiotherapy, wires for pacemaker and as drug delivery microchips (Merchant, 1998). However, gold played a very small role in medicine before the 19th century because it was difficult to dissolve. But some preparations of it were used for the treatment of syphilis and alcoholism (Higby, 1982).

1.3.4 Some Mechanisms of Action of Gold (I) Drugs

1.3.4.1 Ligand exchange reactions

The presence of $Au(CN)_2$ (metabolite of gold compounds) observed in the urine of most patients after the administration of gold drugs and very minute amounts of the administered complex (such as auranofin, solganol etc) suggests that ligand exchange reactions are a possible mechanism by which gold complexes work (Elder *et al*., 1993). These observations thus make gold complexes prodrugs meaning they undergo metabolism *in vivo* and are converted to a form that is active (Shaw III., 1999). Furthermore, $Au(CN)_2$ has been shown to inhibit HIV *in vitro* (Tepperman *et al*., 1994) further confirming that the active component is not the administered compound. Okada

et al (1993) also observed inhibition of HIV by aurothioglucose and aurothiomalate by ligand exchange reactions of the reactive species bis (thiolato) gold (I) and acidic thiol groups exposed on the surface proteins of the virus. Auranofin has been shown to react with cys-34 in serum albumin by ligand exchange reactions that displace the sulfhydryl group (Bernners-Price *et al.,* 1996). Ligand exchange reactions are thus very important when assessing the activity of gold compounds as metallodrugs.

1.3.4.2 Inhibition of reverse transcriptase

Inhibition of cell free RT has also been reported as one of the mechanisms of inhibition of HIV by gold compounds. This has been observed for aurothioglucose and aurothiomalate (Okada *et al*., 1993). Inhibition of RT has also been reported by Tepperman *et al*., 1994 for $Au(CN)_2$. The inhibition of RT can still be attributed to ligand exchange reactions where gold binds to sulfhydryl groups in the active site of reverse transcriptase (Allaudeen *et al*., 1985).

1.3.4.3 Striping of peptides from Class II MHC proteins

Class II MHC proteins are essential for normal immune system function but also drive many autoimmune responses. These proteins bind peptide antigens in endosomes and present them on the cell surface for recognition by CD4+ T cells (Watts, 1997). A small molecule could potentially block an autoimmune response by disrupting MHC-peptide interactions, but this has proven difficult because peptides bind tightly and dissociated slowly from MHC proteins. De Wall *et al* (2006) however demonstrated the use of a high throughput assay to discover a class of noble metal complexes that strip peptides from human class II MHC proteins by an allosteric mechanism. Biochemical experiments

indicate that the metal-bound MHC protein adopts a peptide-empty conformation that resembles the transition state of peptide loading. These metal inhibitors also block the ability of antigen-presenting cells to activate T-cells. This mechanism is possibly how gold (I) drugs affect the progress of RA and therefore other autoimmune diseases.

1.3.4.4 Inhibition of tumour necrosis factor induced HIV-1 replication

NF-κB is a cellular transcription factor found in a large number of cell types and regulates a wide variety of cellular genes including cytokines such as IL-6, IL-8 and tumour necrosis factor (TNF)-α. NF-κB has also been shown to be a potent activator of HIV-1 gene expression by triggering the transcription of viral genes including the transactivator *Tat* resulting in an explosive increase in HIV-1 replication. Down regulation of NF-κB would thus inhibit latently infected HIV and prevent clinical development to AIDS. Chrysotherapy has been shown to reduce the production of IL-6 and 1L-8 in serum and other cells. These are all cytokines under NF-κB regulation. Gold compounds thus possibility act by down regulating NF-κB as shown by the reduction in the production of these cytokines and therefore prevent activation of the transcativator *Tat* gene which in turn prevents explosive increase in HIV-1 replication (Traber *et al*., 1999). The inhibition of NF-κB by anti-rheumatic gold compounds is by modification of cys-159 of IKKβ (IκB kinase) blocking its activity (Jeon *et al*., 2003). This prevents phosphorylation of inhibitory proteins called IκB normally bound to NF-κB in the cytoplasm thereby preventing its dissociation from NF-κB. The undissociated NF-κB will therefore be unable to activate HIV gene transcription in the case of HIV.

1.3.5 Side Effects of Gold Based Drugs

These drugs do come with side effects just like many other therapeutic agents. Compounds such as solganol, myocrisin and gold (I) thioglucose (all polymeric compounds) are only taken up into cells at minimal concentrations and appear to be toxic to human patients at the required dose (Zhang *et al*., 1995). Moreover, gold compounds especially the polymeric ones take up to 2 months to reach a steady state in the blood because of their very long half life (Parish and Cottrill., 1987). Following 10 days of administration, only 70% of the drugs is excreted, further leading to toxicity problems (Jones and Brooks, 1996). The gold is rapidly cleared from the blood and distributed to various tissues like the kidneys where it causes nephrotoxicity. Other adverse effects include mouth ulcers, skin reactions, blood disorders and occasional liver toxicity (Parish and Cottrill., 1987). Toxicity problems are further compounded by the fact that gold compounds are slow acting, requiring treatment for up to 4 to 6 months before a beneficial effect is observed (Jones and Brooks, 1996, Friker, 1996).

Auranofin which is monomeric and administered orally is however tolerated more but then has low efficacy (Jones and Brooks 1996, Friker, 1996). $Au(CN)_2$ is shown to accumulate in cells with relatively low cytotoxicity (Zhang *et al*., 1995).

The aforementioned side effects should therefore be weighed and compared to the benefits before administering gold salts as drugs. It is worth mentioning however that despite the shortcomings; gold based therapy has been popular as the only class of drugs that brings about long lasting remission of disease in arthritis (De Wall *et al.*, 2006, Merchant, 1998) and is tolerated by some patients more than others.

1.4 RATIONALE OF THIS STUDY

With the background that HAART treatments has drawbacks (from causing the development of resistant strains of HIV and the development of latent forms of the virus leading to incomplete eradication, cost of HIV drugs etc), coupled with the alarming statistics of existing and new infections (UNAIDS 2006), continual search for would-be drugs is a necessity. This project was designed on the grounds of screening novel gold containing compounds for anti-HIV activity with the hope of identifying new potential drug leads that could eventually be used as stand alone therapies or in combination with HAART and hopefully with less or non of the problems associated with HAART.

1.5 HYPOTHESIS

Gold containing complexes can inhibit HIV replication *in vitro* and can thus serve as drug leads recommended for further analysis and development to therapeutic agents for the treatment of HIV infection.

1.6 OBJECTIVES

The major objective of this study was to identify novel gold containing lead compounds that have low toxicity to host cells and high efficacy against HIV-1 *in vitro*.

1.7 RESEARCH QUESTIONS

The research questions which were put forward to achieve the objectives of this work were as follows:-

1.7.1 Were the Compounds Stable in Solution?

Stability in solution was important as bioassays were performed in solution. Moreover, in the *in vivo* system, potential drugs have to be in solution for transportation to the relevant sites (cells and organs). Another aspect of stability is storage. Some medicinal compounds loose activity if stored in solution, at the wrong temperature etc. Aspects of stability and maintained biological activity while in solution were evaluated here.

1.7.2 By what Mechanism did the Compounds Inhibit HIV?

For the compounds to be eventually used as drugs, it was important that the mode of inhibition be known especially if the novel drugs would be used in combinationtherapy i.e. HAART. Drugs to be used alongside one another should be chosen properly to avoid administering drugs that are performing similar functions. Direct enzyme assays against vital viral enzymes (reverse transcriptase and protease) required by HIV in its life cycle were targeted. Inhibition of either of these enzymes would label the compound to be either an RT or a PR inhibitor. The anti-reverse transcriptase screen was also used as an initial assay to determine which of the compounds could potentially inhibit the whole virus *in vitro* prior to the bioassays.

1.7.3 Could the Compounds be Taken up by Host cells?

For bioactivity to occur, compounds must be taken up by cells. Uptake was determined by the use of inductively coupled plasma atomic emission spectrometry (ICP-AES). This technique has been established in our laboratory for the confirmation of uptake of other metals previously (Traore and Meyer, 2002). Some gold compounds have been shown to inhibit HIV *in vitro* in direct enzyme assays but were only minimally taken up by cells

and appeared to be toxic to human patients at the required dose (Zhang *et al*., 1995). Gold thioglucose (AuSTg) is an example of this as it inhibited reverse transcriptase in cell free assays but since it and its metabolites could not readily enter cells (which is necessary for its effectiveness against RT), it was shown to be ineffective when used against HIV in cell cultures (Okada *et al*., 1993). It was therefore important not only to confirm uptake but also the concentration of compound taken up by the cells.

1.7.4 Were the Compounds toxic to both HIV Infected and Uninfected Cells?

Cytotoxicity has been defined as the adverse effects resulting from the interference of agents with structures and processes essential for cell survival, proliferation and function (Ekwall, 1983). Some of the current side effect of available ARVs is toxicity. With *in vivo* toxicity comes failure of vital organs such as the liver. It was necessary to verify that the inhibitory concentrations of the compounds were non toxic to immune system cells and cell lines.

1.7.5 If Toxic, by what Mechanism of Death do the Compounds Kill the Cells?

Cell death occurs either by apoptosis or necrosis. Several authors have stated that the induction of apoptosis is a general phenomenon at low doses of chemicals while higher doses lead to necrosis (Gomez-Lechon *et al*., 2002, Lennon *et al*., 1991). Apoptosis is a programmed form of cell death and has been associated with HIV infected cells (Roshal *et al*., 2001). Necrosis is a more spontaneous form of cell resulting in lysis of the cells. Knowing the mode of death was important in understanding whether it was the virus which was causing a cytopathic effect on the cells (for infected cells) or it was the compounds that were being cytotoxic. The mode of cell death seen in acutely infected

cells (i.e. apoptosis) as compared to uninfected cells would also be indicative of successful infection in the case where the cells were infected *in vitro*. The mode of death caused by the compounds was determined by treating both infected and uninfected cells with the compounds. Flow cytometry was used as the detection method.

1.7.6 Did the Compounds Inhibit HIV Replication in cell cultures?

Because the anti-RT and anti-PR assays were direct enzyme assays, we needed to evaluate the ability of the compounds to inhibit replication of the whole virus in infected cell cultures. Determination of HIV replication as such was done by monitoring the effect of the compounds on the cytopathic effect of the virus i.e. through viability assessment. Compounds that inhibit HIV replication should also cause increase in the viability of the infected cells when compared to an untreated control sample. Inhibition of virus in cultures was performed by determining secretion of p24 (core protein of HIV) in infected cell cultures. A decrease in the secretion of this protein in the presence of the compounds as compared to its absence signified inhibition of replication.

1.8 METHODOLOGY

The compounds screened were synthesized by chemists in the AuTEK Biomed consortium using ligands which if conjugated to gold had been reported to have anti-inflammatory activity especially those against rheumatoid arthritis. The transcription factor NF-κB plays a critical role against genes involved in chronic inflammatory diseases (Barnes and Karin., 1997) such as rheumatoid arthritis. The same transcription factor stimulates genes involved in HIV gene expression (Traber *et al*., 1999). These anti-arthritic agents (auranofin, aurothioglucose, aurothiomalate etc) have been shown to

result not only in disease remission in arthritis but to have anti-HIV properties which could be by down regulating NF-κB (Traber *et al*., 1999); a common transcription factor in both disease cases. The novel gold (I) phosphine based complexes containing thiol ligands, DPPE ligands, triethylphosphine thiol and gold (III) nitrogen based ligands were thus screened for anti-HIV activity with the hope that they would inhibit the transcription factor NF-κB or other viral enzymes and thus HIV replication. Initial screening involved stability analysis by ^{31}P NMR and UV-Visible spectroscopy, direct enzyme assays to identify the molecule inhibited and thus mechanism of inhibition i.e. reverse transcriptase or protease inhibition and uptake studies (by ICP-AES) followed by cytotoxicity studies (using tetrazolium dyes and the lactate dehydrogenase (LDH) assays). The cytotoxicity data was confirmed using flow cytometry. The compounds with inhibitory activity on RT and PR and with the least cytotoxicity were further screened in antiviral p24 determination assays using p24 ELISAs.

The principles and procedures of the different assays or techniques applied are provided in chapter 2.

1.9 EXPECTED OUTCOMES

The possible identification of novel chemotherapeutic gold containing compounds that have high efficacy against HIV and low cytotoxicity *in vitro* was envisaged.

CHAPTER 2

MATERIALS AND METHODS

In order to verify the hypothesis that gold compounds have potential as anti-HIV agents, both direct enzyme and cell based analysis were performed. This section elaborates on the materials used and the procedures (including principles for selected methods) followed to validate this hypothesis.

As mentioned in chapter 1 (section 1.8), the gold compounds for bioanalysis were provided by the AuTek Biomed Consortium. The chemists who synthesised the compounds were responsible for confirming accurate synthesis and elucidating compound structure which was achieved through the use of nuclear magnetic resonance (NMR) analysis. Our own analysis with ^{31}P NMR was for the determination of stability in the solvents in which the compounds were dissolved.

Because not all compounds were colourless when in solution and because these would be used in viability assays utilizing tetrazolium and other colorimetric assays that measure absorbance in the presence of the compound, the wavelength at which the compounds may absorb radiation had to be determined. This was done by scanning the ultraviolet and visible range of the spectrum of the compounds in respective solvents in experimental and reference cuvettes of a Cary UV-visible spectrophotometer.

Direct enzyme assays including the anti-reverse transcriptase (RT) and the anti-protease (PR) assays utilising specialized ELISAs and enzyme substrate based assays respectively were utilised to determine anti-HIV activity of the compounds. Lastly, the cell associated analysis including uptake studies (ICP-AES), cytotoxicity studies (using viability dyes

and LDH assay) and antiviral p24 analysis were performed. Figure 2.1 provides a diagrammatic illustration of all the analysis related in this project.

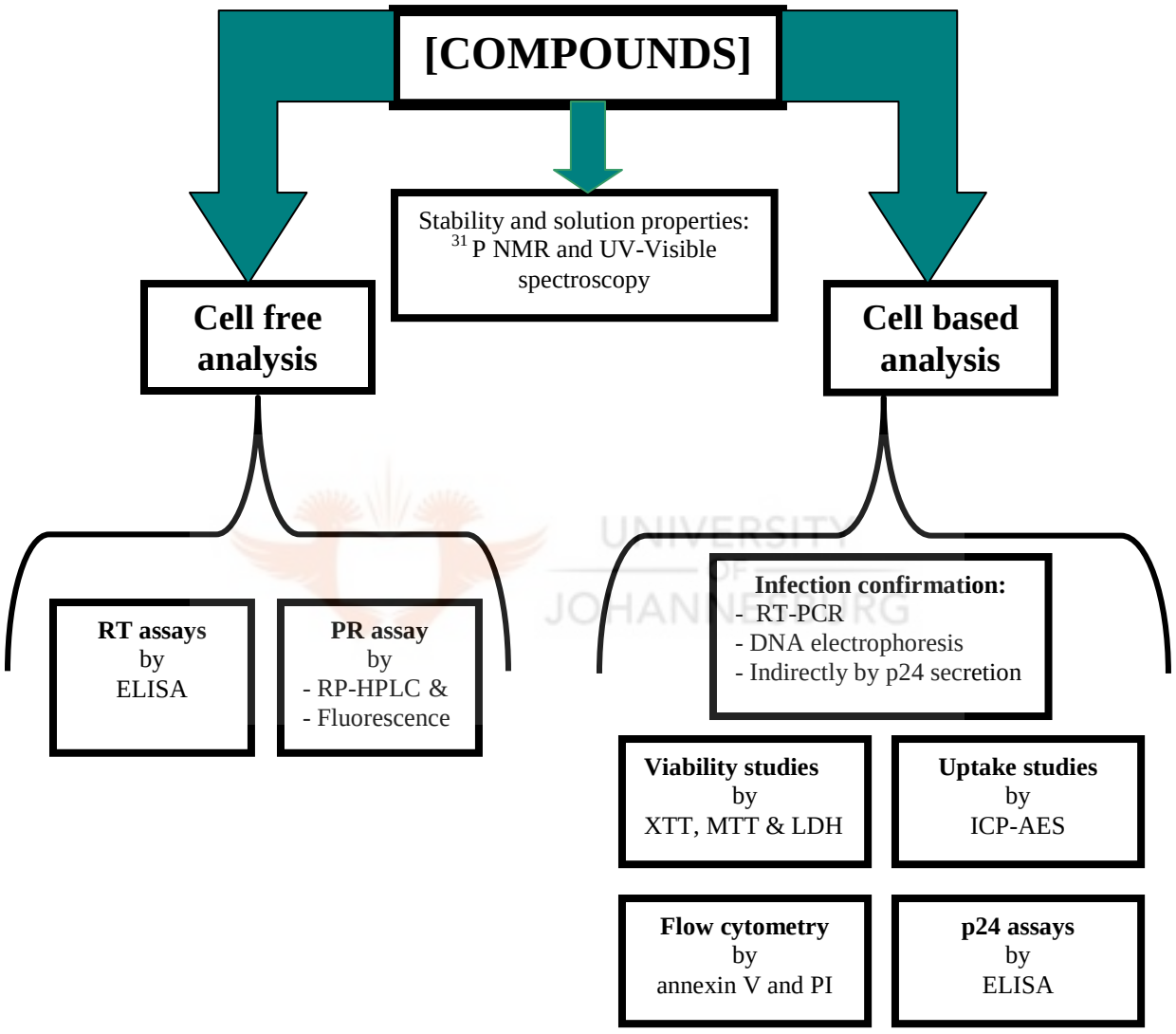


Figure 2.1: Flow diagram representing the main methods and techniques described in this project.

2.1 COMPOUNDS: STABILITY, SPECTRA & SOLUTION PROPERTIES

2.1.1 Types of Compounds

The compounds screened are represented in Table 2.1 and will be referred to from here henceforth as the compounds to mean all 16 of them (including complexes and ligands). As mentioned earlier, the ligands are those moieties without a gold atom while gold complex or compound would refer to ligands with gold atom(s) attached.

The compounds include gold (I) phosphine complexes (TTC3, TTC10, TTC17, TTC24) with imine (TTL3, TTL10) and amine ligands (TTL17, TTL24) respectively, bis (diphenylphosphino) ethane (DPPE) complexes (EK207, EK208, EK219, EK231), triethylphosphine thiol ligand based complexes (MCZS1, MCZS2 and MCZS3) and a nitrogen based gold (III) complex (KFK154b). Complexes TTC3, TTC10, TTC17, TTC24, EK207, EK208, EK219, EK231, MCZS1, MCZS2 and MCZS3 all have gold (I) phosphine ligands with differences in the ligand type as just indicated. The ligands were included in the screens to verify that it is the gold in the gold complex and not the ligand that has activity as has been shown by other authors (Chircorian and Barrios, 2004, Sutton *et al*., 1972). The ligands serve to transport the metal to their active site were bioactivity which could be through ligand exchange etc occurs. It has been shown that it is not the administered gold compound that has effect on disease but a metabolite of it hence these drugs are prodrugs (Shaw, 1999).

Phosphine gold and triethylphosphine thiol ligands were chosen for coordinating the gold nucleus around as these are soft ligands and result in more relatively stable complexes that could still undergo ligand exchange reactions due to their preference for sulfhydryl

Table 2.1: Structure of gold complexes* and ligands analyzed for anti-HIV-1 activity.**

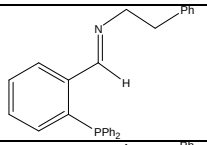
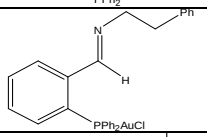
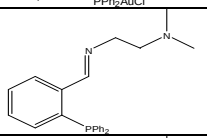
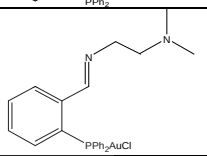
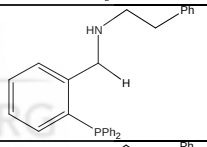
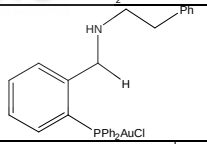
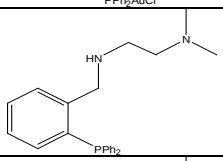
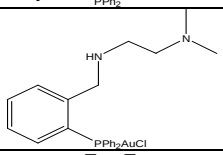
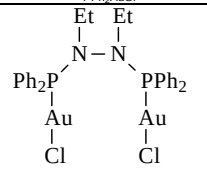
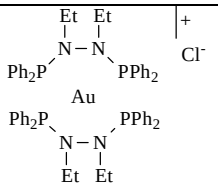
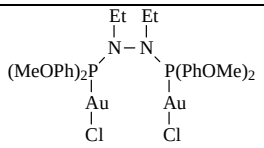
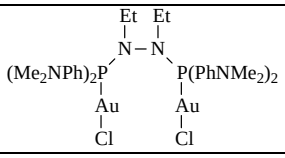
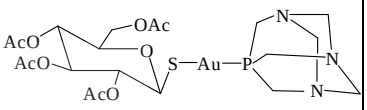
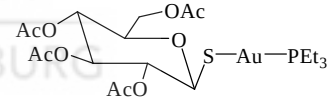
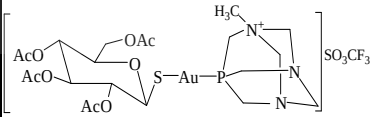
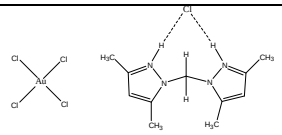
No	Sample code & Mwt (g/mol)	Name	Structure
1	**TTL3**** (393.46)	(2-diphenylphosphanyl-benzylidene)-phenethyl	
2	**TTC3*** (635.10)	Benzyl-(2-diphenylphosphanyl-benzylidene)-phenetyl-amine gold(I)chloride	
3	**TTL10**** (360.43)	*N'*-(2-diphenylphosphanyl-benzylidene)-*N*,*N*-dimethly-ethane-1,2-diamine	
4	**TTC10*** (592.85)	*N'*-(2-diphenylphosphanyl-benzylidene)-*N*,*N*-dimethly-ethane-1,2-diamine gold(I) chloride	
5	**TTL17**** (395.48)	2-diphenylphosphanyl-benzyl)-phenethyl-amine	
6	**TTC17*** (627.90)	2-diphenylphosphanyl-benzyl)-phenethyl-amine gold (I) chloride	
7	**TTL24**** (362.45)	*N'*-(2-diphenylphosphanyl-benzyl)-*N*,*N*-dimethyl-ethane-1,2-diamine	
8	**TTC24*** (594.87)	*N'*-(2-diphenylphosphanyl-benzyl)-*N*,*N*-dimethyl-ethane-1,2-diamine gold chloride	
9	**EK207*** (921.34)	Bis(diphenylphosphino)-1,2-diethylhydrazine di(gold chloride)	

Table 2.1 continues:

No	Sample code & Mwt (g/mol)	Name	Structure
10	**EK208*** (1145.42)	Bis [bis (diphenylphosphino)-1,2-diethylhydrazine] gold chloride	
11	**EK219*** (1045.47)	3.7 bis (di (4-methoxyphenyl) phosphine)-1,2-dimethyl hydrazine di(gold chloride) (6)	
12	**EK231*** (1109.74)	bis(di(N,N-dimethyl aniline)phosphine)-1,2-dimethyl hydrazine di(gold chloride) (4)	
13	**ZS1/MCZS1*** (717.49)	(2,3,4,6-tetra-*O*-acetyl-1-thio-*B*-D-glucopyranosato-S-)(1, 3, 5-triaza-7 phosphaadamantane) gold (I)	
14	**ZS2/MCZS2*** (678.49)	(2,3,4,6-tetra-*O*-acetyl-1-thio-*B*-D-glucopyranosato-S-) (triethylphosphine) gold (I)	
15	**ZS3/MCZS3*** (881.59)	(2,3,4,6-tetra-*O*-acetyl-1-thio-*B*-D-glucopyranosato-S-)(1, 3, 5-triaza-7 phosphaadamantane)gold (I) (+1) trifluoromethanesulphonate(-1)	
16	**KFK154b*** (610.59)	Tetra-chloro-(bis-(3,5-dimethylpyrazolyl)methane)gold (III)chloride	

** The following compounds (codes only), TTL3, TTL10, TTL17, TTL24 are ligands used for the gold complexes denoted TTC3, TTC10, TTC17 and TTC24. ZS1, ZS2 and ZS3 also denoted MCZS1, MCZS2 and MCZS3 represent different batch names. The code names are initials of the chemist who synthesized the compounds.

groups in enzymes (Parish and Cottrill, 1987). The only gold (III) complex (KFK154b) contains a nitrogen based ligand as nitrogen donors make relatively stable ligands for gold (III) termed hard ligands. It is also worthy to note that changes in ligands and

stereochemistry of the ligand coordinated with the gold nucleus have resulted in altered biological activity (Shaw, 1979), the reason for which these newly coordinated compounds were tested.

2.1.2 UV-Visible Spectra of compounds

A scan of all the compounds over UV-Visible (200-800 nm) using a Cary UV-Vis spectrophotometer from Varian was performed to identify the wavelength at which maximum absorption occurred. It was necessary to perform this scan so as to avoid wavelength interferences with viability dyes and other colorimetric bioassays whose analysis were based on absorbance at particular wavelengths. This spectrum also represents compound stability when done over time in a particular medium (DMSO or a physiological buffer).

2.1.2.1 UV-visible spectra in dimethylsulfoxide (DMS0)

The compounds were dissolved in DMSO for stability analysis. DMSO was chosen as solvent of choice as the gold complexes sufficiently dissolve in it. Moreover, this solvent has the least inhibitory effect on HIV-1 reverse transcriptase compared to other solvents such as methanol and ethanol (Ghee *et al*., 1991). Generally, DMSO also adequately dissolves organic compounds and metallodrugs such as the ones tested here as compared to aqueous solvents. The stability of the compounds in DMSO gives an indication of their longevity (half-life) in solution, which is necessary if the compounds are to be used in drug screens.

A spectrum of the compounds was obtained immediately on dissolution in DMSO. The same sample was stored at 4 ºC and after 24 hrs, another spectrum was obtained and then

later at 1 week with the samples still maintained at 4 ºC. The initial spectrum on immediate dissolution gave an indication of the wavelength at which the compounds' chromophore absorbs while the subsequent time study spectra (at 24 hrs and 1 week) were compared to this for stability assessment of the complexes in DMSO. Changes in absorbance intensity which could either be an increase from the normal (hyperchromic shift) or a decrease from the normal (hypochromic shift) as well as changes in wavelength maxima which could either be a wavelength decrease (hypsochromic shift) or increase (bathochromic shift) all served as indicators of instability.

2.1.2.2 UV-visible spectra in a physiological media

The stability of the complexes in a physiological buffer (phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH = 7.4) was investigated spectrophotometrically by kinetic measurements over time. One hundred micromolars of a concentrated DMSO solution of each of the gold complexes was prepared in PBS and the UV-visible spectra of the sample obtained immediately, 40 mins later and every 2 hrs after the initial scan with the compounds maintained at 37 ºC.

The concentration required to detect a spectrum of the gold complex by using a UV-visible spectrophotometer is in the micromolar range (compared to mg quantities needed for NMR). Stability testing in a physiological buffer could therefore be done only by UV-visible spectroscopy as the concentration required for NMR (20 mg/ml) could not dissolve properly in aqueous media. Improper dissolution would mean poor resolution in NMR peaks.

Initial stability studies were performed at 4 ºC as mentioned before (2.1.2.1). We also had to perform the assay at 37 ºC (in DMSO) and this could still have been done by use of

UV-visible spectroscopy. However, UV-visible spectroscopy is a less sensitive method for determining stability compared to NMR spectroscopy because it typically contains far fewer features and therefore lower information content (Field *et al.,* 1995). Also, the higher temperature of 37 ºC meant possible decomposition could occur which might not be detected by UV-visible spectroscopy. Stability of the complexes at this higher temperature (37 ºC) was therefore performed with the use of the more sensitive NMR detection method.

2.1.3 Stability of Gold Complexes by ^{31}P NMR

NMR is useful in studying the structures of molecules in the liquid state and in solution. Many nuclei have magnetic properties that arise from the spin of the nucleus. It is therefore possible to observe the NMR spectra given by many different nuclei including ^{1}H, ^{13}C, ^{19}F and ^{31}P.

Gold does not have a useful NMR nucleus since 100 % abundant ^{197}Au has a quadrupolar ground state (Shaw, 1999) such that gold NMR spectroscopy is very difficult to obtain. Therefore NMR characterization of gold compounds depends on the presence of other magnetic nuclei such ^{1}H, ^{13}C and ^{31}P. ^{31}P NMR was used for stability assessment and the experiment was conducted over time using a 300 MHz Varian NMR instrument (Oxford). Twenty milligram per milliliters of each gold complex was dissolved in deuterated DMSO and a spectrum obtained immediately on dissolution. The compounds were further incubated (37 ºC) for 24 hours after which another spectrum was obtained and later at 1 week. The analysis was done in the presence of a phosphoric acid reference (for calibration) which peaks at 0 ppm. The chromatogram obtained on immediate dissolution was used as a baseline for checking any changes in subsequent spectra collected

following different incubation times. The shifts or absence of shifts in the peaks on graphs obtained with the differently treated samples (immediately analyzed or stored at 37 ºC prior to analysis) were then compared and noted as a measure of stability or instability as peak shifts indicate bond breaking or formation of new bonds as the case may be.

Analysis of compounds dissolved in a physiological buffer could not be performed by ^{31}P NMR as mentioned earlier as the minimum concentration at which the compounds could be detected by NMR (20 mg/ml) could not sufficiently dissolve in a physiological buffer.

2.2 DIRECT ENZYME ASSAYS

The direct enzyme assays performed were anti-HIV-1 reverse transcriptase and anti-HIV-1 protease. These assays give an idea into the mechanism by which the compounds inhibit HIV i.e. if they are reverse transcriptase or protease inhibitors. Procedures for these assays are describes in this section.

2.2.1 The Reverse Transcriptase (RT) Assay

The reverse transcriptase assay (Roche Diagnostics, Mannheim, Germany) is a colorimetric assay where the enzyme activity is determined after treatments in the presence or absence (untreated control) of different concentrations of the different compounds. This assay gives a quantitative measure of the RT activity. Figure 2.2 represents the principle of this technique which takes advantage of the ability of RT to synthesise DNA, starting from the template/primer hybrid poly (A) x oligo $(dT)_{15}$. Digoxigenin and biotin labeled nucleotides are incorporated into one and the same DNA molecule as it is freshly synthesized by RT. The detection and quantification of newly

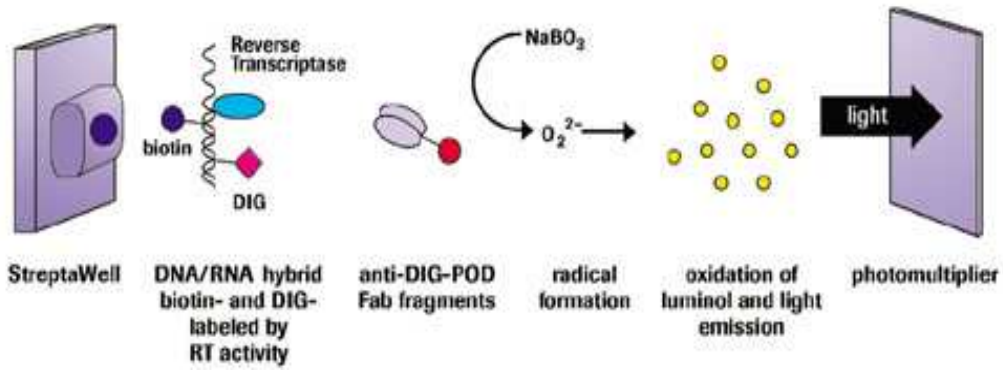


Figure 2.2: Test principle of the reverse transcriptase colorimetric assay. (Taken from the Roche Diagnostic catalogue, https://www.roche-applied-science.com/servlet/RCConfigureUser?URL=StoreFramesetView&storeId=10151&catalogId=10151&langId=-1&countryId=za (URL active 31/12/07).

synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. The direct enzyme assay was performed according to the manufacturer's instructions by transferring 20 µl (0.2U) of purified recombinant HIV-1 RT (Merck, Darmstadt-Germany) and 20 µl of reaction mixture (reconstituted template-template/primer hybrid poly (A).Oligo $(dT)_{15}$) and diluted nucleotide (tris- HCl (50 mM, (pH 7.8) with DIG-dUTP, biotin-dUTP and dTTP)) to microfuge tubes containing 20 µl of gold compounds (6.25, 25 and 250 µM) dissolved in DMSO (maximum DMSO concentration was 1.5%) and diluted with lysis buffer. This was followed by a 1 hr incubation at 37 ºC. The samples were then transferred to appropriate wells of a streptavidin coated plate followed by another 1 hr incubation (37 ºC). The plate was washed 5 times with 250 µl of wash buffer and the buffer completely removed before adding 200 µl of anti-DIG-POD working solution (200 mU/ml). A further 1 hr incubation (37 ºC) followed by 5 rinses using wash buffer was performed. An ABTS substrate solution (200 µl) was transferred

to all wells of the plate and incubated at room temperature (15-25 ºC) until sufficient color development (green) for photometric detection was attained (15 min after). An untreated control used contained enzyme only with an equivalent amount of DMSO used in the test samples (1.5%) while a positive control was a plant extract for which anecdoctal evidence had shown decrease mortality and mobility in HIV positive patients. The plate was read on a Synergy plate reader (BioTek, Analytical & Diagnostic Products (ADP), Africa) at 405 nm and a reference wavelength of 492 nm used. The data was analysed using the Gen5^{Tm} software (ADP, South Africa) and the percentage inhibition was calculated based on the formula: 100 – [(Test reagent Abs / untreated control Abs) x100)] where Abs = absorbance.

2.2.2 The Protease Assay

The protease assay was performed using both a reverse phase high performance liquid chromatography (RP-HPLC) and fluorescence substrate based detection methods.

2.2.2.1 The RP-HPLC method

The S-1000 protease assay kit (Bachem Bioscience Inc. King of Prussia, PA, UK) was used for inhibition screening in the RP-HPLC protease assay. This assay was performed at compound concentration of 83 µg/ml. Table 2.2 is a representation of the molar concentrations obtained from coverting 83 µg/ml and they differ as the compounds have different molecular weights.

The proteolytic activity of HIV-1 PR was assessed by using the synthetic peptide substrate (His-Lys-Arg-Val-Leu-(NO_2) Phe-Glu-Ala-Glu-Ala-Met-Ser-NH_2) provided in the kit following experimental procedures recommended by the manufacturer.

Table 2.2: Concentrations of compounds used in the protease assay.

Compound	Concentration (µM)	Compound	Concentration (µM)
TTL3	209.93	EK207	89.65
TTC3	131.97	EK208	72.11
TTL10	229.17	EK219	79.01
TTC10	139.33	EK231	74.43
TTL17	208.86	ZS1/MCZS1	115.12
TTC17	131.55	ZS2/MCZS2	121.74
TTL24	227.89	ZS3/MCZS3	93.69
TTC24	138.85		

Because no appreciable hydrolysis of the substrate was obtained using the positive control inhibitor (acetyl pepstatin (AP), modifications were made to the manufacturer's protocol according to other literature sources. These were increased incubation times (from 15 mins to 1 hr, Tewtrakul *et al.,* 2003), and an increase in the percentage of TFA required to stop the reaction (from 0.8% to 5%, Pazhanisamy *et al*., 1996). The substrate is cleaved between Leu and Phe and these products were separated by RP-HPLC. The resultant products should generate two peaks that elute earlier than the substrate peak. The assay was performed at a final volume of 121 µl containing 10 µl of substrate, 1 µl HIV PR and 10 µl gold compounds (83 µg/ml final concentration) in assay buffer (100 mM NaOAc pH 4.7, 300 mM NaCl, 4 mM EDTA). The reaction was carried out for 1 hour at 37 °C and was terminated by adding 5% TFA. The supernatant obtained by centrifuging at 12,000 rpm for 5 minutes was analysed by RP-HPLC on a C_{18} column using SPD-M10A photodiode array (Shimadzu, Shimadzu Corporation, Tokyo, Japan). Ten microlitres of the reaction mixture was injected into the HPLC column and gradient elution performed with 0.1% TFA in water and 0.1 % TFA in acetonitrile at a flow rate of 1.0 ml/minute over 35 minutes (initial time used was 15 minutes but was increased to 35

minutes so as to increase the cleaning time of the column and increase resolution of peaks). A blank run was performed after 3 samples to allow for cleaning of the column so as to further increase resolution. The elution profile was monitored at 220 nm. A negative control sample included the substrate and enzyme only. Percentage inhibition was calculated using the formula: Control A – Sample A/Control A x 100 where A = Area of the peak on chromatogram.

2.2.2.2 HIV-1 PR fluorogenic assay

This assay makes use of a fluorogenic HIV protease substrate 1 with structure (Arg-glu-(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys-(DABCYL)-Arg, (Sigma Aldrich, Missouri USA). This substrate is a synthetic peptide sequence that contains a cleavage site (Tyr-Pro) for HIV protease as well as two covalently modified amino acids for the detection of cleavage (Matayoshi *et al*., 1990). One of the modifications is the addition of the fluorophore [EDANS, 5-(2-aminoethylamino)-1-naphthalene sulfonate] to the glutamic residue. The other modification is the addition on an acceptor chromophore (DABCYL, 4'-dimethlyaminoazobenzene-4-caboxylate) to the lysine residue. The modified amino acids are on opposite sides of the cleavage site. Spatial orientation and overlap of the DABCYL absorbance with the EDANS emission permits resonance energy transfer between the two moieties and quenching of the EDANS fluorescence at 490 nm occurs. However, when the peptide is cleaved by the HIV protease, the DABCYL group is no longer proximal to the fluorophore and emission at 490 nm can not be detected. A gold compound that inhibits HIV protease therefore prevents this cleavage and therefore allows for quenching such that the EDANS fluorescence signal is diminished.

The assay was performed according to procedures by Lam *et al*., 2000. The substrate was dissolved in DMSO to make a 1mM stock. The stock fluorogenic substrate was diluted to 16 µM using assay buffer (0.1M sodium acetate, 1 M NaCl, 1 mM EDTA, 1mM DTT and 1 mg/ml BSA, pH 4.7). An aliquot of the substrate (16 µM, 49 µl) and 1 µl of HIV-1 PR solution (1 µg/µl; Bachem, Switzerland) were added to the reaction mixture in an assay buffer in the presence or absence (untreated control) of compounds to a final reaction volume of 100 µl. This mixture was incubated at 37 ºC for 1 hr in black 96 well fluorescence assay plates (Scientific group, South Africa). A 10µg/ml of pepstatin A (Bachem-UK) was used as a positive control for inhibition of HIV-1 protease. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Synergy microplate spectrofluorometer (BioTek, Analytical & Diagnostic Products, South Africa). A blank treatment consisted of assay buffer only. Data was analysed using the Gen5^{Tm} software (ADP, South Africa) and the percentage inhibition calculated based on the formula: 100 – [(Test reagent RFU / untreated control RFU) x100)] where RFU = relative fluorescence units.

2.3 CELL ASSOCIATED ANALYSIS

The direct enzyme assays provide information on the effect of the compounds on some viral enzymes but since the enzymes are located in cells, cell based analysis are required to determine the effect of the compounds in culture on the whole virus. Cell culture reagents were obtained from Highveld Biologicals (Sandringham, South Africa) unless otherwise stated.

2.3.1 Cells

Cells used included both primary cells (peripheral blood mononuclear cells denoted PBMCs) and continuous cell lines: CEM.NKR-CCR5 (courtesy of Dr.Alexandra Trkola, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; Howell *et al*., 1985; Lyerly *et al*., 1987) and PM1 (courtesy of Dr. Marvin Reitz was obtained from the NIH AIDS Research and Reference Reagent Program., Lusso *et al*., 1995). HIV infected and uninfected cells were analysed to be able to compare the effect of compounds on both the host cell and pathogen.

2.3.1.1 Primary cell isolation

The use of human subjects for specimen collection (in this case blood) required ethics approval. This was obtained from the University of the Witswatersrand (because Helen Joseph were the blood was collected is a teaching hospital of this University and the University of Johannesburg). Following ethics approval and signing of an informed consent form by the donor, blood was collected into vacuette EDTA (anti-coagulant) blood tubes (Beckman Coulter, California USA) from both seronegative and seropositive individuals. In the case of infected cells, blood was obtained from donors attending the AIDS Clinic at the Helen Joseph Hospital (Johannesburg, South Africa), specifically from participants who were asymptomatic and not on ARVs to prevent interference between compounds to be analysed and ARVs. The use of peripheral blood mononuclear cells (PBMCs) which are primary cells composed of different cells types (monocytes, T-cell, Nk cells etc) mimics the in *vivo* situation as the compounds will finally be used as such if they proved to be inhibitory. PBMCs collected from infected patients were termed chronically infected cells. Once collected, the blood was allowed to stand for between 1

and 2 hours in a biological safety cabinet to separate the cells from plasma. The top plasma layer was collected into 2 ml cryovials and later used to confirm the HIV status of the individual. PBMCs in the lower layer were separated by ficoll gradient centrifugation following a 1:1 dilution with warm (37 ºC) RPMI 1640 medium. The cells recovered from the gradient were washed with RPM1 medium to remove platelets. Five millilitres of ACK (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2-7.4) was added to the pellet and allowed to stand for 5 minutes (ACK lyses any red blood cells present) after which the ACK was washed off. The cells were then resuspended in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% heat-inactivated (56°C, 30 min) foetal bovine serum (FBS) and antibiotics that consisted of penicillin G (10 mg/ml), streptomycin sulphate (10 mg/ml), fungizone (25 µg/ml) and 1% gentamycin sulphate (GS) referred to as complete medium from here henceforth. PBMCs were stimulated with PHA-P (4 µg/ml) and IL-2 (20 U) at a concentration of $1x10^6$ cells/ml before treating with compounds for bioassays. Only the HIV infected PBMCs were stimulated with IL-2 as this cytokine is decreased by HIV infection (Lee *et al* 1996, Maggi *et al* 1994).

2.3.1.2 Continuous cell lines

The CEM.NKR-CCR5 cell line is a lymphoblastoid cell lined obtained from transducing CEM.NKR to be able to express CCR5 (Trkola *et al.,* 1999), a coreceptor required by HIV for successful infection. These cells were cultured in complete RPMI media at 37 ºC in a 5% CO_2 incubator and seeded every two days in a 1:3 ratio by adding fresh complete medium to the culture.

The PM1 cell line obtained by transforming a neoplastic T-cell line Hut 78 (Lusso *et al.*, 1995) to display CCR5 on its surface making them susceptible to infection by X4 and R5

isolates and therefore ideal for expansion of progeny virus was also used. These cells not only support replication of X4 and R5 tropic viruses but also secrete IL-2 (Dittmar *et al.*, 2001) a cytokine necessary for cell proliferation. The cells were cultured in complete RPMI medium and split 1:5 every 2 days. When *in vitro* infection was required, the cells were maintained for at least two weeks in culture, which is time enough for proper expression of the CCR5 coreceptor (Trkola *et al*., 1999), prior to infection.

Both cells lines are suspension cell lines allowing for ease of manipulation compared to adherent cell lines.

The use of continuous cells lines reduces costs (the use of PHA and IL-2 for stimulation is eliminated) and time required for isolation and use of primary cells (Lusso *et al.*, 1995). Moreover, it is difficult to grow primary cells in culture and interperson variation between isolates does not allow for reproducibility in experiments (Trkola *et al.,* 1999). There is however the extra requirement of infecting these cells *in vitro*. The next section describes how this was achieved. Continuous cell lines that were infected *in vitro* were termed acutely infected cells to indicate a recent infection.

2.3.2 Acute Infection and Confirmation there of.

The cell lines chosen both have CCR5 as coreceptor and therefore can be infected with the HIV-1 subtype C isolate DU 151 as subtype C isolates have a preference for CCR5 (Peeters *et al*., 1999). To obtain infection, 1×10^6 cells/ml were exposed to 500 $TCID_{50}$ of virus (Du 151- an isolate of two phylogenetically distinct subtype C strains (Coetzer *et al*., 2007). Ten µg/ml of polybrene (Sigma Aldrich, Missouri, USA) was added to assist with infection and the cells were then centrifuged at 1200 × *g* for 3 hours. Polybrene neutralizes negative charge repulsion on the cell surface making the binding of virus

particles to cell surfaces less limiting (Le Doux *et al.*, 2001) while spinoculation (centrifugation) enhances the adsorption of HIV-1 to infectious cells through virus binding (O'Doherty *et al*., 2000).

After centrifugation, the virus/cells/polybrene mix was incubated overnight at 37 ºC. Excess virus and polybrene were washed off two times with warm RPMI medium and the pellet resuspended in complete medium to a concentration of 2 x10^5 cells/ml. These were used in bioassays i.e. viability studies, p24 based experiments and flow cytometry. Compound treated cells were incubated at 37ºC for a further 7 days to allow for viral replication as well as viral and compound spread throughout the culture before reactions were stopped. As reported by Bedoya *et al*., (2001) and Walder *et al.,* (1997), antiviral screens are usually performed after a 4-14 days incubation period post *in vitro* infection. We choose 7 days of incubation for all bioassays. The seven days was specifically chosen as this time allows room for viral replication and spread in culture (Trkola *et al*., 1999). Cells that were infected *in vitro* were termed acutely infected cells and here we only infected the cell lines *in vitro* as infected primary cells were collected from seropositive people (hence no acutely infected primary cell assays are reported). In addition, the percentage of CCR5 coreceptors on primary cells is lower than in the continuous cell lines (Basson, 2005) thus restricting *in vitro* infection of the primary cells compared to the cell lines. Moreover, the growth kinetics of continuous cell lines following cryo-storage remains unchanged compared to that of primary cells (Basson, 2005).

2.3.2.1 DNA isolation

To confirm successful infection of cells, DNA was isolated and used in a real time polymerase chain reaction (RT-PCR) to determine the presence of the *gag* gene of HIV.

One ml of $1x10^6$ cells/ml was washed with PBS and transferred to a 500 µl microcentrifuge tube. Lysis buffer (60 µl) consisting of 10 mM Tris pH 8.3, 50 mM KCl, 0.5% (v/v) Tween-20 in PBS pH 7.4 and 50 µg/µl Proteinase K (Promega, MI, USA) was added to the cells. The mixture was incubated overnight in a water bath at 56ºC. The DNA extract was then mixed, heated at 96ºC for 10 min to inactivate proteinase K and used for RT-PCR.

2.3.2.2 Real time polymerase chain reaction (RT-PCR)

Real time PCR is a technique where fluorescent probes, specific to a target gene (e.g. HIV *gag*) are employed to monitor the product formed (Vitone *et al.*, 2005). It is a sensitive, specific and reproducible assay offering improvement for the quantification of viral DNA compared to conventional PCR (Casabianca *et al.*, 2004). HIV-1 specific primers SK462 (5'-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3') and SK 431 (5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3') (IDT, Iowa, USA) were used. A 25 µl volume RT-PCR assay was performed containing 12.5 µl of 2 × SensimiX, 1 µl of each primer, 1 µl DNA (5 ug/ul), 0.5 µl Cyber green and 9 µl PCR H_2O (Sigma Aldrich, Missouri, USA). The nucleosense mix was analysed on a Rotor-Gene Light Cycler-RG-3000A (Corbett Research, Sydney, Australia). The reaction was initiated with activation of the Taq enzyme for 10 mins at 95 ºC followed by 3 cycles of melting for 10 s at 94 ºC, annealing 55 ºC for 10 s and extension at 72 ºC for 10 s. Subsequently, 42 cycles were conducted in three temperature steps: 10 s at 94 ºC, 10 s at 60 ºC and 10 s at 72 ºC. The amplified DNA was identified by a characteristic melting curve.

2.3.2.3 Agarose gel electrophoresis

This technique is important as it allows for the visualisation of the PCR product obtained and for the estimation of its size using molecular weight markers which are run alongside the PCR product. Agarose gels were prepared by dissolving 1 g of agarose powder (Whitehead Scientific, South Africa) in 50 ml of 1 X TBE buffer (final agarose concentration being 2 %). Ten times TBE buffer contained (0.6 M Tris base, 0.9 M boric acid, and 25 mM EDTA). The mixture was microwaved for about 2 mins until the agarose was dissolved. The liquid was left to cool to about 60 ºC, and 3 µl of ethidium bromide (Whitehead Scientific, South Africa) at 8 mg/ml added to it. The mixture was poured into a gel cast and a Teflon comb inserted into the unset gel to form wells for sample loading. Once the gel was set, the comb and casting pieces were removed and the gel placed into an electrophoresis tank (Shelton Scientific, MI USA). The TBE tank buffer containing 17.5µl ethidium bromide (8 mg/ml) was poured into the electrophoresis tank. DNA (2 and 5 µg/µl) was loaded onto the wells of the agarose gel and ran for 1 hr 30 mins at 80 V from the cathode (negative electrode) to the anode (positive electrode) as DNA migrates in this direction. The ethidium bromide intercalates in the DNA and aids in visualising the position of DNA bands as it fluoresces under UV light. The gel was then visualized using a UV source and an image of it obtained using an image analyser (Bio-Rad Laboratories, Milan, Italy).

2.3.3 Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) for assessment of Gold Uptake.

Uptake studies were done by means of ICP-AES using an ICP emission spectrometer, the Liberty 110 from Varian (Palo Alto, USA). ICP-AES is a fast multi-element technique

that uses an ICP source to dissociate a sample into to its constituent atoms or ions exciting them to a level where they emit light of a characteristic wavelength such that they can be detected (Figure 2.3). This assay was used to verify that the cells actually take up the gold complexes by checking for the emission of gold in the spectrum. Uptake is related to bioactivity as poor uptake will result in limited bioavailability and distribution and hence inactivity *in vitro*. The characteristic wavelength (242.8 nm) at which gold absorbs was used and serves as an indication that the complex as a whole was taken up by the cells.

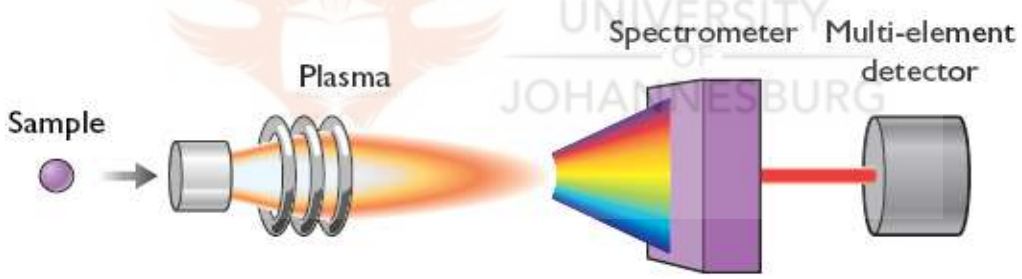


Figure 2.3: Schematic of an ICP-AES instrument.
http://www.research.philips.com/technologies/misc/matanalysis/downloads/icp-aes.pdf (Active on 19/11/07)

Cells (uninfected PBMCs and CEM.NKR-CCR5) were incubated with compounds at three different concentrations of 12.5, 25 and 50 µM. The minimum DMSO concentrations in the final treatments (and for all bioassays) were maintained at 0.5% and below as these concentrations have been shown to have little effect on the viability of

cells. Seven days after incubation, the cells were washed (470 x *g*, 5 minutes) 3 times with PBS (prepared in Milli Q deionized water). The cells were resuspended in analytical grade nitric acid (10 % in deionized water). The nitric acid serves to disrupt the cells and releases the gold taken up into the supernatant. This process was aided by heating to 56 ºC for 30 minutes. The cell debris was discarded after centrifuging for 10 minutes at 10,000 rpm. The supernatant was then analysed for gold at 242.8 nm by ICP-AES. Standard solutions of gold were prepared by appropriate dilutions of a gold standard (1, 10 and 50 ppm) using analytical grade nitric acid (10 %) in deionized water from a stock standard (1000 ppm gold solution, Alfa Aesar, Ward Hill USA). The use of the gold standards ensures that the readings obtained from the treated samples actually represent gold. Using a pneumatic pump, samples were channeled into a nebuliser where they were converted to fine aerosols by a steam of argon. After passing through a spray chamber to remove unfavourable large droplets, the aerosols enter the plasma through an injector. The gold atoms gain enough energy to ionize and the resulting ions are in turn raised to excited electronic states. They then produce characteristic line spectra measured by a spectrophotometer and the signal monitored on a computer. Instrumental operation conditions were: plasma flow 15 L/min, auxiliary flow 1.5 L/min, power 1.3 W and pump speed 25 rpm.

2.3.4 Cytotoxicity Assays

As defined by Ekwall (1983), cytotoxicity is the adverse effects resulting from the interference of agents with structures and processes essential for cell survival, proliferation and function. It was therefore necessary to determine the cytotoxic concentration of the compounds prior to any host cell based antiviral assays once stability

and uptake had been performed. It is important that inhibitory compounds should be non cytotoxic to human cells as bioactivity would be impossible or limited if the compounds are toxic. Cytotoxicity studies were done on the primary cells (PBMCs) and the continuous cells lines (CEM.NKR-CCR5 and PM1) by the use of viability dyes XTT (sodium 3'-[1-[(phenylamino)-carbonly]-3,4-tetrazolium]- bis (4-methoxy-6-nitro) benzene-sulfonic acid), MTT (3-(4, 5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide), lactate dehydrogenase (LDH) cytotoxicity detection assays and by flow cytometry using annexin V and propidium iodide. An increase in viability in HIV infected cells compared to an untreated control for a particular compound was used as an indirect suggestion that the compound inhibits the replication of the virus. The assumption is that successful replication of virus lowers host cell numbers such that any increase in viability would be due to compound effects. It could also mean that the compound stimulates proliferation (just like PHA) without necessarily inhibiting viral replication.

2.3.4.1 Viability dye assays

The viability dye assays used were XTT (Roche Diagnostics, Mannheim, Germany) and MTT (Sigma Aldrich, Missouri, USA).

The XTT assay (Roehm *et al.,* 1991) involves a tetrazolium salt and is used in colorimetric analysis of cell viability and proliferation by normal activated T cells and cytokine dependent cell lines. XTT is cleaved by dehydrogenase enzymes of metabolically active cells to yield a highly colored formazan product, which is water-soluble.

MTT (Mosmann, 1983) is also a tetrazolium salt that has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. It is chemically reduced in cells to a colored formazan product. This conversion is presumably accomplished by NADPH and NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge *et al*., 1993). The product formed is insoluble in water and needs an additional solubilisation step. The assay therefore detects living but not dead cells and the signal developed depends on the degree of activation of the cells.

The compounds were dissolved in DMSO to 1 mg/ml stocks prior to experiments. The compound stocks were further diluted with complete medium to concentrations of 400 µM each. Into appropriate wells of a 96 well plate, 100 µl of complete medium was transferred followed by addition of 100 µl of diluted compound. The compounds were then serially diluted in growth medium at a 1:2 ratio giving concentrations of 200, 100, 50, 25, 12.5, and 6.25 µM respectively. One hundred microlitres of CEM.NKR-CCR5/PM1 cells (in the log phase of growth) maintained and cultured in complete medium at 37 ºC and in a 5% CO_2 humidified atmosphere were then added to the test compounds in the 96 well plate to a final concentration of 1 $x10^5$ cells/ml. The final concentration of compounds in the well was thus 100, 50, 25, 12.5 and 6.25 µM. Control wells included, untreated cells (cells and medium only), compound controls (for subtraction of any intrinsic absorbance of the compounds) and blanks of medium only. Following 7 days of exposure of the cells to the compounds, 50 µl of XTT (Roche Diagnostics, Mannheim, Germany) working solution containing N-methly dibenzopyrazine methyl sulfate (PMS) and XTT (1:50) was added to the test wells. The plate was incubated for 4 hours at 37 ºC and read at 450 nm (reference wavelength of 690

nm) on a Synergy spectrophotomer (ADP, South Africa). A similar procedure was followed for acutely infected cells but cells were used after overnight culture with virus.

Where MTT (Sigma Aldrich, Missouri USA) was used as the viability dye, the cells (PBMCs, CEM.NKR-CCR5 or PM1) were plated similarly as above, following 7 days of incubation with the compounds, 20 µl of MTT (5 mg/ml in PBS) was added to the test wells and the plate incubated at 37 ºC for 4 –24 hours depending on the cell type. CEM.NKR-CCR5 cells were incubated for 4 hrs while PBMCs and PM1 cells were incubated for 24 hrs with the dye due to the inadequate level of formazan generation that was observed for PBMCs and PM1 cells after 4 hrs. It has been reported that generation of formazan is affected by the type of cell line and the time of incubation (Alley *et al.*, 1988). Fifty microlitres of acidified isopropanol (1 ml of 1 M HCl: 9 ml propanol) was used to solubilise formazan crystals (formed when MTT is reduced) prior to reading the plates. Viability was determined by reading absorbances on a plate reader at a wavelength of 540 nm (reference 690 nm). Percentage viability was calculated using the formula.

$$\text{Viability (\%)} = \frac{\text{Abs Sample - Abs medium}}{\text{Abs control - [A] medium}} \times 100$$

Where Abs = absorbance. All experiments were performed at least three times in duplicate.

2.3.4.2 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) release is used as an indicator of cell membrane damage. This differs from the use of MTT and XTT which are indicative of detrimental intracellular effects on mitochondria metabolic activity. The test principle is represented in Figure 2.4. Culture supernatant was collected and the LDH activity determined using

the reaction mixture from the LDH cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany). A total of 1 x 10^5 cells/ml were seeded in 96 well plates together with gold compounds at concentrations of 6.25-100 µM. Following 24 hrs of exposure to the cells, the plates were centrifuged at 250 x *g*. Fifty microlitres of culture supernatant was carefully aspirated from the plate into corresponding wells of an optically clear 96 well flat bottom microtitre plate. To this, 50 µl of LDH reaction mixture was added and the absorbance read immediately at 490 nm (reference wavelength 690 nm). Total cellular LDH was obtained by treatment with 0.1% triton x-100 (v/v) and set as 100% cytotoxicity. Background and negative controls was obtained by LDH measurement of

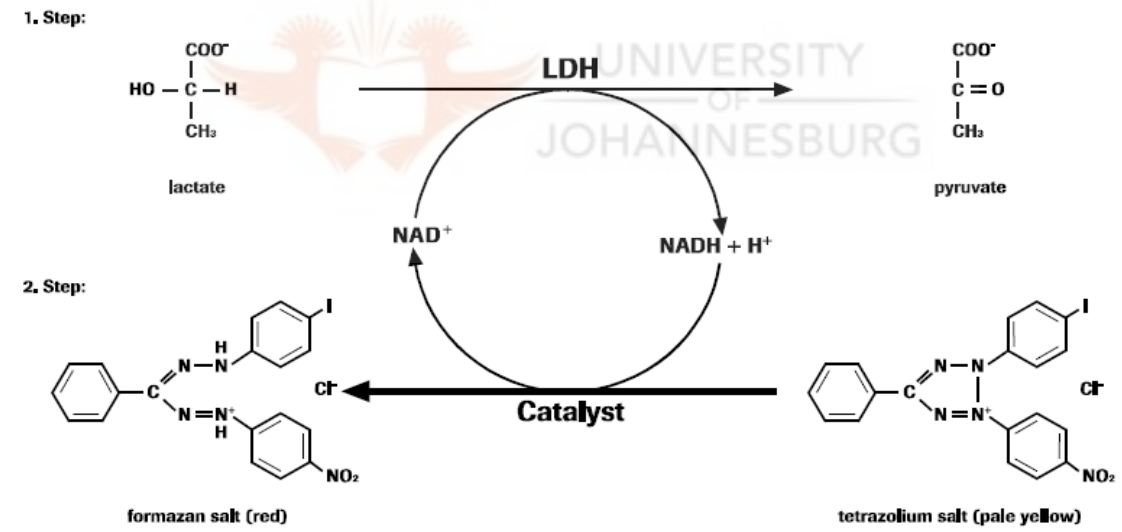


Figure 2.4: LDH cytotoxicity assay test principle.
The LDH activity is determined in an enzymatic test where in the first step; NAD+ is reduced to NADH/H+ by LDH catalysed conversion of lactate to pyruvate. In a second step, the catalyst (diapharose) transfers H/H+ from NADH/H+ to the tetrazolium salt INT which is reduced to formazan. An increase in plasma membrane damage leads to an increase in LDH activity in the culture supernatant which is directly proportional to water soluble formazan dye formed. Figure taken from the LDH cytotoxicity detection kit catalogue (No. 11 644 793 001)

assay medium and untreated cell medium respectively. Data from control and treated cells was calculated as percentage cytotoxicity using the following formula:-

$$\text{Cytotoxicity (\%)} = \frac{\text{Abs Sample} - \text{Abs medium}}{\text{Abs100\% - Abs medium}} \times 100$$

Where Abs sample, Abs medium, Abs100% denote the absorbance of the sample, medium control and triton X-100 control respectively. All experiments were done in triplicate. In the XTT, MTT and LDH assays, the data was analysed using the Gen5^{Tm} software (ADP, South Africa).

2.3.4.3 Flow cytometry

In addition to the use of tetrazolium dyes and the LDH cytotoxicity kit for viability or cytotoxicity determination, fluorescent dyes were utilized to assess viability by flow cytometry. Table 5 (appendix section B) indicates comparative characteristics of viability assays used in this project. Additional information from flow cytometry is knowledge of the mechanism of cell death which could either be apoptosis or necrosis. The annexin V and propidium iodide kit (Beckman Coulter, California, USA) was used for this purpose. Annexin V stains cell surface phosphatidylserine (PS) indicating apoptotic cells while propidium iodide indicates necrotic cells as it stains DNA in damaged cells. Cells that are negative for both annexin V and propidium iodide are viable cells. Following 7 days of incubation of the cells with compounds, supernatant was collected for further analysis such as p24 (in the case of infected cells) while the rest of the cells were washed (500 x *g*, 5 minutes) with ice cold PBS and transferred to plastic flow tubes (BD Biosciences, California, USA). The cell pellet was resuspended in 100 µl of binding buffer followed

by the addition of 1 µl of annexin V-FITC solution and 5 µl of propidium iodide working solution. After gentle mixing, the tubes were kept on ice in the dark for 15 minutes followed by the addition of 400 µl of ice cold binding buffer. Controls included a solvent control (0.5% DMSO) while annexin positive and PI positive controls were obtained by fixing cells in ice cold methanol for 5 mins followed by permeabilizing by exposure to 0.1% triton x-100 in PBS for 5 minutes. The annexin positive, the PI positive as well as unstained cell controls were used for compensation and quadrant specification. The controls and samples suspended in 500 µl of binding buffer were subjected to flow cytometric analysis with 10,000 events collected for each sample within 30 minutes of treatment. Flow cytometric profiles were determined using a FACSCalibur and the data analysed using the Cell Quest Pro software (both fromBD Biosciences, California, USA).

2.3.5 HIV-1 p24 Antigen Assay

This assay together with the viability assays gives an idea of the replicative activity of whole virus in the presence or absence of the compounds. This assay could also be used to ascertain successful *in vitro* infection of cells. The p24 ELISA assay was performed using supernatant collected 7 days after treating chronically infected PBMCs (5 x 10^6cells/ml) and acutely infected CEM.NKR-CCR5 (1 x 10^5cells/ml) cells with the compounds. A final compound concentration of 25 µM was plated with these cells in 24 well plates. The experiments were stopped on the seventh day by collecting 500 µl of cell free supernatant from each treatment. The supernatant was stored (-20 ºC) or analyzed immediately for the presence of HIV-1 p24 antigen using the HIV-1 p24 assay kit (Beckman Coulter, California, USA) while the cells were stained and analysed by flow

cytometry. Two hundred microlitres of this supernatant and standards were transferred to murine monoclonal coated 96-well plates and 20 µl of lysis buffer (triton X-100, dipotassium EDTA, tween-20, thimerosal 0.05%) added to lyse any cells present. Controls included a positive control consisting of complete medium and 50 µl antigen reagent (provided in the kit) while a negative control was complete medium only. After incubating (1 hr $\pm$ 5 minutes, 37 ºC), the wells were washed 5 times with wash buffer and 200 µl of biotinylated human anti-HIV IgG was added to the wells and another hr of incubation followed. This was followed by another wash step (5 times) following which 200 µl of SA-HRPO working solution was added to the wells followed by another 30 $\pm$ 2 minutes of incubation. In a final step, 200 µl of a substrate reagent (tetramethylbenzendine-TMB) was added. The reaction was stopped by the addition of 50 µl of Coulter stopping reagent (CSR, 4N H_2SO_4) and the absorbance measured on a plate reader (Synergy BioTek, ADP, South Africa) within 30 minutes at 450 nm. The concentration of p24 was determined based on a standard curve constructed from authentic p24. Percent viral inhibition was calculated by comparing the p24 values for the test substance (treated infected cells) with the p24 values for untreated infected cells. In parallel, the effects of various treatments on cell viability were also examined as described in section 2.3.4.

2.4 STATISTICAL ANALYSIS

Statistical analyses were performed using Microsoft Excel 2003 and Graphpad Prism (San Diego, CA USA). The following statistical calculations were performed for direct enzyme assays and cell based assays.

Mean: This refers to the average value of a data set i.e. the sum of all the data divided by the number of variables.

Standard deviation (SD): This indicates measures of deviation or spread of values from the mean of treatments in a population or multiset of values.

Coefficient of variation (CV): This is the ratio of the standard error for an estimate to the mean value of the estimate.

P values: This is a probability value ranging from zero to one. This value is used to determine the difference between sample means if the means were the same. If the p value is <0.05, then there is a significant difference between two populations but if it is > 0.05 then the differences are not significant.

CC50: This is the concentration of a compound at which 50% of cells are killed.

CHAPTER 3

RESULTS

The biochemical analysis of potential chemotherapeutics involves multiple analysis including the determination of stability in solution as well as stability as a measure of shelf life. Stability in DMSO and in a physiological buffer was determined by UV-Visible and ^{31}P NMR spectroscopy.

Reverse transcriptase and protease are key enzymes in the life cycle of HIV and are thus targets for drug development against the virus. Current HIV drug combinations (HAART) include both RT and PR inhibitors and the use of these have led to decreases in morbidity and mortality rates in HIV patients. It was therefore important that the compounds be tested against RT and PR in cell free antiviral assays. Though other mechanisms of inhibition such as viral entry, inhibition of integrase etc exist, the RT and PR screens were used to gain an indication of the mechanism by which the compounds inhibit HIV and to prioritise certain compounds for further analysis for our purposes. Further analyses were thus assays to determine the inhibition of whole virus in cell cultures in the presence of compounds and this was the p24 antigen antiviral assay.

The ultimate use of the test compounds (Table 2.1) by people necessitated the conducting of cell based assays. These included uptake studies (ICP-AES), cytotoxicity studies (MTT, XTT, LDH assays, and flow cytometry) as well as the antiviral p24 antigen assays.

This chapter covers results obtained following the *in vitro* screening of the sixteen compounds (refer to Table 2.1 for the identity of the compounds) as potential anti-HIV agents by the use of both cell free and bioassays (both infectious and non infectious) as mentioned above. Each result is the mean of at least three independent experiments.

3.1 SPECTROSCOPIC ANALYSIS OF SOLUBILITY & STABILITY

A scan of all the compounds was performed to determine the wavelength at which maximum absorbance occurred while selected compounds from each of the group of compounds provided i.e. gold (I) phosphine based complexes, DPPE based complexes and triethlyphosphine thiol ligand based complexes were tested for stability in DMSO and in a physiological buffer. Because the compounds had similar stereochemistry, the results obtained from one member could thus be related to the others.

3.1.1 UV-Vis Spectroscopy

The electronic spectra of the compounds over the UV-Visible range (200-800nm) were performed to identify the portion of the spectrum where the gold complexes absorb. This spectrum collected over time also served to indicate stability both in DMSO and in a physiological buffer (PBS). The wavelength at which a particular compound absorbs is important to eliminate misinterpretation of data for points where other biological analysis measure absorbance at the same wavelength (e.g. MTT at 540 nm). None of the gold complexes analysed absorbed in the range for which the tetrazolium dyes MTT and XTT were read (refer to section A - appendix Figures 1, 2 and 3 for spectra of the different complexes). It was also observed that the complexes were quite stable in the organic solvent (DMSO) over a 1 week period following dissolution as depicted by consistency

in their spectra (Section A-appendix Figures 1, 2 and 3). The gold (I) phosphine complexes' chromophores all peaked in the range of 256 and 259 nm except for EK231 which peaked at 304 nm.

The stability of 3 of the gold complexes (TTC3, EK207 and KFK154b) in a physiological buffer (PBS, pH = 7.4) was monitored on dilution and 40 mins after being in solution at room temperature. It was observed that the complexes were hydrolysed over time (0 to 40 mins) as seen in Table 3.1 and Figure 4A (appendix Section A) observed by a hypochromic shift (decrease in absorbance intensity). Over a 24 hr period, complete hydrolysis was observed for TTC3 and EK207 (Table 3.1, Figure 4 B – appendix section A for actual graph) as the peak flattens out completely seen by a disappearance of the initial peak.

Table 3.1: Stability of gold compounds in a physiological media (PBS, pH = 7.4).
Stock solutions of three gold compounds (in DMSO) were further diluted in PBS and a kinetic spectrum obtained 40 mins after and every 2 hrs until 24 hrs for TTC3 and EK207. Changes in initial absorbances were seen with the type of shift being a hypochromic shift for all 3 gold compounds. ND = not determined.

Gold complex	Initial absorbance intensity	40 mins later	24 hrs later	Type of shift
TTC3	0.1	0.05	0.02	Hypochromic
EK207	0.45	0.38	0.2	
KFK154b	0.56	0.5	ND	

3.1.2 ^{31}P NMR Studies for Assessment of Stability

All gold complexes were synthesized by chemists who used NMR to confirm the structure of the synthesized compounds. For bioactivity analysis, the gold compounds were dissolved in DMSO and stability in this solvent needed to be determined. The stability of the compounds was determined by ^{31}P NMR.

When using NMR for stability analysis one expects spectra taken at different time points to exhibit peaks at the same position in the spectra irrespective of when the spectra was collected. If peaks shift from say x ppm to y ppm, this is interpreted as possible decomposition of the compound (ligand or complex).

The spectrum obtained on immediate dissolution was compared to that taken 24 hrs, 1 week and 4 months later (Table 3.2). The gold (I) phosphine complexes analysed (TTC3, TTC10) were stable over a 1 week to 4 months period in solution with no decomposition observed for either the ligands or the gold complexes. The observed chemical shift from 34.4 to 39.1 ppm after 24 hrs is not relevant as there is a corresponding shift in the peak down stream from 25.5 to 30.2 ppm (Table 3.2, please refer to appendix section A, Figure 5 for actual graph). The change observed was therefore just a shift in the scale and not decomposition of the gold complex. EK207 initially peaks at 87 ppm and presented remarkable stability after 24 hrs unlike EK231 (both DPPE gold complexes) which initially peaks at 82.9 and later results in other peaks observed at 86.9 and 77 ppm by 24 hrs (Table 3.2, appendix section A Figures 6 and 7). EK231 thus starts undergoing breakdown by 24 hrs and continues to 1 week. By 1 week EK207 was also noted to be unstable with smaller peaks at 1, 23 and 31 and 89.6 ppm (Table 3.2, appendix section A, Figure 6). In Table 3.2, ND refers to not done and this was applicable to complexes that were shown to be already unstable by 1 week in solution i.e. EK207 and EK231.

Table 3.2: ^{31}P NMR peak shifts of selected gold complexes.
The gold complexes were dissolved in deuterated DMSO and a spectrum taken immediately, 24 hrs, 1 week and 4 months later. The shifts in NMR spectra are represented in ppm.

		^{31}P NMR (ppm)			
Type of compound	**Code**	**30 Mins**	**24 hrs**	**1 week**	**4 months**
Gold (I) phosphine compounds	TTC3	33	32.9	32.9	33
	TTC10	34.4	39.2	33.9	33.9
Bis (diphenylphosphino) ethane (DPPE) gold complexes	EK207	87	87	87 89.6	ND
	EK231	82.9	82.9 86.9 77.5	82.9 86.9 77.5	ND
Triethylphosphine thiol ligand based gold complexes	MCZS1	-47.9	-47.755	-47.3	ND

Actual graphs are provided in the appendix (Figures 1-4) for complexes with changes in their spectra

No major changes were observed in the ^{1}H NMR spectrum of all the complexes except for that of MCZS1. The ^{31}P NMR spectrum is stable (Table 3.2) but on looking at its ^{1}H NMR spectrum, there is a triplet (between 5.5.and 5.6 ppm) and a duplet peak (5.5 and 5.9 ppm) which started appearing after 24 hrs and are even more visible by 1 week. New peaks are also shown to emerge between 4.7 and 4.8 ppm for this same complex (actual graph in section A of appendix, Figure 8).

3.2 DIRECT ENZYME ASSAYS

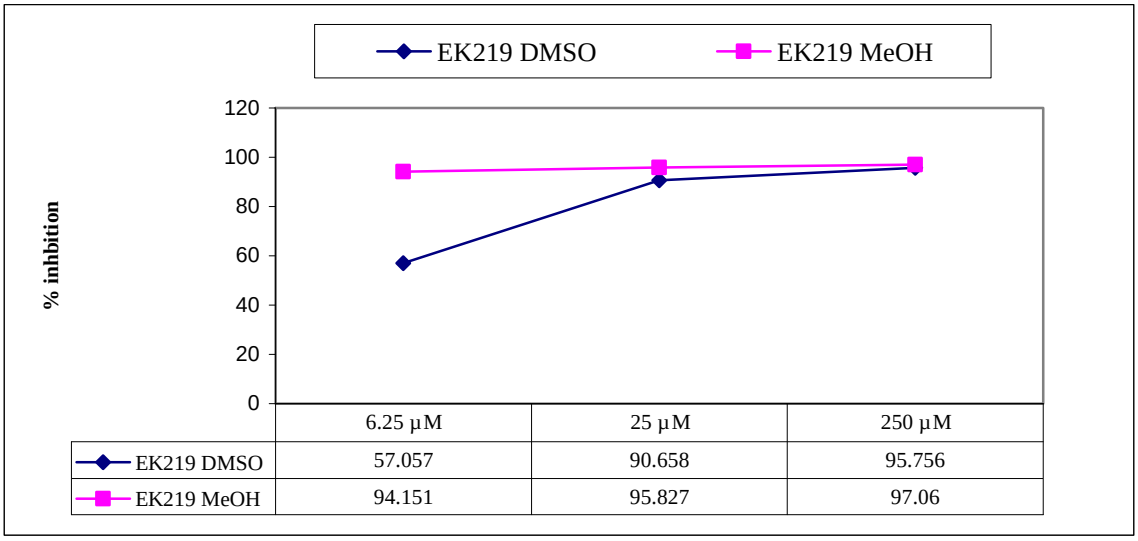
3.2.1 Reverse Transcriptase Assay

DMSO is always the solvent of choice when screening compounds (natural or synthetic) as potential drugs. It is preferred for drugs screening because most products completely dissolve in it. Because compounds were dissolved in DMSO for analysis, we had to

confirm that the concentration of DMSO used did not significantly inhibit RT. Methanol is a solvent that could have been used in dissolving the compounds as well as such, the effect of these two solvents on RT was compared. The effect of solvent on RT was evaluated by determining changes in inhibition observed when sample EK219 in the presence of the same concentrations of DMSO (1.5%) and methanol were used compared to one with neither solvent. There was generally an increase in the inhibition of RT in the methanol fraction compared to the DMSO fraction (Figure 3.1). Ghee and his coworkers (1991) observed that DMSO at 10% demonstrated the least inhibition (30%) on RT compared to methanol and ethanol. DMSO was thus the solvent of choice in dissolving the compounds for RT assays and all other bioassays and the concentration of DMSO was kept below 1.5% in RT assays and less than 0.5% in bioassays.

The use of DMSO for dissolution had a shortcoming in that the compounds could not be left in DMSO for too long (> 1 month) as there was a possibility of the complex associating with DMSO. Here we demonstrate that gold complexes stored in DMSO could not inhibit RT (Figure 3.2) while freshly made up gold complexes inhibited the enzyme (Figure 3.3).

Three concentrations of freshly made up compounds (6.25, 25 and 250 µM) were tested in the cell free reverse transcriptase assay using a colorimetric kit from Roche (Figure 3.3). The percentage inhibition was calculated relative to an untreated control using the formula 100-(Abs Sample/Abs control)*100. A positive inhibitor of RT designated KI (known inhibitor) and negative controls of lysis buffer and reaction mixture only were



	6.25 µM	25 µM	250 µM
EK219 DMSO	57.057	90.658	95.756
EK219 MeOH	94.151	95.827	97.06

Figure 3.1: The effect of solvent on RT activity.
The effect of solvent on RT was determined by comparing its effect on an untreated sample with no solvent to one of sample EK219 containing the same amount of DMSO and methanol (1.5%). The methanol fraction generally inhibited RT more compared to the DMSO fraction.

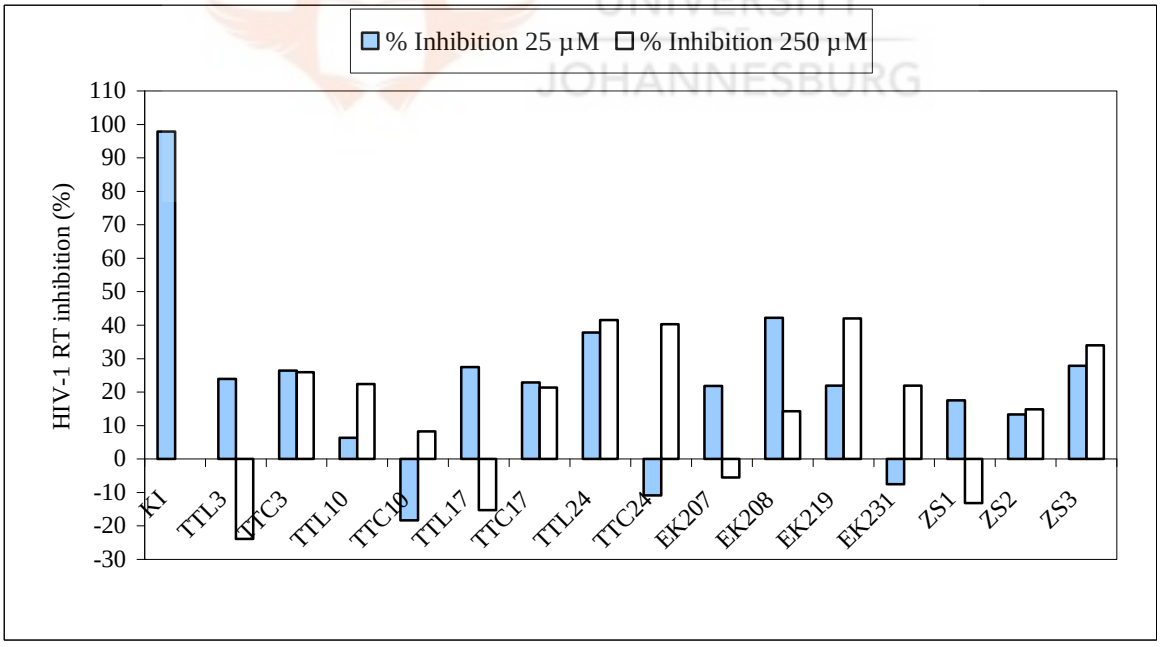
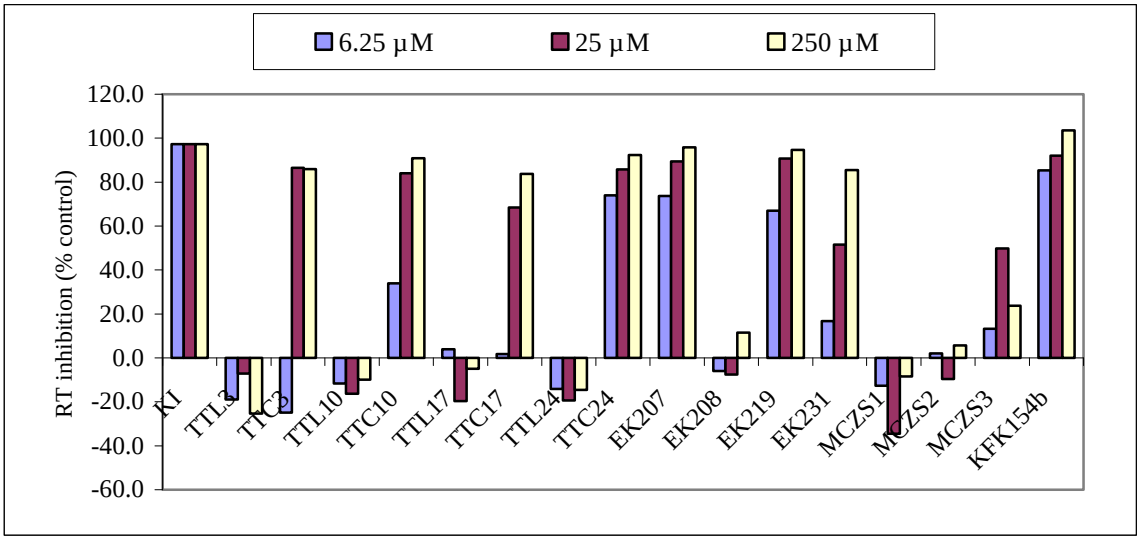


Figure 3.2: The effect of gold compounds on RT.
A stock concentration of each compound (20 mg/ml) was diluted and tested for RT after > 1 month in solution stored at 4 ºC. HIV-1 RT inhibition of gold compounds at concentrations of 25 µM and 250 µM are represented. The percentage inhibition is calculated relative to an untreated control containing enzyme only. A positive control (KI) tested demonstrated 98% inhibition while none of the compounds was capable of significant inhibition ($p>0.05$).



	% Inhibition			p- values		
	6.25 µM	25 µM	250 µM	6.25 µM	25 µM	250 µM
KI	97.33	97.33	97.33	**<0.05**	**<0.05**	**<0.05**
TTL3	-18.98	-7.16	-25.31	**0.026**	0.21	0.305
TTC3	-24.96	86.56	85.97	0.321	**0.005**	**0.004**
TTL10	-11.64	-16.28	-9.92	0.093	0.214	0.352
TTC10	33.98	83.97	90.92	0.281	**0.007**	**0.0003**
TTL17	3.86	-19.70	-4.97	0.182	0.113	0.269
TTC17	1.81	68.45	83.79	0.466	**0.012**	**0.002**
TTL24	-14.13	-19.34	-14.61	**0.014**	0.175	0.117
TTC24	74.03	85.70	92.33	**0.001**	**0.005**	**0.0003**
EK207	73.65	89.38	95.87	**0.011**	**0.004**	**0.0003**
EK208	-5.92	-7.58	11.56	0.317	0.296	0.124
EK219	66.99	90.79	94.67	**0.013**	**0.004**	**0.0004**
EK231	16.79	51.51	85.52	0.237	**0.046**	**0.0001**
MCZS1	-12.68	-34.59	-8.41	0.159	0.067	0.257
MCZS2	2.05	-9.57	5.65	0.387	0.277	0.365
MCZS3	13.20	49.87	23.70	0.136	**0.012**	0.22
KFK154b	85.28	92.07	103.52	0.026	**0.002**	**0.001**

Figure 3.3: The effect of compounds on RT at concentrations of 6.25, 25 and 100 µM.

The compounds were treated with a reaction mixture of biotin and digoxiginin labeled nucleotides in the presence of RT. Synthesis of DNA in the presence of the compound prevents or allows the formation of a coloured product representing RT inhibition or lack of it respectively. Most gold complexes significantly ($p<0.05$) inhibit RT except for EK208, MCZS1, MCZS2 and MCZS3 and the ligands (TTL3, TTL10, TTL17, and TTL24). KI stands for a known inhibitor of RT. Control samples contained an equivalent amount of DMSO (1.5%) as found in the test samples. This percentage of DMSO in the sample is not significantly inhibitory.

included. The positive control is a plant product that has been shown to inhibit RT *in vitro* and according to anecdotal evidence has led to improved health in HIV infected patients (Basson, 2005). A dose dependent inhibition of RT by gold complexes TTC3, TTC10, TTC17, TTC24, EK207, EK219, EK231, KFK154b and not the ligands and gold complexes EK208, MCZS1, MCZS2 and MCZS3 (Figure 3.3) was observed. This is understandable as a possible mechanism of action of gold complexes inhibiting RT is through ligand exchange reactions with sulfhydryl groups on the enzyme (Allaudeen *et al*., 1985), a reaction not possible between ligand and RT. Furthermore, it has been shown that it is the gold in the gold complex that causes activity and not the ligand (Chircorian and Barrios, 2004).

For the RT inhibition analysis, it was therefore necessary to dissolve the compounds just prior to experiments and this was maintained for all other bioassays to avoid possible decomposition of the gold complexes.

3.2.2 HIV-1 Protease Assay

HIV-1 protease is the enzyme responsible for maturation of viral particles into infectious virions. This enzyme has therefore been a major target for anti-HIV drugs and presented here are results obtained following the screening of the gold complexes for anti-protease activity. Two different methods were employed namely a reverse phase high performance liquid chromatograph (RP-HPLC) method and a fluorogenic method.

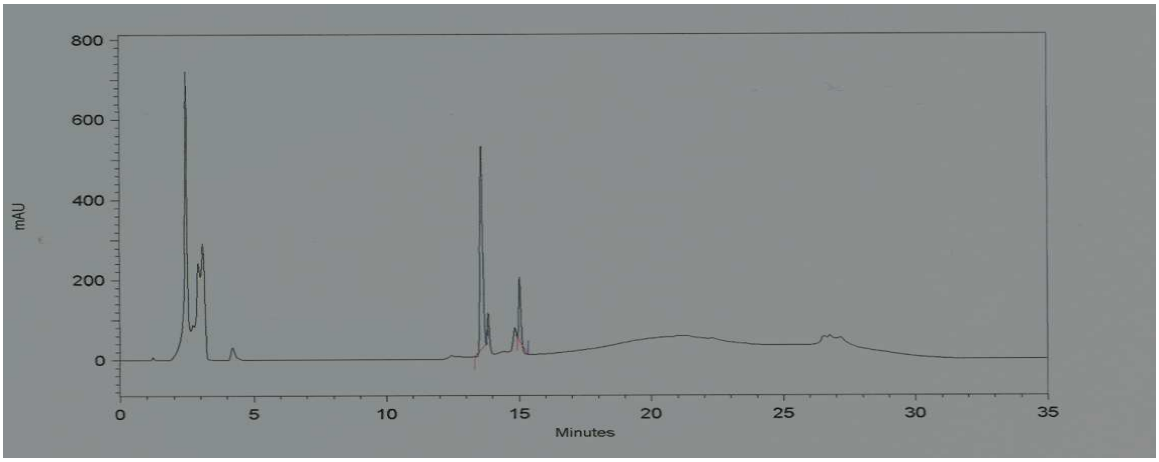
3.2.2.1 RP- HPLC

The S-1000 kit (Bachem Bioscience Inc. King of Prussia, PA, UK) was used for anti-HIV-1 protease inhibition screening. Employing this kit and HPLC as detection method

for our complexes is relatively new and required troubleshooting. Modifications to the protocol included increasing the incubation and HPLC run times and also increasing the TFA concentration for stopping the reaction. Figure 3.4 is a representation of how the RP-HPLC profile of control samples appears on a chromatogram. In Figure 3.4A the reaction of the enzyme and substrate results in the elution of a product peak with retention time of 14.2 mins while that of the substrate peak is at 15.07 mins. The earlier peaks at 2.5 mins on both chromatograms are solvent peaks (water, acetronitrile) while the peak at 4 mins in Figure 3.4B is the DMSO peak. In Figure 3.4 B, the product peak at 14.2 mins has a diminished area as this sample was treated with a known inhibitor of HIV-1 protease (acetyl pepstatin). A reduction in the peak area relative to an untreated control is representative of inhibition.

The effect of DMSO on HIV-1 protease was investigated first because of reports that this chemical could possibly affect the dimeric structure of the functional protease (Wan *et al*., 1996). Wan and colleagues observed a 30% loss in activity when the PR enzyme was analysed in the presence of 4.5% DMSO. We also observed a reduction in HIV-1 protease activity when DMSO at 8.3% (percentage calculated using the protocol provided in the kit) was added to a control sample of enzyme and substrate only (Figure 3.5). There was a 29% decrease in HIV-1 protease activity at this concentration of DMSO (Figure 3.5). All the compounds inhibited HIV-1 PR when the peaks were integrated in relation to control sample peaks (Figure 3.6). TTL3 and ZS2 inhibited HIV-1 protease the most (89 and 97% inhibition respectively). But because the stop reagent (in this assay) contained only 0.8% trifluoroacetic acid (TFA) (as recommended by the kit), the possibility that the reaction continued was assumed to be a possible cause for the high

A



B

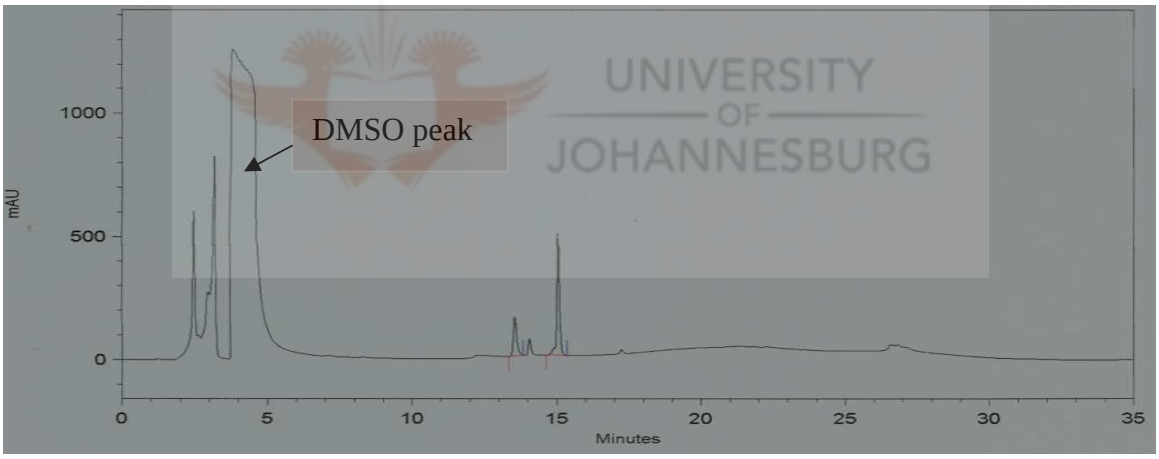


Figure 3.4: HPLC profile of the interaction of the HIV-1 PR enzyme with its substrate.

Figure 3.4A represents a profile of the enzyme and substrate only while B indicates the enzyme, substrate and a known inhibitor of HIV-1 PR (acetyl pepstatin). Both samples were incubated for 1 hr at 37 ºC and the RP-HPLC profiles obtained. The substrate and its hydrolysate were detected at 220 nm and their retention times were 15.07 and 14.2 mins respectively. The peak at 14.2 mins is the product hydrolysate (NO_2)-Phe-Glu-Ala-Glu-Ala-Met-Ser-NH_2) peak. A reduction in the product peak area in B is indicative of inhibition of HIV-1 protease. The peaks at 5 mins and below are solvent peaks (e.g. DMSO).

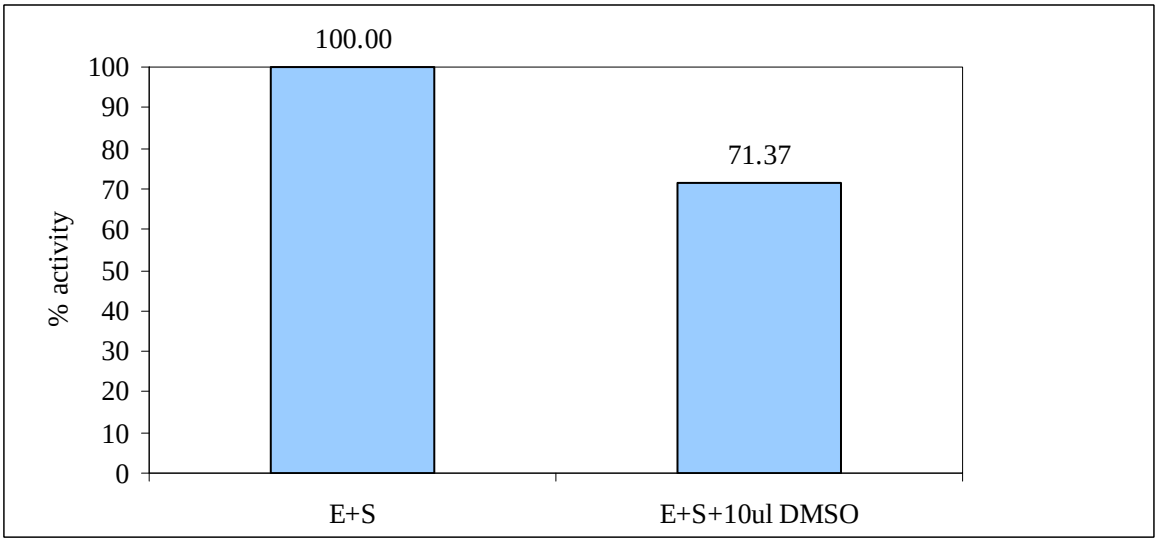


Figure 3.5: The effect of DMSO on HIV-1 PR.
There was a 29 % decrease in the proteolytic activity of the enzyme in the presence of 8.3% DMSO. This should therefore be considered when calculating the percentage inhibition of the test compounds (dissolved in DMSO).

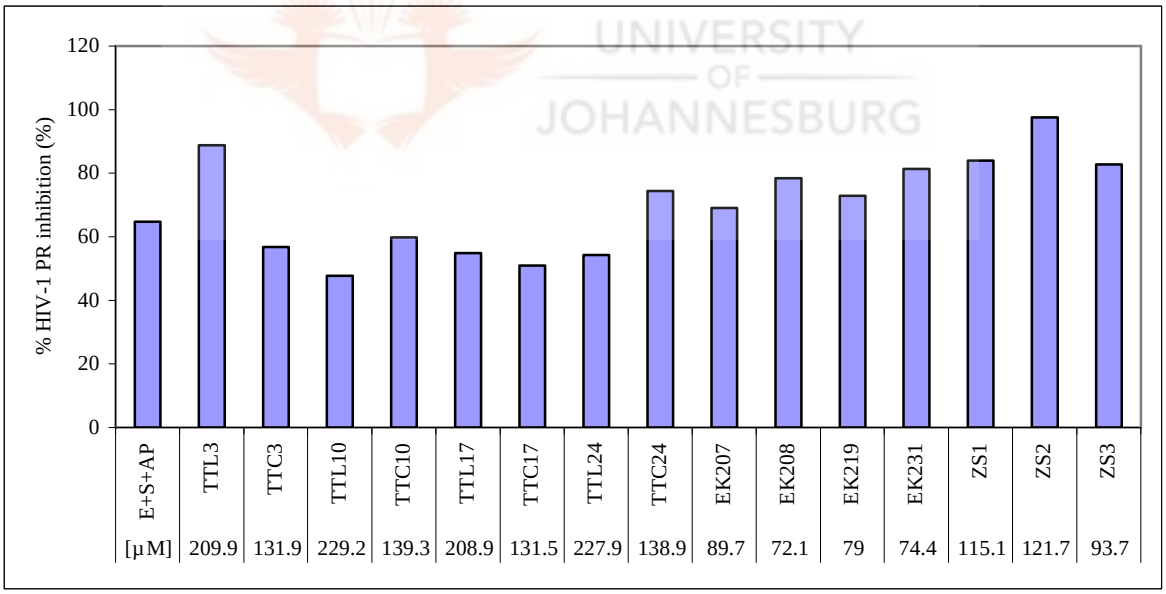


Figure 3.6: The effect of gold complexes on HIV-1 PR.
Percentage inhibition was calculated using the formula (Control A - Sample A/Control A x100 where A=peak Area). The compounds were screened at 83 µg/ml (micromolar concentrations [µM] are shown on graph). All the compounds screened showed inhibition of 50% and above with TTL3 and ZS2 having values close to 100% (89 and 97% respectively). Percent inhibitions are relative to untreated control with equal amount of solvent used in test samples. E = enzyme, S = substrate and AP = acetyl pepstatin (known inhibitor).

percentage inhibition observed. Pazhanisamy *et al.,* (1996) recommends that a final TFA concentration of 5 % be used to stop this type of reaction. According to the Kromasil Analytical products catalogue (www.kromasil.com); metal impurities in analytes are capable of interacting with silica columns resulting in poor resolution of peaks. Similar observations have been made by some members in the AuTEK group suggesting that gold compounds can interact with silica columns such as the C_{18} column used in this analysis. These interactions could result in interference with the HPLC profiles leading to false positive results. Only manufacturer related publications of using the HPLC-PR inhibition protocol exist with conditions being 15 mins incubation, 0.8% TFA stop reagent. The above fact coupled with the protocol troubleshooting issues (incubation time, HPLC run times), golds' possible interference with C_{18} columns and the inadequate stop reaction meant we had to find another way of confirming the perceived inhibition of PR by all our compounds (including ligands).

3.2.2.2 The HIV-1 protease fluorogenic substrate assay

As mentioned before, it was necessary to confirm the results obtained from the RP-HPLC assay because of the shortcomings that were observed. A fluorogenic HIV protease substrate I (Sigma Aldrich, Missouri USA) and a recombinant HIV-1 protease enzyme from Bubendorf (Switzerland) were employed for this purpose. Interaction of the enzyme and substrate produces a product that fluoresces and inhibition of this reaction results in a decrease of the relative fluorescence obtained as a result of quenching. This assay has more than just manufacturer based publications to confirm its reliability. Four of the twelve gold complexes (EK208, MCZS1, MCZS3 and KFK154b) significantly inhibited HIV-1 PR with inhibitions of > 50% at 100 µM (Table 3.3). The concentration of the

gold complexes tested in this and the RP-HPLC assay were comparable as seen in Figure 3.6. In this case as just reported, only four of the complexes(Table 3.3) inhibited PR and not all as seen with the RP-HPLC assay (Figure 3.6) indicating that the shortcomings mentioned in the RP-HPLC assay did actually influence its reliability.

Table 3.3: The effect of gold complexes on HIV-1 protease.
The gold complexes, fluorogenic substrate and HIV-1 PR enzyme were incubated at 37 ºC for 1 hr. The relative fluorescence unit (RFU) was obtained using a microplate reader at an excitation wavelength of 355 nm and emission of 460 nm. The mean obtained is from duplicate treatments of 5 repeats. [] = concentration. Complexes EK208, MCZS1, MCZS3 and KFK154b (100 µM only) significantly inhibit HIV-1 PR with > 50 % inhibition. Other significant p values are highlighted e.g. for TTC17 and TTC24 etc. KI is a known protease inhibitor in this case acetyl pepstatin.

Gold complexes	**[ ]**	**RFU**	**SD**	**% Inhibition**	**p values**
Untreated control		12.1	2.7	0	
KI	10 µg/ml	2.7	1.6	**77.6**	**0.01**
TTC3	25 µM	10.4	2.7	14.1	0.28
	100 µM	10.5	2.6	13.7	0.13
TTC10	25 µM	11.4	2.3	6.3	0.52
	100 µM	9.7	2.1	20.1	0.01
TTC17	25 µM	7.6	1.4	**37.1**	**0.01**
	100 µM	6.3	1.1	**47.3**	**0.01**
TTC24	25 µM	9	2.2	**25.7**	**0.01**
	100 µM	6.7	1.9	**45.0**	**0.01**
EK207	25 µM	11.9	2.8	1.6	0.45
	100 µM	11.8	2.1	3.3	0.82
EK208	25 µM	8	2.3	**34.1**	**0.09**
	100 µM	4.7	1.3	**60.8**	**0.004**
EK219	25 µM	11.7	2.3	3.6	0.45
	100 µM	10.4	3.1	13.6	0.41
EK231	25 µM	13.5	2.7	-12.4	0.10
	100 µM	12.2	2.9	-1.2	0.87
MCZS1	25 µM	9.1	2.7	**25.3**	**0.01**
	100 µM	4.6	2.7	**61.8**	**0.01**
MCZS2	25 µM	10.8	3.1	11.1	0.13
	100 µM	10.9	2.8	10.4	0.19
MCZS3	25 µM	7.6	3.4	**37.1**	**0.01**
	100 µM	3.4	0.8	**71.5**	**0.001**
KFK154b	25 µM	10.3	4.4	14.7	0.23
	100 µM	4.5	2.1	**62.7**	**0.001**

3.3 CELL ASSOCIATED ANALYSIS OR BIOASSAYS

3.3.1 Uptake Studies by ICP-AES

Uptake of complexes by host cells was confirmed using ICP-AES. Table 3.4 shows complex uptake by PBMCs and CEM.NKR-CCR5 cells represented by the amount of gold (ppm) as detected by the plasma in the ICP-AES instrument. The cells were treated with the gold complexes at starting concentrations of 12.5, 25 and 50 µM. The gold concentration taken up is represented in ppm and µM (Table 3.4). It should be noted that the conversions to µM show gold to be below the starting compound concentration as expected. This is because only gold uptake was analysed as representative of gold complex uptake. An untreated control sample of cells only had -0.04 and -0.01 ppm or no gold at all following analysis. At 12.5 µM, the supernatant from EK208 and EK219 treated PBMCs recorded, gold uptake of -0.55 and -0.330ppm. Because the instrument has a 0.05 ppm threshold limit for gold detection, this observation is not due to the fact that the gold complexes were not taken up but because the concentration of gold was below the threshold limit for detection by the instrument. At 25 and 50 µM, there was a decrease in uptake of the gold complexes in the CEM.NKR-CCR5 cell line compared to the PBMCs. This could be attributed to the fact that the gold complexes were found to be toxic to the CEM.NKR-CCR5 cell line and not to the PBMCs as will be shown later in the cytotoxicity studies (section 3.3.3). Overall, the treatment of the cells with gold complexes TTC24, EK231, and ZS3 resulted in the highest uptake of gold in both cell types with EK231 being the highest (Table 3.4). In some cases (EK231 in PBMCs), there was a decrease in gold uptake at 50 µM compared to the uptake at 25 µM of gold complex. A possible reason could be because there was increased cytotoxicity (or a

decrease in proliferation) at the higher concentration of 50 µM hence less cells were available and thus the concentration of complex take-up decreased.

Table 3.4: The uptake of gold complexes by PBMCs and CEM.NKR-CCR5 cells. The cells were treated with gold complexes only at 3 concentrations (12.5, 25 and 50 µM) and incubated for 7 days at 37 ºC under 5% CO_2. The excess gold complexes were washed off and the cells lysed with 10 % nitric acid. The supernatant was analysed for the presence of gold (an indication of the complex being taken up) using ICP-AES.

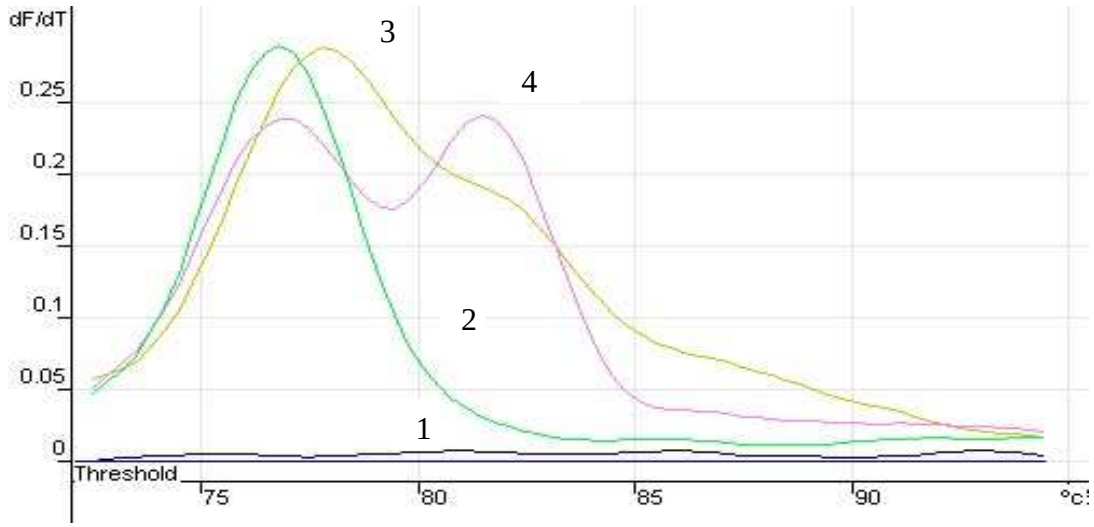
Gold Complexes	**PBMCs**						**CEM.NKR-CCR5**			
	12.5 µM		**25 µM**		**50 µM**		**25 µM**		**50 µM**	
Au uptake	**ppm**	**µM**	**ppm**	**µM**	**ppm**	**µM**	**ppm**	**µM**	**ppm**	**µM**
Cells	**-0.04**						**-0.01**		**-0.01**	
TTC3	0.15	0.24	0.26	0.42	0.24	0.38	0.12	0.19	0.23	0.37
TTC10	0.42	0.71	0.30	0.51	0.33	0.56	0.19	0.32	0.33	0.56
TTC17	0.49	0.78	0.89	1.42	0.66	1.05	0.33	0.53	0.63	1.00
TTC24	1.13	1.90	4.74	7.97	1.78	2.99	0.36	0.61	0.97	1.63
EK207	0.33	0.36	0.08	0.09	0.73	0.79	0.01	0.01	0.03	0.03
EK208	-0.55	-0.48	0.26	0.23	0.68	0.59	0.07	0.06	0.13	0.11
EK219	-0.30	-0.29	0.31	0.30	0.67	0.64	0.03	0.03	0.20	0.19
EK231	**2.95**	**2.66**	**5.45**	**4.91**	**3.28**	**2.96**	**0.85**	**0.77**	**2.62**	**2.36**
ZS1/MCZS1	0.15	0.21	0.23	0.32	0.39	0.54	0.08	0.11	0.08	0.11
ZS2/MCZS1	0.06	0.09	0.15	0.22	0.20	0.29	0.02	0.03	-0.01	-0.01
ZS3/MCZS1	2.13	2.42	1.42	1.61	1.42	1.61	0.35	0.40	0.47	0.53
KFK154b							0.07	0.11	0.08	0.13

3.3.2 Infection Confirmation

The use of cell lines as *in vitro* models during drug development studies has several requirements. Firstly, uptake of the compounds by cells had to be confirmed as reported

in 3.3.1. Once uptake had been confirmed, cytotoxicity studies were performed on both cell lines and primary cells (achieved by MTT, XTT, LDH and flow cytometry). Lastly, the effect of non toxic compounds on viral replication (looking at p24 productivity) was exploited. The use of cells lines as opposed to primary cells (which could be obtained already infected) had the added requirement for the cells to be infected *in vitro*. Once "infected", *in vitro* confirmation of infection was necessary. This was done using RT-PCR and agarose gel electrophoresis. HIV-1 infected DNA peaked at a melting temperature (Tm) of 81.5 ºC as shown in Figure 3.7 (4) while the non template control (NTC) had a maximum peak at 76.8 ºC (2) corresponding to fluorescent signals from primer dimers. DNA from an uninfected control sample (3) also peaked at 77.8 ºC representing primer dimers while a water control sample (1) had no major peak as expected.

The RT-PCR product from the infected cells was run on a 2 % agarose gel alongside molecular weight markers (Figure 3.8, lane 1) to further confirm the presence of the 142 bp *gag* gene of HIV-1. If there had been successful infection, this gene should have been amplified by the specific primers used in the RT-PCR experiment. Lanes 2-5 show bands representative of this gene (Figure 3.8). In lane 6, an uninfected negative control sample is shown with primer dimers seen at around 80 bp.



1	water only	75.3	80.8	86	93
2	NTC	76.8	85.5		
3	uninfected CEM.NKR-CCR5	77.8			
4	infected CEM.NKR-CCR5	77	81.5		

Figure 3.7: RT-PCR melt peaks of DNA isolated from acutely infected CEM.NKR-CCR5.

Cells were treated with HIV-1 subtype C viral isolate Du 151 at a concentration of 500 $TCID_{50}$. Infection was enhanced using polybrene (10 µg/ml) and the mixture centrifuged for 3 hrs, in the process known as spinoculation. Following overnight culture (37 ºC), excess virus was washed off. DNA was isolated 7 days post infection and used for infection confirmation by RT-PCR. Melting curves were converted to melting peaks by plotting the derivative of fluorescence with respect to temperature (dF/dT) against temperature. 1 = water, 2 = non template control (NTC), 3 = uninfected control, 4 = DNA from HIV-1 infected cells

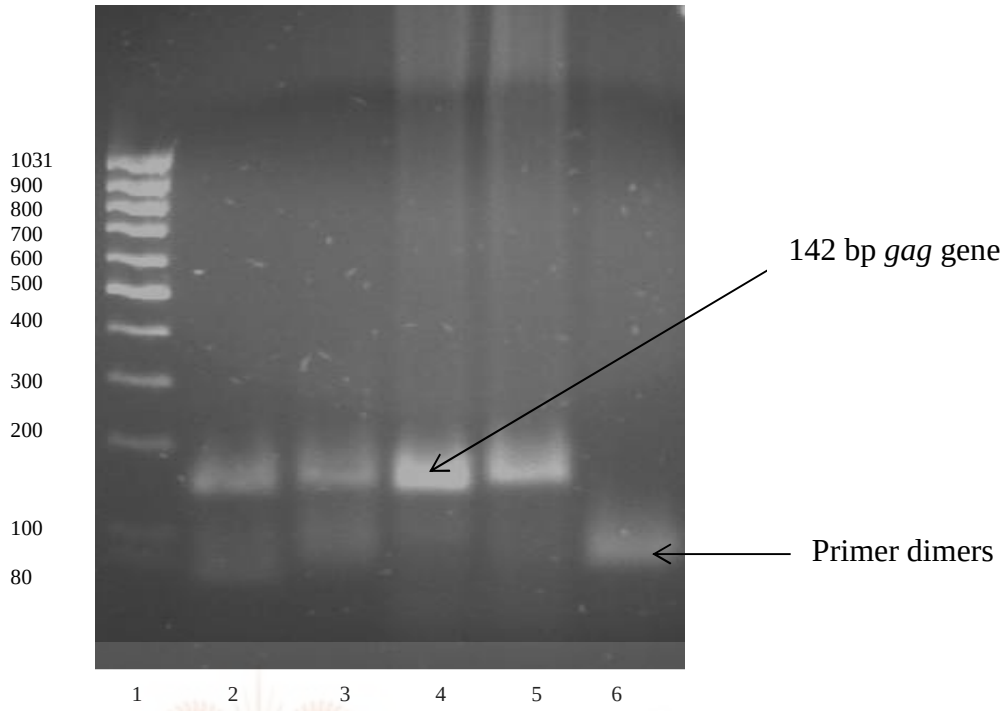


Figure 3.8: Infection confirmation of CEM.NKR-CCR5 cells
A 2 % agarose gel showing from lanes 1 molecular weight markers in base pairs (bp), lanes 2, 3, 4 and 5 amplicons from infected CEM.NKR-CCR5 cells. Lane 2 and 3 were loaded with less DNA (2 µg/µl) starting material compared to lane 4 and 5 (5 µg/µl). In lanes 2-5, a 142 bp band is visible representing the *gag* gene. Lane 6 is an amplicon from DNA isolated from uninfected cells with no visible band at 142 bp while a lower band seen around 80 bp represents primer dimers.

3.3.3 *In vitro* Cytotoxicity Assays

Once uptake had been established, *in vitro* cytotoxicity assays were performed to determine non cytotoxic doses of the compounds to both the primary cells and the cells lines. XTT and MTT tetrazolium dyes were used to determine *in vitro* proliferation and viability over a 7 day period while the LDH assay was used for cytotoxicity determination over 24 hrs as it assesses plasma membrane integrity over short incubation

times. The reason for the short incubation times being that the LDH enzyme once released into the supernatant, is not very stable after 48 hrs.

Finally flow cytometry was used not only to check for viability but also the mode of death where the compounds were cytotoxic. Flow cytometry has the advantage of being more sensitive and was therefore employed as a confirmatory assay.

3.3.3.1 Viability/proliferation assessment using the tetrazolium salts MTT and XTT

Viability of cells in the presence of compounds is indicative of non toxicity of the compounds to the cells; an ideal situation for potential drug candidates. Different concentrations of the compounds (1-200 µM) were tested for toxicity and the concentrations that were non cytotoxic and clinically relevant (1-50 µM – Traber *et al*., 1999, Yoshida *et al.,* 1998) were chosen for antiviral tests. The range of concentrations tested was also related to that in patients on chrysotherapy e.g. gold sodium thiomalate (a gold (I) complex) reaches blood concentrations of 5-25 µM while auranofin reaches 0.1-0.5 µM (Georgiou, 2002).

The advantage of XTT over the MTT dye is that it is less time consuming as no solubilisation step is involved. However, these dyes gave similar patterns when the formazan crystals formed by MTT were appropriately solubilised.

The viability of the CEM.NKR-CCR5 cell line following 7 days of treatment with the compounds at concentrations of 1-200 µM is represented in Figure 3.9 A. The gold complexes (except EK231 and KFK154b) demonstrated an overall cytotoxic effect (CC_{50s} being TTC3=4.4µM, TTC10=2.3 µM, TTC17=2.4 µM, TTC24=3.9 µM, EK207 <1 µM, EK208<1 µM, EK219=9 µM, ZS1=13 µM, ZS2=14 µM, ZS3=3.3 µM) on these

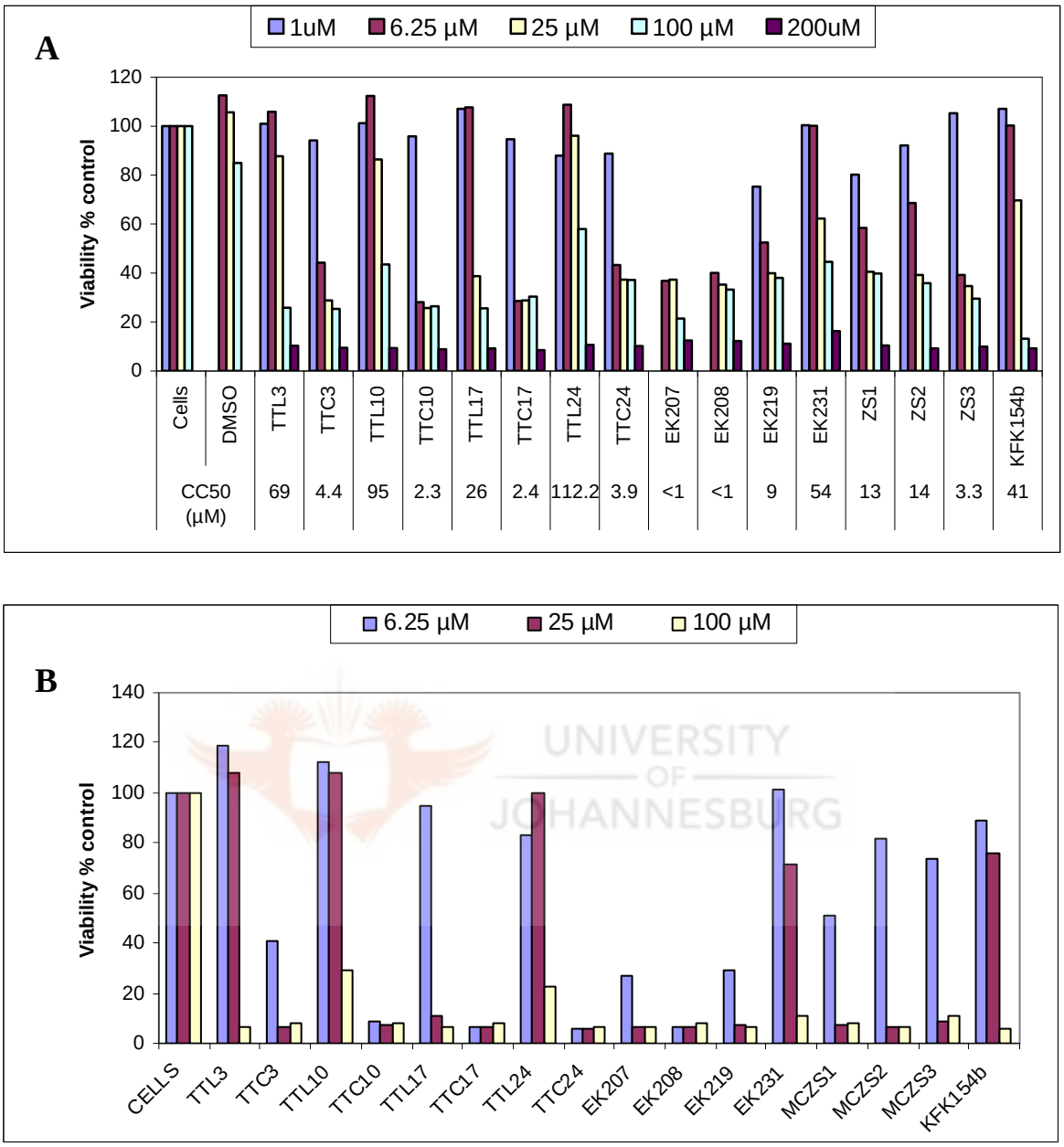


Figure 3.9: The effect of compounds on the viability of uninfected (A) and infected (B) CEM.NKR-CCR5 cells.

Following 7 days of incubation of the cells with the compounds at various concentrations (1-200 µM), viability was determined using XTT. The ligands (TTL3, TTL10, TTL17, TTL24) and gold complexes EK231, KFK154b were the least cytotoxic as seen from their high CC_{50} values (A) compared to the other gold complexes. Similarly in B, ligands and gold complexes EK231 and KFK154b were the least toxic with a 6 µM concentration being the least toxic concentration for most of the compounds. CC_{50} is defined as the concentration at which the cells were 50% viable. The DMSO concentration used was < 0.5%.

cells compared to the ligands (TTL3=69 µM, TTL10=95 µM, TTL17=26 µM and TTL24=112.2 µM). Gold complexes EK231 and KFK154b were the least toxic with CC_{50s} of 54 µM and 41 µM respectively. EK207 and EK208 were the most cytotoxic of the gold complexes with CC_{50s} of less than 1 µM while TTL17 was the most cytotoxic of the ligands (CC_{50}=26 µM) and TTL24 (CC_{50} =112.2 µM) the least cytotoxic.

In the infected CEM.NKR-CCR5 cell population (Figure 3.9B), a similar pattern was observed with gold complexes EK231, KFK154b and the ligands being less toxic. However, there was an overall decrease in viability in the infected cells compared to the uninfected at the concentrations tested. The cytopathic effect of virus might contribute to this decreased viability observed in the infected cells. The CC_{50}s were not determined as only three concentrations were screened due to limitations in the volume of blood that could be collected from one patient for these assays. According to the Graphpad Prism Software that was used for determining CC_{50}, at least 5 concentrations have to be tested to accurately determine the CC_{50}.

The need to screen another cell line for cytotoxicity prior to antiviral p24 studies was necessary following the observations made on the CEM.NKR-CCR5 cell line where the compounds were found to be toxic. The compounds were screened for toxicity using the PM1 cell line (a cell line that supports infection and replication of HIV-1) and MTT. A similar finding was observed for this cell line (Figure 3.10) when compared to the CEM.NKR-CCR5 cell line (Figure 3.9) though different dyes were used (the comparison between dyes is dependent on the fact that both are tetrazolium dyes that function on the same principle i.e. determine viability depending on metabolic activity).

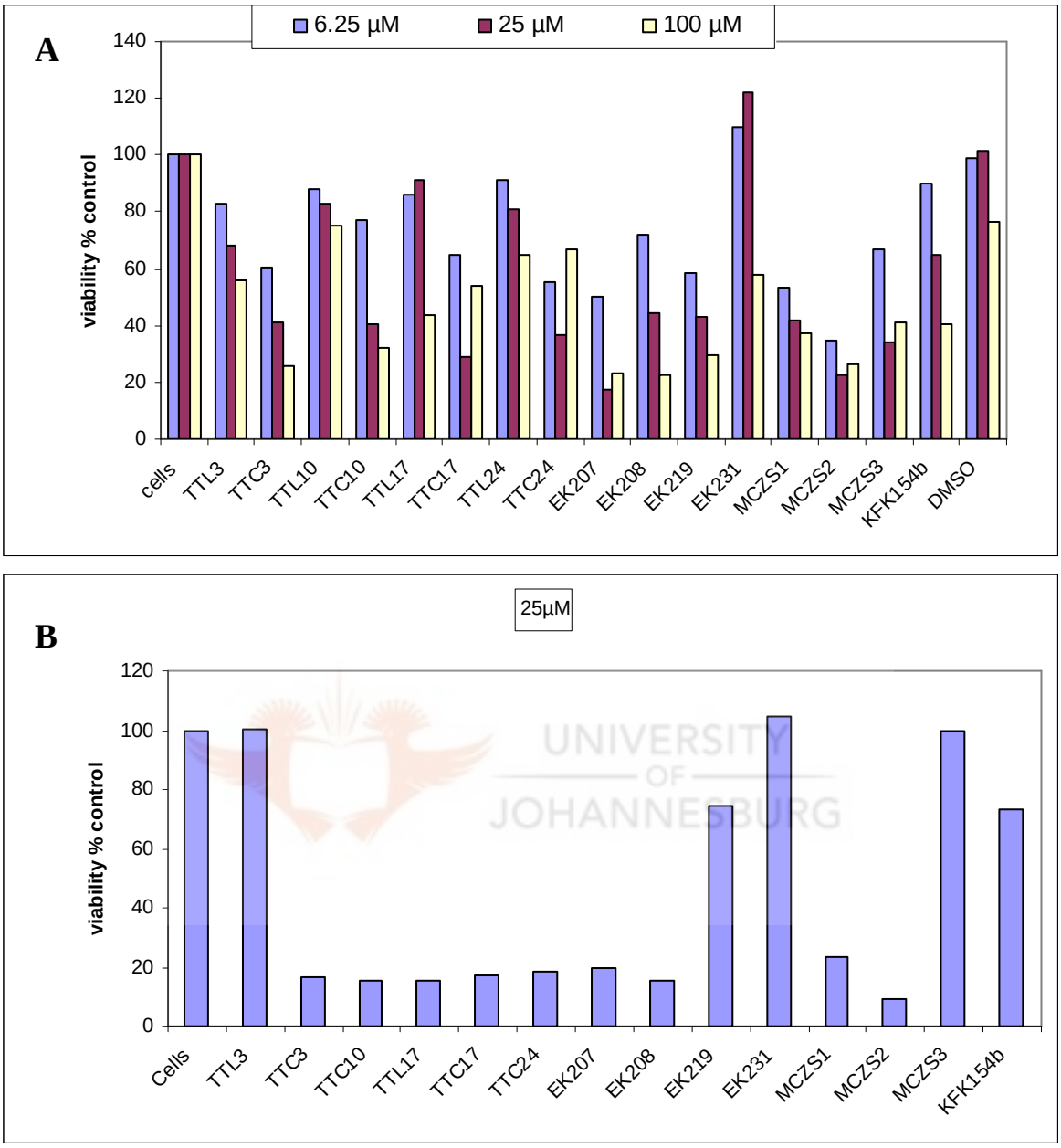


Figure 3.10: Effect of compounds on the viability of uninfected PM1 (A) and acutely infected PM1 (B) cells.

After 7 days of incubation with the compounds (6.25-100 µM), cell viability was determined using MTT. The ligands were the least toxic (A) compared to the gold complexes with EK231 and KFK154b being the least toxic of the gold complexes. In B, EK219 and MCZS3 increased proliferation at 25 µM compared to the same concentration in A.

The ligands and gold complexes EK231, KFK154b were the least cytotoxic while the rest of the gold complexes had cells varying cytotoxicities at the various concentrations tested. This was observed for both the uninfected (Figure 3.10A) and the infected (Figure 3.10B – 25 µM only) cell populations. The ligands were the least toxic to the uninfected cells at 25 µM than they were to the acutely infected cells. Overall, the gold complexes were more toxic to the infected than the uninfected PM1 cells at 25 µM. There was a dose dependent increase in cytotoxicity with increasing concentration of the compounds from 6.25 to 100 µM (Figure 3.10A). EK219 and MCZS3 appear to restore viability at 25 µM in the infected cells (Figure 3.10B) when compared to the uninfected PM1 cells (Figure 3.10A). Only one concentration (25 µM) was tested for the infected cells as this was clinically relevant and appropriate for the antiviral assays (p24, and RT assays).

In vitro cytotoxicity data for both the CEM.NKR-CCR5 and PM1 cell lines both indicate that the ligands and gold complexes EK231 and KFK154b are the least cytotoxic.

Cytotoxicity studies on PBMCs was also conducted at concentrations of 6.25 -100 µM. PBMCs isolated from both seronegative and seropositive individuals were screened as these cells gave an *ex vivo* representation of HIV uninfected or infected cells. In Figure 3.11A and B, viability data for uninfected and infected PBMCs is presented respectively. There was a dose dependent decrease in viability from 6.25 to 100 µM except for EK208 for which increasing concentration led to increasing viability. This compound might thus stimulate proliferation of the PBMCs at these concentrations. The tetrazolium dyes indicated that most of the compounds were toxic to cell lines but not to PBMCs. The

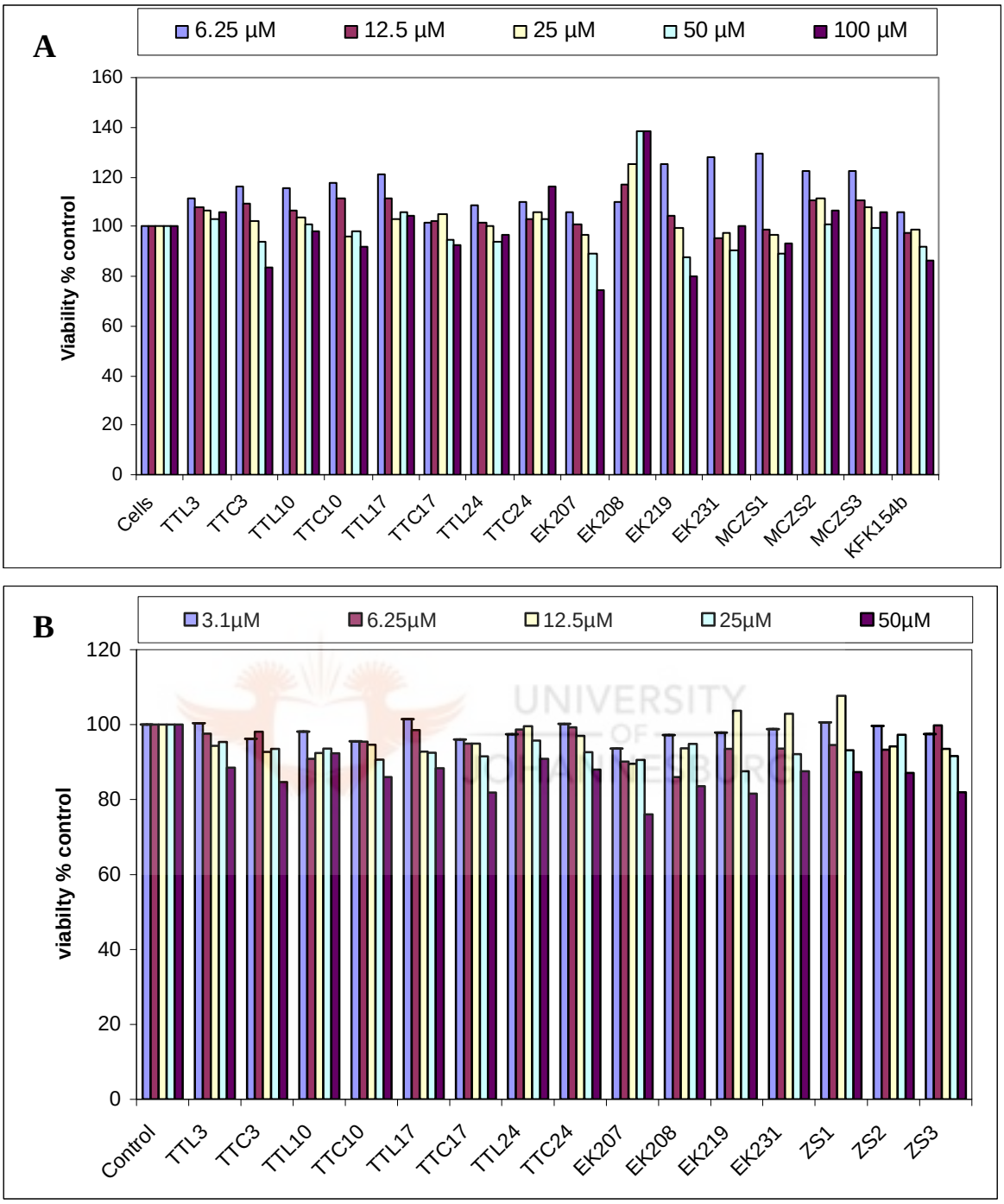


Figure 3.11: Effect of compounds on the viability of uninfected (A) and chronically infected PBMCs (B) cells. After 7 days of incubation with the compounds (6.25-100 µM) at 37 ºC and 5% CO_2, viability was tested using the MTT dye. None of the gold complexes or the ligands were toxic to the PBMCs.

percentage coefficient of variation is far more than 10% in the XTT assays with uninfected and infected CEM.NKR-CCR5 and uninfected PM1 (appendix Table 1 and 2), > 10% for infected PM1, uninfected and infected PBMCs in the MTT assays (appendix section B, Table 3). This indicates high variation between duplicate repeats, a common phenomenon with tetrazolium based viability dyes which usually give high standard deviations. Moreover, dye-based assays have drawbacks such as high background, a limited linear response range, and lack of reproducibility. In addition, some human cell lines metabolize tetrazolium dyes very inefficiently, and in some cases such dyes are toxic to cells (e.g. MTT, Hertel *et al*., 1996). This is another reason why flow cytometry was necessary. The variation in CV values was not as high as with these dyes (appendix section B Tables 1-4).

3.3.3.2 The LDH assay

In vitro cytotoxicity was not only evaluated using MTT and XTT but by LDH assay as well. MTT and XTT determine detrimental effects on mitochondria and metabolic activity while the LDH test measures the effects of the compound after short incubation times and reports on the damage to or leakage of plasma membranes of cells. A shorter incubation time (24 hrs) was employed for this assay unlike with the tetrazolium dyes (7 days) as the released LDH is not very stable for long periods of time (> 48 hrs). LDH is an enzyme present in the cytoplasm of cells and plasma membrane damage leads to release of this enzyme into the culture supernatant. The assay was performed using an LDH cytotoxicity kit (Roche Diagnostics, Mannheim, Germany). This assay measures the amount of LDH released into culture supernatant. The assay was performed on uninfected cells only just to confirm the toxicity observed using the tetrazolium dyes with

the assumption that the complexes would be even more toxic to infected cells due to the combined cytopathic effect of virus and compounds as seen with the tetrazolium dyes. An overall observation was non toxicity of the compounds on the primary cells while the majority of the gold complexes tested were toxic to the cell lines PM1 and CEM.NKR-CCR5 confirming the XTT and MTT data.

The LDH released from cells incubated with EK231 and KFK154b (Figure 3.12) was the least for both cell lines further confirming that these gold complexes were the least toxic as seen in the XTT and MTT data (Figure 3.9 and 3.10). EK208 was the most toxic of the gold complexes evident from the high percentage toxicity (> 50% for all 3 concentrations of 5, 25 and 100 µM) in both the CEM.NKR-CCR5 and PM1 cell lines (Figure 3.12). This is in agreement with the percentage viability seen in Figures 3.9 and 3.10 where its CC_{50} was < 1 µM in the case of uninfected CEM.NKR-CCR5. The ligands as expected were the least toxic at these concentrations (Figure 3.12B).

It was however noticed that for some gold complexes, increasing concentration led to decreasing percentage cytotoxicity e.g. TTC3, TTC17 TTC24, MCZS2 etc (Figure 3.12B). This could possibly be due to some form of interference of high concentration of the gold compounds with LDH (in the supernatant) preventing the expected reaction from occurring.

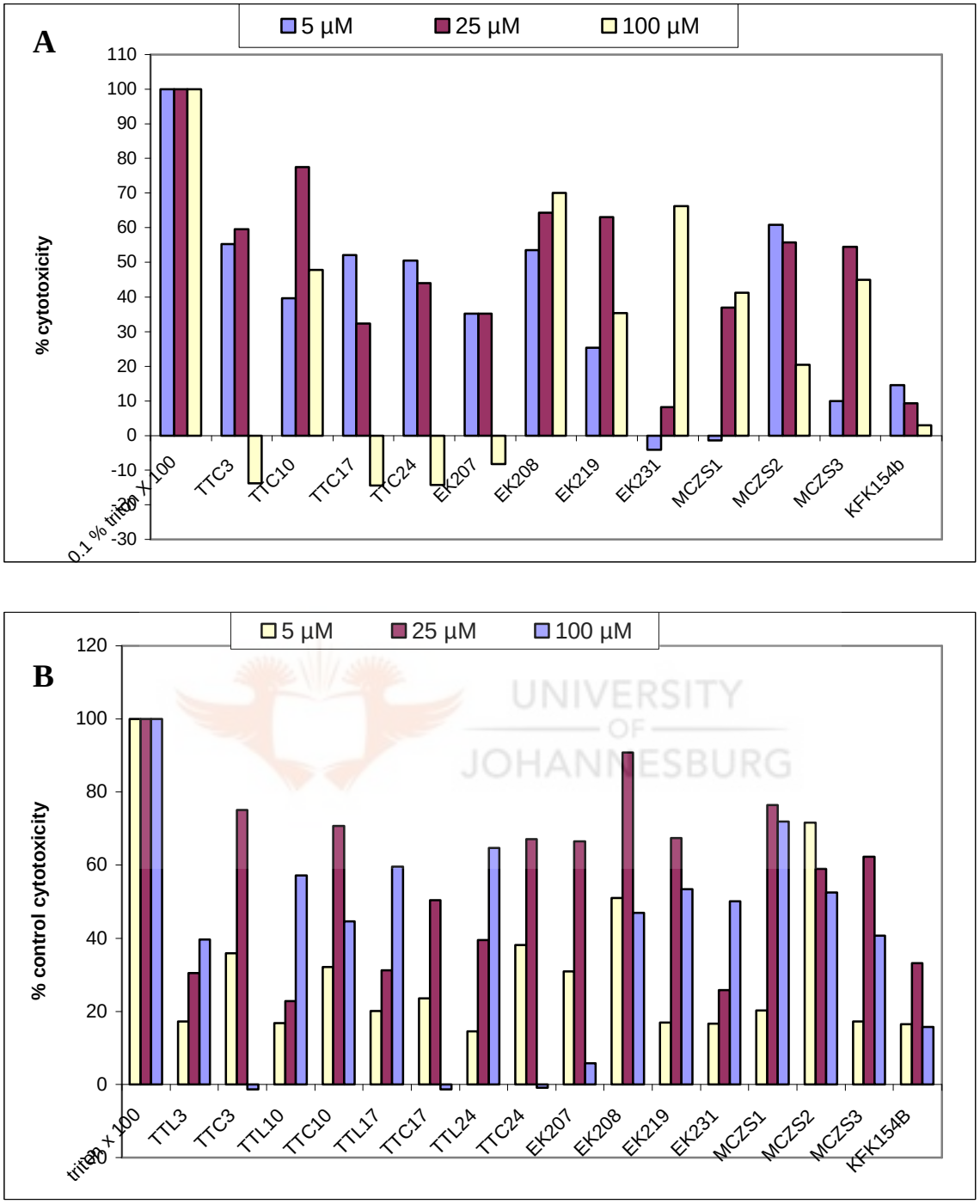


Figure 3.12: Effect of gold complexes on LDH release in CEM.NKR-CCR5 (A) and PM1 cell lines (B).

The cells were treated with the compounds for 24 hrs at 37 ºC and 5% CO_2. LDH release was measured relative to a control sample treated with 0.1% triton x-100 for maximum release of the enzyme.

The release of LDH from the PBMCs (indicated in Figure 3.13) was less compared to that observed for the cell lines (Figure 3.12). The LDH released from cells treated with complex EK208 is above 50% indicating cytotoxicity for all three concentrations tested (Figure 3.13). This is contrary to data obtained using MTT (Figure 3.11) where there was no toxicity but rather proliferation. Because of the short incubation time (24 hrs) used in the LDH assay compared to the MTT assay (7 days), a direct comparison between the data provided by these assays is not entirely valid. For longer compound incubations (in the XTT and MTT assays), there is always the possibility of the cells adjusting to the compounds leading to increased proliferation after 24 hrs.

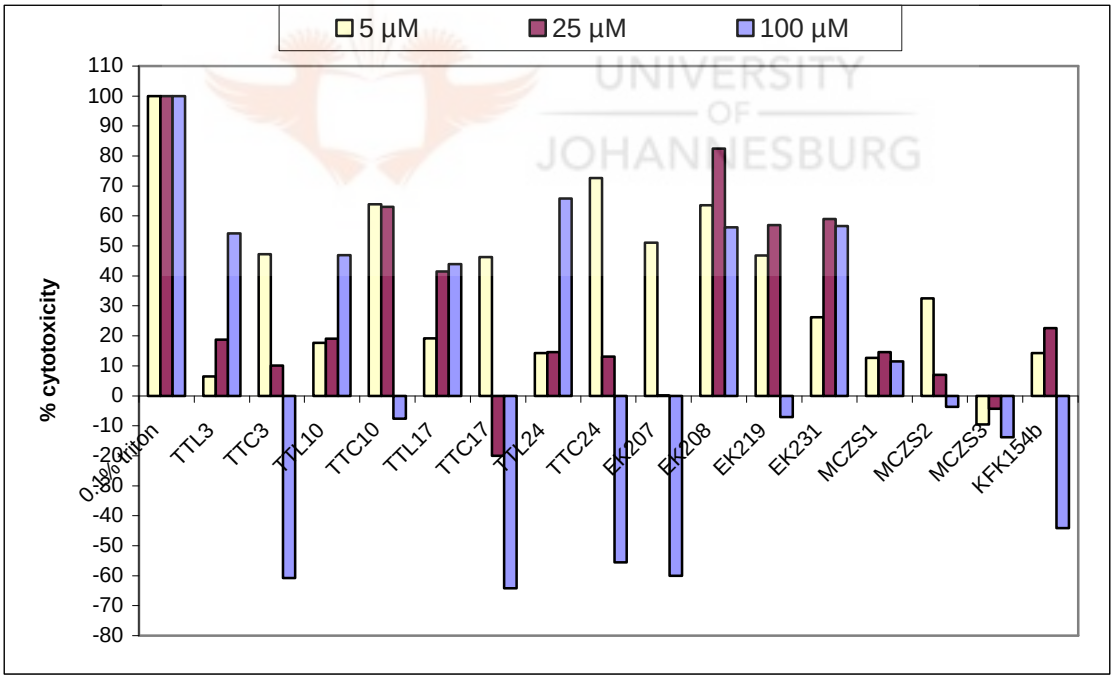
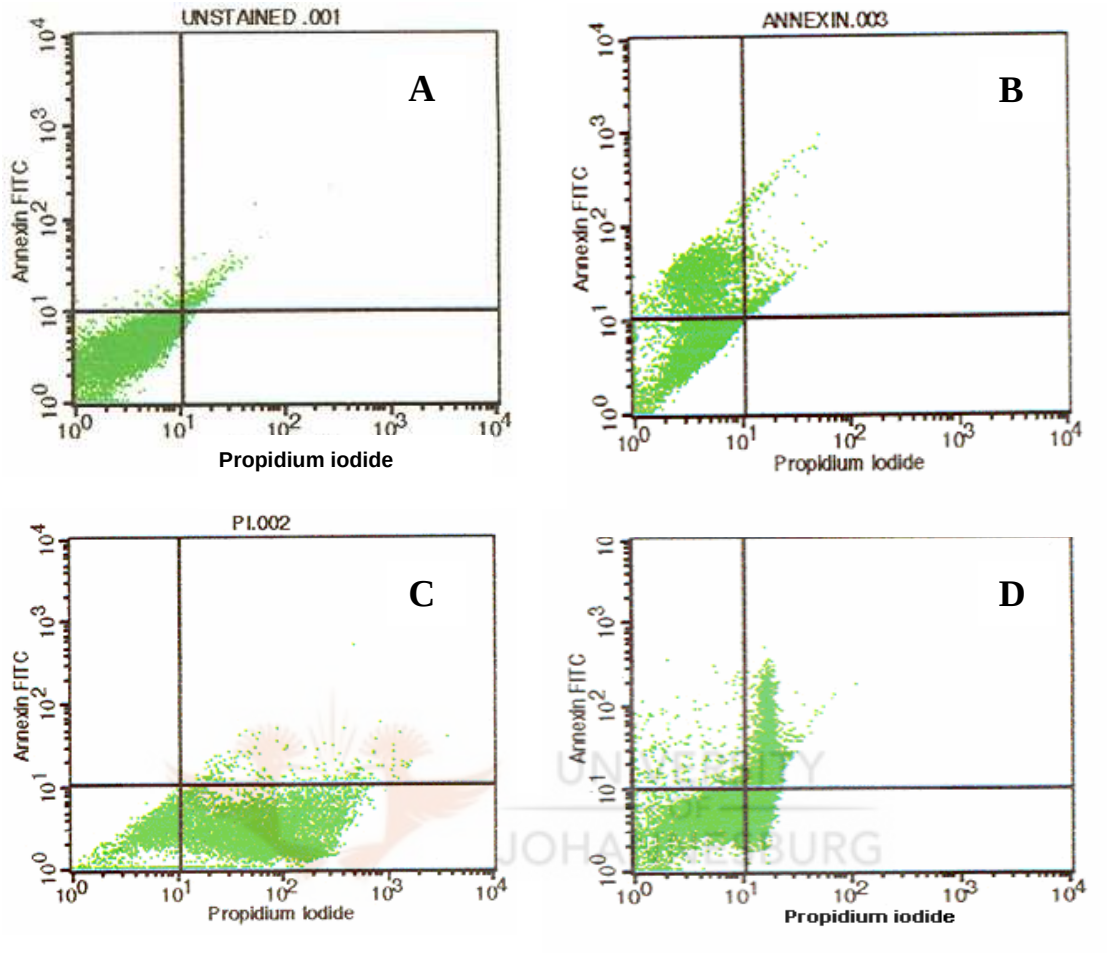


Figure 3.13: Effect of gold complexes on LDH release in PBMCs.
The cells were treated with the compounds for 24 hrs at 37 ºC and 5% CO_2. LDH release was measured relative to a control sample treated with 0.1% triton x-100 for maximum release of the enzyme.

3.3.3.3 Flow cytometry

Flow cytometry was used not only to determine viability but also the type of cell death in situations where the compounds were toxic. This technique was also used as confirmatory methodology assay for cell viability as the shortcomings of the tetrazolium dyes (such as the need for solubilisation in the case of MTT, the fact that some compounds have intrinsic absorbances that had to be subtracted and also the possibility of some compounds reducing the dyes) were eliminated. Figure 3.14 is a representation of controls that were employed for setting instrument parameters on the FACSCalibur (BD Biosciences, California, USA) also known as quadrant specification. The controls in Figure 3.14 were obtained using uninfected PBMCs. These controls include unstained cells (lower left quadrant (LL) - Figure 3.14A), annexin positive/apoptotic cells (upper left (UL) - Figure 3.14B), propidium iodide positive/necrotic cells (lower right (LR) - Figure 3.14C) and a treatment including both dyes (Figure 3.14D). Figure 3.14E, represents the identity of each quadrant. Quadrant specification allows for proper instruments settings and calibration leading to the placement of each cell population in their respective fluorescence quadrants with no resulting overlap. It was only following appropriate quadrant specification that data was collected for the rest of the gold compound treated samples.



UL	UR **E**
LL	LR

UL = upper left/ early apoptosis
UR = upper right/late apoptosis
LL = lower left / viable
LR = lower right/necrotic

Figure 3.14: Apoptosis/necrosis controls for flow cytometry.
Untreated PBMCs were incubated alongside treated cells for 7 days at 37 ºC. Prior to sample analysis, an unstained sample (A) was analysed followed by an annexin positive (B), a PI positive (C) and lastly a sample containing both annexin and PI (D) for quadrant specification. E represents the identity of each quadrant.

In Figure 3.15 A and B, the effect of the compounds (25 µM) on uninfected and infected CEM.NKR-CCR5 cells is shown respectively. As mentioned earlier, just one concentration (25 µM) was chosen for flow cytometry analysis and antiviral screening as this was a clinically relevant concentration and because the compounds had been shown to inhibit RT at this concentration. In A, it can be seen that the ligands (TTL3, TTL10) and gold complexes EK207, EK231 and KFK154b allow for cell viabilities above 50%. Most of the cells treated with gold (I) phosphine complexes (TTC3, TTC10, TTC17, TTC24) are in the early apoptotic phase of cell death while the DPPE gold complex treated cells are in late apoptosis. In the untreated (no compounds added) sample of cells, the viability is slightly lower (58%) than in the presence of TTL3, EK231 and KFK154b. The level of cell death because of factors like overcrowding (cells cultured for 7 days) or non specific binding of annexin to rapidly dividing untreated cells (Dillon *et al*., 2001) might be responsible for this observation. Any additional cell death or increase viability might thus have been due to the effect of the compounds. In the infected cell population (Figure 3.15B), there was an observed increase in early apoptotic cell death. The ligands (TTL3, TTL10 and TTL24) and gold complexes EK231, ZS2 and KFK154b were the least toxic to these cells while TTL17 and the rest of the gold complexes were toxic. In general, there was an overall decrease in viability in the infected (Figure 3.15B) population with death being by apoptosis compared to the uninfected population of cells (Figure 3.15A).

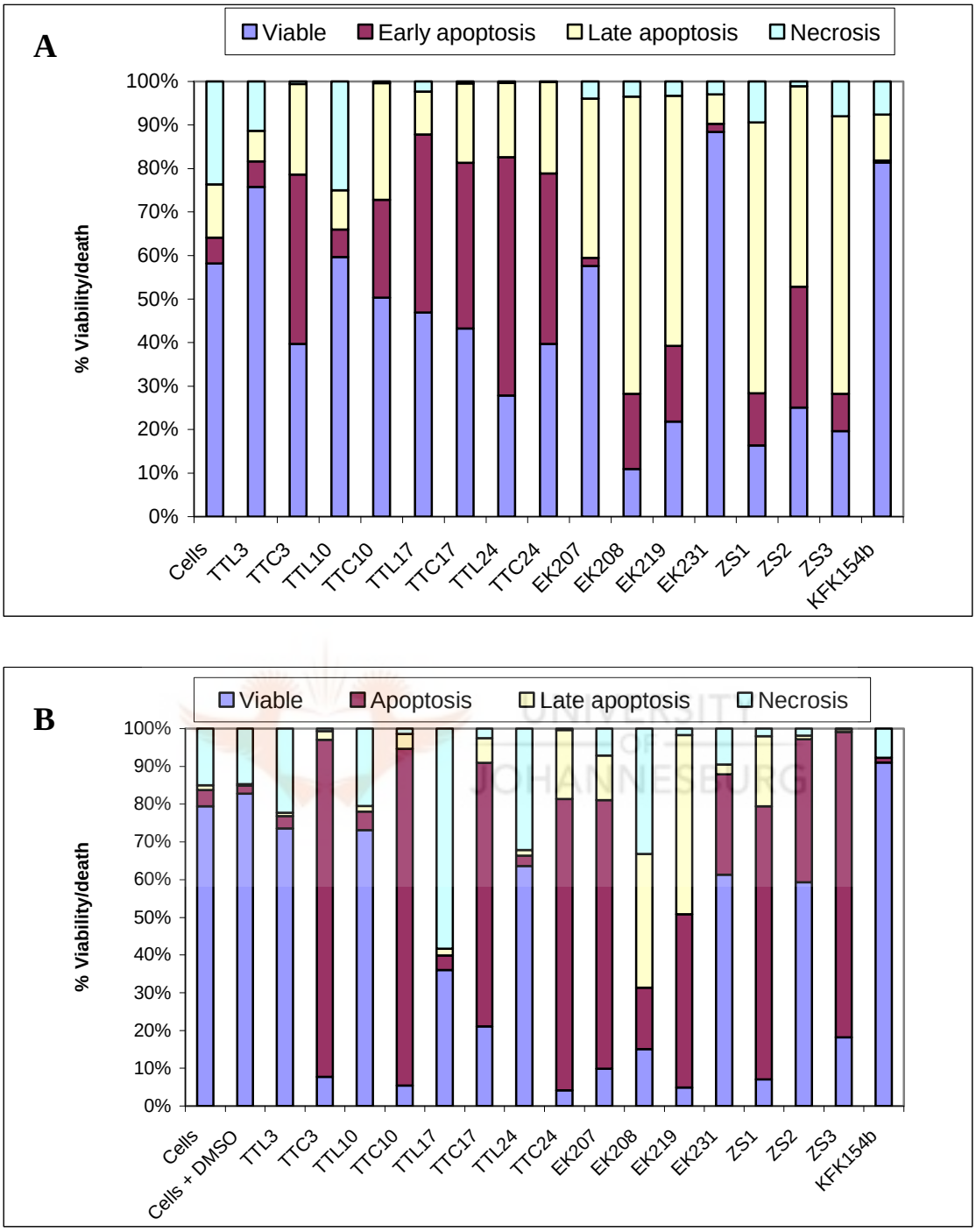


Figure 3.15: The effect of gold compounds (25 µM) on the viability of uninfected (A) and infected (B) CEM.NKR-CCR5 cells.

CEM.NKR-CCR5 cells were incubated with gold compounds at 25 µM final concentration for 7 days at 37 ºC under 5% CO_2. DMSO (0.5%) and untreated cells were included as controls. Apoptosis and necrosis was assessed using the annexin V antibody and propidium iodide kit.

The type of cell death caused by the compounds on the PM1 cell line was also evaluated on the uninfected population only (Figure 3.16). It was assumed that a similar or greater effect would be observed with the infected population due to viral cytopathic effects. All treatments lowered cell viability while EK231 and KFK154b were the least toxic relative to the untreated control sample. All the other gold complexes induced viabilities below 30% ($p<0.05$) indicating a significant population of cell death in these samples. There was a shift towards late apoptosis as the mechanism of cell death.

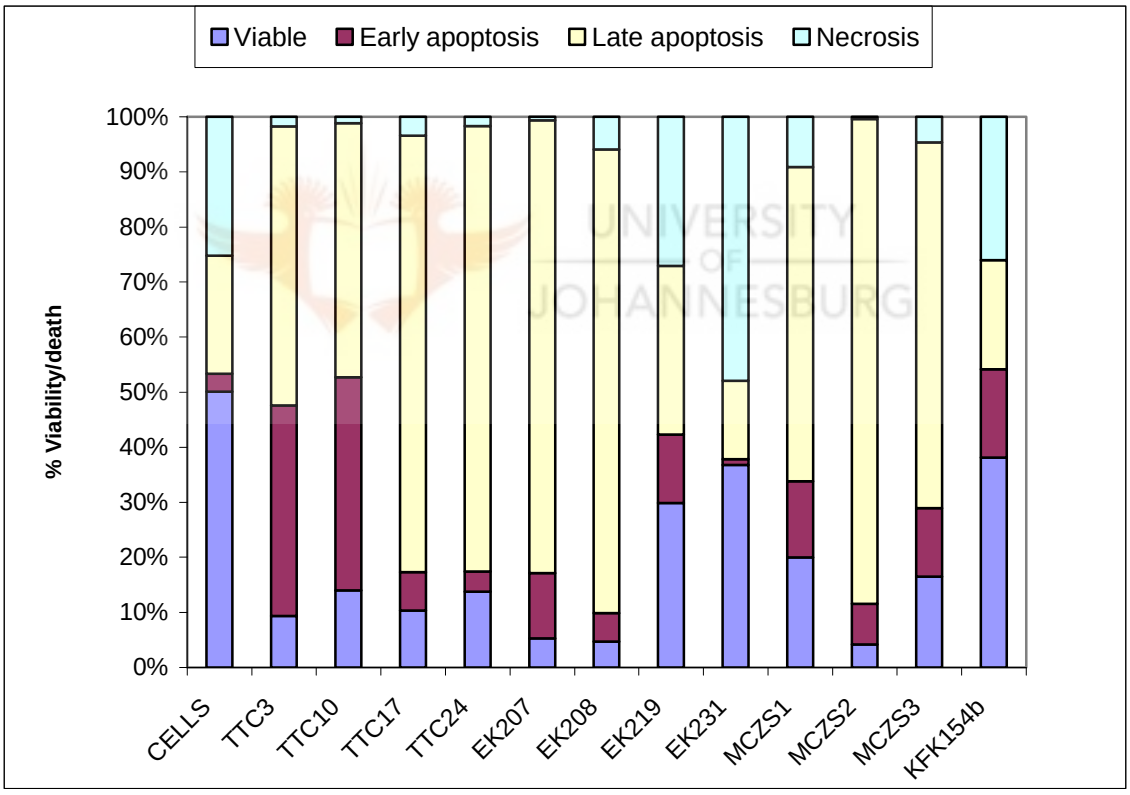


Figure 3.16: The effect of gold complexes (25 µM) on the viability of uninfected PM1 cells.
PM1 cells were incubated with gold complexes at 25 µM for 7 days at 37 ºC under 5% CO_2. Apoptosis and necrosis was assessed using the annexin V antibody and propidium iodide dye.

In Figure 3.17A and B flow cytometry results obtained for uninfected and infected PBMCs are presented respectively. In Figure 3.17A, treated and untreated cells were generally viable. In the case of the infected cells (Figure 3.17B), the viability in the presence of most of the compounds except MCZS1 (42%) and MCZS3 (43%) were above the 60% mark. In the control cells (Figure 3.17), the percentage viability was lower for the untreated infected cells (73.5%) than the untreated uninfected cells (84%) presenting a shift toward early apoptosis. In the DMSO (< 0.5%) control, there is no significant difference in viability compared to the untreated cells. There were less apoptotic cells and more necrotic cells for uninfected PBMCs while for the infected PBMCs more apoptotic cells were recorded.

Flow cytometry data demonstrates that while routine viability assays (MTT, XTT) show cells to be viable, they may already be transitioning to some form of cell death as seen for MCZS1 and MCZS3. This observation was made by Traore and Meyer in 2002. At the same time, when comparing the CV values obtained from repeats in the flow cytometry data (Table 4 appendix section B), differences in standard deviations are not > 10 for majority of the complexes as was seen with the MTT and XTT assays (Table 1, 2 and 3 – appendix section B) indicating minor differences between duplicate repeats.

3.3.4 Antiviral p24 Antigen Assays

The secretion of p24 into the culture supernatant following treatment of both chronically infected and acutely infected cells was measured using the p24 ELISA kit from Beckman Coulter (California, USA). The reduction of p24 following treatment, compared to an untreated control is indicative of an antiviral effect of a particular compound. Compounds tested were those that were non cytotoxic (Figure 3.17) and had been shown to be

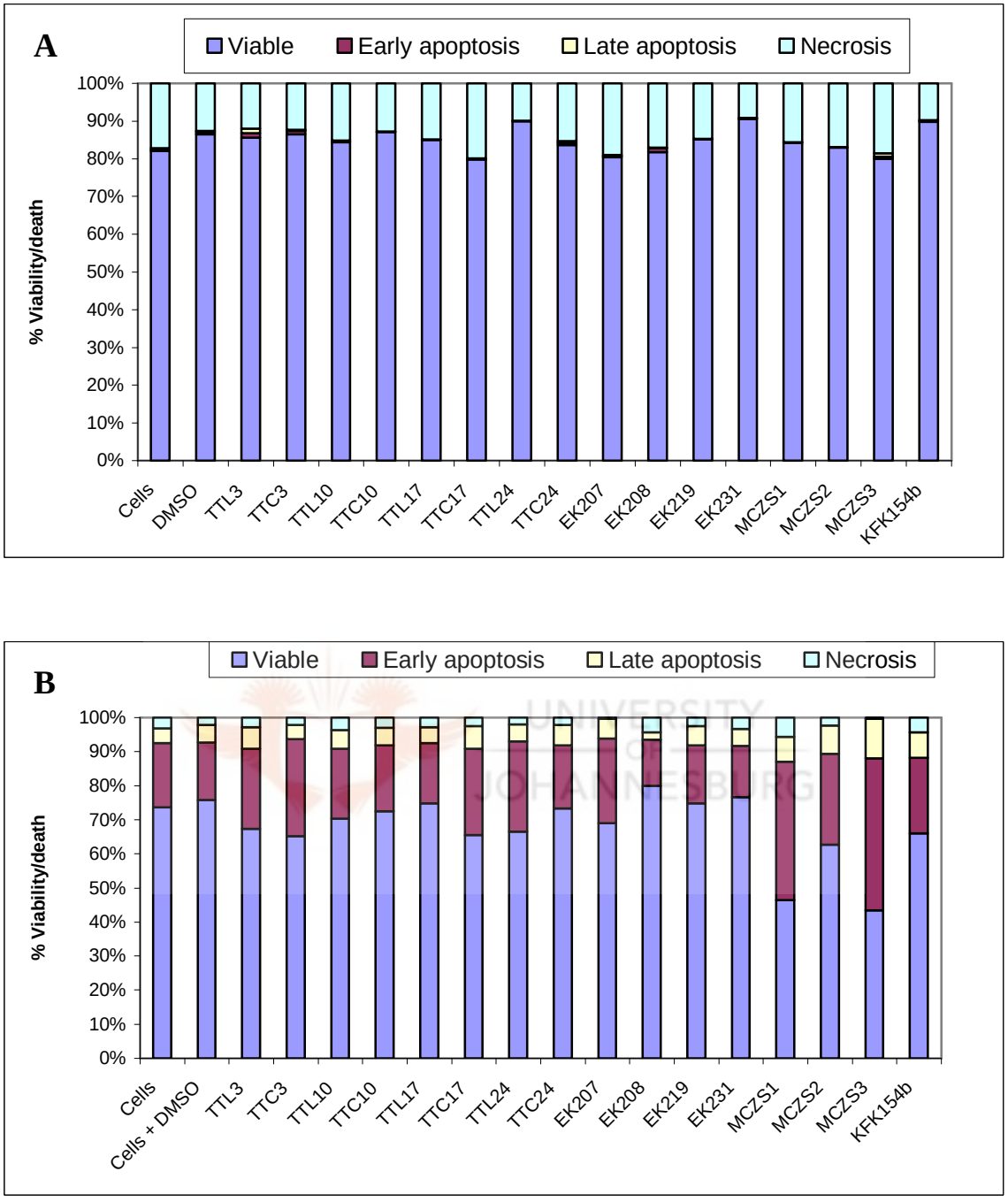


Figure 3.17: The effect of gold compounds on the viability of uninfected (A) and infected (B) PMBCs.

PBMCs were incubated with gold compounds (25 µM) for 7 days at 37 ºC under 5% CO_2. DMSO (0.5%) and untreated cells were included as controls. Apoptosis and necrosis was assessed using the annexin V antibody and propidium iodide. These results represent the mean of 3 experiments with samples treated in duplicate per experiment.

inhibitory to RT in direct enzyme assays (Figure 3.3) i.e. some and the gold complexes. The antiviral effect of the gold complexes on acutely infected CEM.NKR-CCR5 is shown in Figure 3.18. EK231 significantly reduced p24 at a non cytotoxic concentration of 25 µM (p value = 0.0042).

In the case of the chronically infected PBMCs, none of the complexes significantly inhibited p24 as seen from the p-values indicated (Figure 3.19) suggesting that the Au compounds may have an effect in early infection (prior to the chronic phase). This possibility is supported by the data collected during acute infection (Figure 3.18).

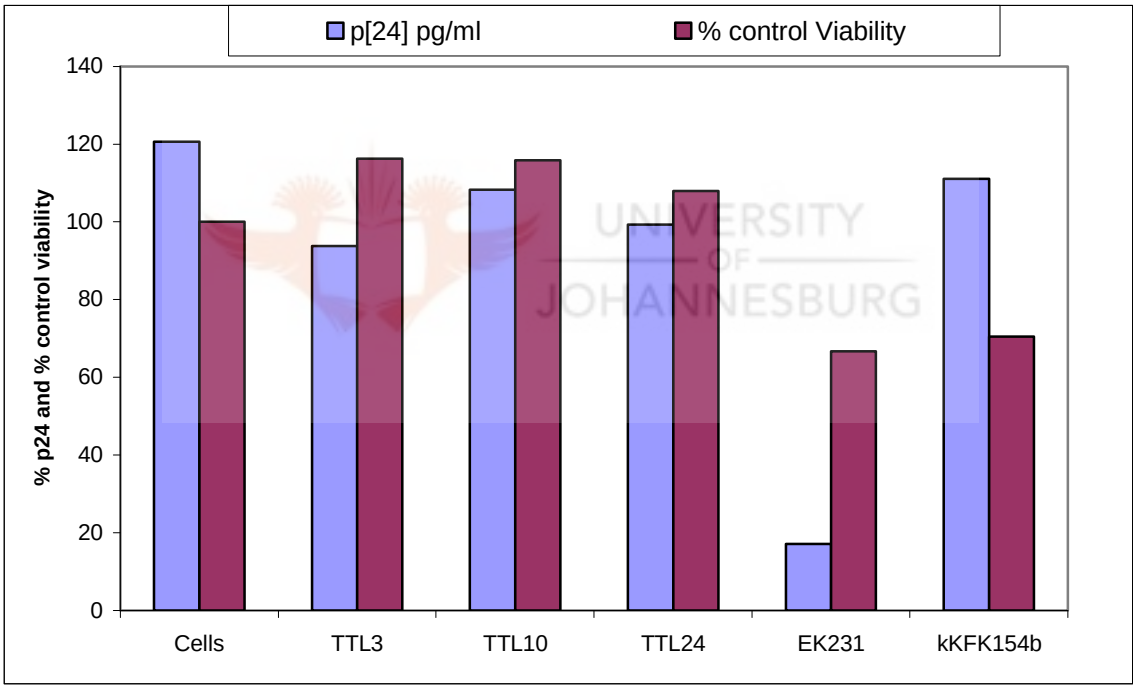
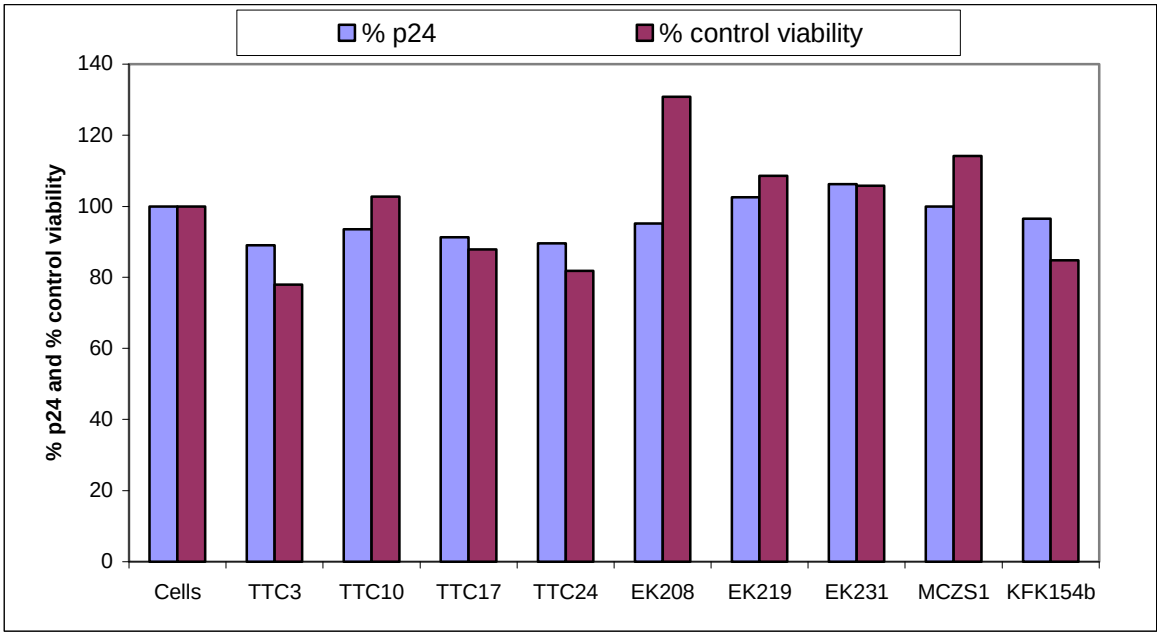


Figure 3.18: The effect of gold complexes (25 µM) on the secretion of p24 in CEM.NKR-CCR5.
CEM.NKR-CCR5 cells were infected overnight with DU-151 (500 $TCID_{50}$). The cells were then incubated with the gold complexes for 7 days at 37 ºC under 5 % CO_2. Cell viability was assessed using the MTT viability assay while HIV-1 replication was monitored with the HIV-1 p24 ELISA kit. EK231 was shown to significantly ($p<0.05$) inhibit p24 secretion.



% p24	cells	TTC3	TTC10	TTC17	EK208	EK219	EK231	MCZS1	KFK154b
Average	100	89.28	97.49	91.14	95.24	97.75	102.44	99.96	99.42
Stdev		17.68	22.40	8.13	11.50	13.24	13.52	19.54	24.08
p- values		0.48	0.89	0.26	0.62	0.83	0.82	1.00	0.98

Figure 3.19: The effect of gold complexes (25 µM) on the secretion of p24 in PBMCs. PHA and IL-2 stimulated PBMCs from an HIV-1 infected individual were incubated with the gold complexes for 7 days at 37 ºC under 5 % CO_2. Cell viability was assessed using the MTT viability assay while HIV-1 replication was monitored with the HIV-1 p24 ELISA kit. No significant inhibition of HIV-1 replication was noted for any of the complexes tested.

3.4 SUMMARY OF RESULTS

Out of the twelve complexes tested, eight inhibit RT (Figure 3.3) and 4 of them significantly inhibit HIV-1 PR (> 50%) in direct enzyme assays (Table 3.2). However, the concentration (100 µM) at which the four complexes inhibit PR are not in the clinically relevant range (Georgiou, 2002, Traber *et al.*, 1999, Yoshida *et al.*, 1998). Since this assay was cell free, it is possible that administration of these gold complexes at

this concentration could allow for attainment of clinically relevant concentrations in a whole cell scenario.

The gold complexes (except EK231 and KFK154b) and the ligands are toxic to cells lines and not to the primary PBMCs. This cytotoxicity or lack of it was confirmed using flow cytometry as seen in Figures 3.15, 3.16 and 3.17 respectively. Antiviral p24 assays using chronically infected PBMCs did not reveal any significant decrease in the protein for any of the complexes tested. A possible explanation for this is the fact that the p24 secretion in asymptomatic patients usually diminishes to undetectable levels following. seroconversion as a result of formation of immune complexes with antibody present (Rodriguez-Iglesias *et al*., 1992). In the CEM.NKR-CCR5 cell line, EK231 significantly decreases the p24 secretion to 17 pg/ml compared to the untreated cells (121 pg/ml). This was a non cytotoxic concentration of 25 µM (Figure 3.18) were viability was 67%. No significant reduction of p24 was observed in the chronically infected cells (Figure 3.19) suggesting that the compounds might be effective in early acute infection as seen with the CEM.NKR-CCR5 cells (Figure 3.18).

CHAPTER 4

DISCUSSION

The medicinal applications of gold for various ailments such as rheumatoid arthritis (Parish and Cotrill, 1987) over time and the continuous need for new therapy for some of these diseases prompted the formation of project AuTek with the Biomedical group involved in anti-cancer, anti-malaria and anti-HIV screening. The focus in this thesis was on the *in vitro* anti-HIV screening of some novel gold complexes under the AuTEK consortium. This was stimulated by literature reports on the anti-HIV effect of some gold complexes by various authors namely: Okada *et al* 1999, Shapiro and Masci, 1996, Tepperman *et al*., 1994 to name but a few. The shortcomings of HAART therapy such as the development of drug resistant viral strains (Chen *et al.*, 2004), the alarming statistics of current and new HIV infections (UNAIDS, 2006) also stimulated the search for novel possible therapeutic leads. The project aimed to identify complexes that had minimal toxicities to host cells and high efficacy against HIV with the goal to identify novel anti-HIV agents to supplement or possibly replace existing drugs. Sixteen compounds were screened with 12 of them being gold complexes and 4 being ligands of some of the complexes (Table 2.1). The screening of these compounds was performed in both cell free and cell based or bioassays.

The *in vitro* analysis performed revealed that the gold complexes were generally stable in DMSO when stored at 4 ºC or at 37 ºC for over 1 week. They started undergoing hydrolysis immediately when diluted in a physiological buffer. Unlike the ligands and complexes EK231 and KFK154b, the rest of the gold complexes were found to be toxic

to cell lines and not to primary cells. Inhibition of HIV-1 activity by RT inhibition was observed for 7 of the gold (I) phosphine complexes and a gold (III) complex while 4 of the gold complexes also inhibited PR with inhibitions of more than 50%. EK231 significantly reduced p24 secretion at a non cytotoxic concentration.

4.1 STABILITY AND SOLUTION PROPERTIES

Stability could be determined by the use of both NMR and UV-visible spectroscopy. The latter however is less sensitive as it typically contains far fewer features (bands) compared to NMR and therefore lower information content (Field *et al*., 1995). It was however used to determine the stability of the complexes in a physiological buffer as the concentration at which the compounds were required for analysis by NMR could not be dissolved in a physiological buffer. In the case of UV-visible spectroscopy, micromolar quantities dissolved in DMSO were further diluted in the physiological buffer for analysis.

Selected complexes representative of each group i.e. gold (I) phosphine complexes (TTC3 and TTC10), DPPE complexes (EK207 and EK208) and triethylphosphine thiol complexes (MCZS1) were tested for their stability in DMSO by ^{31}P NMR. The gold (I) phosphine complexes (TTC3, TTC10) remained stable over time (1 week and up to 4 months) as their ^{31}P NMR spectra remained unchanged. The bridged EK207 complex was observed at 87 ppm and breakdown products at 1, 23 and 31 ppm (Table 3.2, appendix section A Figure 6) being mostly oxides of the ligand (DPPE) which is less stable than the gold complex and slowly undergoes solvent dependent autooxidation to the mono and bisphosphine oxidized forms (Bernners-Price *et al*., 1986). The peak at 89 ppm after 1 week is possibly as a result of the formation of a gold (III) complex (noticed

by colour change) resulting from the oxidation of the gold (I) complex to gold (III). This is in line with observations made by Brenner-Price *et* al (1986) where ^{31}P NMR of some DPPE complexes in DMSO and other solvents including methanol, ethanol and acetone resulted in no breakdown of the complex after 24 hrs with samples being stable for at least 4 days. It was therefore appropriate to observe additional peaks at 1 week in solution. In the ^{31}P NMR spectrum of EK231, the complex initially peaks at 82.9 ppm. By 24 hrs, there were 2 new peaks at 86 ppm and 77.5 ppm. The peak at 86 ppm is likely the tetrahedral gold (I) complex normally expected at 84 ppm (Table 3.2, appendix section A Figure 7). A 4 months spectra of complexes EK207 and EK231 (Table 3.2) was not obtained as there was visible decomposition by 1 week such that it was unnecessary to perform the 4 months analysis.

The ^{1}H NMR was obtained for all the complexes and remained unchanged except for that of MCZS1 (section A, appendix – Figure 8). A triplet (between 5.5 and 5.6ppm) and duplet (between 5.8 and 5.9 ppm) peak is observed. Differences in the original peak (between 4.8 and 4.9) and new peaks (between 4.7 and 4.8) are also seen. The identity of these peaks is not yet known. It is however possible that these were as a results of certain groups in the compound being hydrolysed as the DMSO peak was shown to also increase in area over time suggesting water uptake from the atmosphere as DMSO is hygroscopic (absorb water from the atmosphere).

The UV-visible spectra obtained for the complexes indicates that the chromophore present in the complexes absorbed maximally in the UV section of the spectrum with most of them having maximum absorbances in the range 256-259 nm except for EK231 which had a maximum absorbance at 304 nm (appendix section A, Figure 5). The

presence of phenyl groups in their structure is possibly the cause of this absorbance. None of the complexes absorbed in the visible region and therefore do not interfere at the wavelengths at which the colorimetric assays (XTT and MTT) that we employed absorb. A scan of the compounds on immediate dissolution, 24 hrs and 1 week later did not show any changes in the spectra i.e. there was neither a hypsochromic shift (shift to a lower wavelength) or a bathochromic shift (increase in wavelength) indicating there were no new bonds being formed or broken e.g. conjugation of double bonds will cause an increase in the wavelength at which the chromophore absorbs while protonation of an aromatic ring for example causes a shift to a lower wavelength.

Stability in a physiological buffer (PBS) for 3 of the complexes (TTC3, EK207 and KFK154b) was done by the use of UV-Visible spectroscopy. It was observed that the complexes experienced rapid hydrolysis over time (Figure 3.4 A and B appendix section A) seen as a hypochromic shift in the original spectrum (decrease in absorbance intensity). A possible reason for this is the result of the rapid charge transfer of

Cl $\longrightarrow$ Au(I) (in the case of TTC3 and EK207) or Cl $\longrightarrow$ Au(III) (in the case of KFK154b) and this hydrolysis process depends on the progressive detachment of chlorine atoms (Calamai *et al*., 1997). Chlorine is a good leaving group (Allaudeen *et al.*, 1985) and therefore was replaced by water molecules in aqueous media resulting in the hydrolysis observed. This is a possible indication that in the physiological milieu, the compounds would be able to undergo the necessary reactions necessary for bioactivity.

The decomposition of gold from the complex or a possible association with DMSO (Schröter *et al*., 1997) was also noted in the RT assays when experiments were done with compounds that had been in solution for over a month (Figure 3.2) as compared to when

compounds that were freshly made up (Figure 3.3) were used. Freshly made up solutions of the gold complexes inhibited RT but not solutions prepared from a DMSO stock (20 mg/ml) of over a month and stored at 4 ºC. Because a possible mechanism for RT inhibition is through ligand exchange reactions between sulfhydryl groups on the enzyme and the gold complex be it gold (I) or gold (III) (Allaudeen *et al*., 1985), this reaction will not be possible if the gold complex is already in a dissociated state or the compound is associated with DMSO.

In the highly concentrated stock (20 mg/ml), it was observed that there was oxidation of gold evident by transition of the stock from lighter to darker colours. It was therefore necessary to prepare fresh samples each time or alternatively, a stock solution of the compounds dissolved in DMSO could be made and samples frozen at -20ºC until needed for bioanalysis.

It is worth noting that the employment of soft ligands for gold (I) (such as sulphur and phosphorous) and hard ligands for gold (III) (such as nitrogen) during the synthesis of the compounds aids in stability by increasing the shelf life of the gold compounds (Parish and Cottrill, 1987) as the choice of ligand helps in stabilising the gold centre. Though these ligands stabilize the compounds, they are not so tightly bound such that metabolic reactions can not occur (Parish and Cottrill, 1987).

4.2 EFFECT OF DMSO ON RT AND PR

DMSO is commonly used for the dissolution of synthetic and natural products in drug screening assays. These metallodrugs undergo complete dissolution in this solvent compared to aqueous media and were therefore preferred. The concentration of DMSO should however be kept to the minimum (0.5% and below) in cell based assays as higher

concentrations affect viability of the cells. The use of DMSO as solvent (in our purposes) required that its effect on HIV-1 enzymes (RT and PR) be evaluated. DMSO has the least effect on RT inhibition compared to other solvents (methanol and ethanol) that could have been used (Ghee *et al*., 1991). We observed an increase in RT inhibition of the methanol fraction of complex EK219 compared to the DMSO fraction when the same concentration of solvent was utilized (Figure 3.1). Also according to Wan *et al* (1996), DMSO of 4.5% could affect the dimeric structure of HIV-1 protease thereby decreasing the activity of the enzyme. For biological purposes (especially cell associated analysis), the DMSO concentration was kept as minimal as possible (<0.5%) and an equivalent amount of DMSO (in samples) was included in the untreated control (no compound) so as to cancel out any false inhibition or increased activity recorded as a result of the use of DMSO.

4.3 DIRECT ENZYME ASSAYS

4.3.1 RT Activity

The gold complexes (except EK208, MCZS1, MCZS2 and MCZS3 and the ligands) inhibited RT at the 3 concentrations tested as well as the gold (III) complex (KFK154b) shown in Figure 3.4. Interestingly enough, MCZS2 (auranofin) which has been reported to have anti-HIV activity *in vivo* in an arthritis patient being treated for psoriatic arthritis by increasing the patient's CD4+ count (Shapiro and Masci, 1996) did not inhibit RT *in vitro*. This implies that auranofin probably acts by a mechanism other than inhibition of RT. On the other hand, the inhibitory compounds may be better inhibitors of RT because chlorine (attached to the Au – please refer to structures in Table 2.1) makes a better leaving group than the tetraacetylthioglucose moiety of auranofin (MCZS2) and its

analogues i.e. MCZS1 and MCZS3 (Allaudeen *et al*., 1985) in ligand exchange reactions. Ligand exchange reactions with the complexes that have an easily accessible chlorine atom (TTC3, TTC10, TTC17, TTC24, EK207, EK219, EK231 and KFK154b) were therefore favoured as all these gold complexes inhibited RT. This is a structure activity relationship. Other authors (Tepperman *et al*., 1994, Okada *et al*., 1993) have also reported the inhibition of RT in direct enzyme assays by gold compounds.

4.3.2 PR Assays

The Bachem S-1000 kit was initially used for anti-protease screening by means of RP-HPLC. According to the manufacturer's protocol, the assay was supposed to be performed for 15 minutes and stopped with 10 µl of 10% TFA (0.8% final concentration) in water. According to Lam *et al* (2000) incubations times of up to 1 hr have been used and the final TFA concentration for stopping the reaction was 5.5% TFA (Pazhanisamy *et al*., 1997). With the initial protocol, all the complexes and even the ligands were shown to inhibit PR (Figure 3.6). Because of a possible reaction between silica present on the HPLC C_{18} column and gold present in the samples as well as differences with the manufacturer's protocol and available publications (Lam *et al*., 2000, Pazhanisamy *et al*., 1996), PR data needed to be confirmed using another detection system.

The fluorogenic assay which has more than just manufacturer based publications was used to ascertain the effect of the gold complexes on HIV-1 PR. Four of the gold complexes tested (EK208, MCZS1, MCZS3 and KFK154b) significantly inhibited HIV-1 PR at 100 µM (Table 3.3). Observations by Chircorian and Barrios (2004) have indicated chrysotherapeutic effect on some lysosomal cysteine proteases which contain cysteine in their active site. It is therefore possible that our compounds act by a similar mechanism

which is ligand exchange in the case of the gold (I) compounds i.e. (EK208, MCZS1 and MCZS3). EK208, MCZS1, and MCZS3 did not however inhibit HIV-1 RT. KFK154b (a gold (III) compound) appears to inhibit both HIV-1 PR and RT. It would be interesting to know specifically how this compound interacts with HIV-1 PR as ligand exchange with thiol groups is the possible mechanism by which it inhibits RT for either gold (I) or gold (III) complexes (Allaudeen *et al*., 1985). If the active protease contains thiols in its active site then a possible mechanism of inhibition of PR by KFK154b would be by ligand exchange as well.

Auranofin (MCZS2) did not inhibit either RT or PR. This compound has been shown to lead to remission of AIDS in an HIV+ patient not on ARVs by significantly increasing the patients' CD4+ count (Shapiro and Masci, 1996). It therefore might inhibit HIV by an alternative mechanism (other than RT or PR inhibition) such as modification of a surface component of the virus (Okada *et al*., 1993). Compounds having a thiol ligand such as MCZS2 and its analogues (MCZS1 and MCZS3) have been implicated in such reactions as the thiol ligand undergoes ligand exchange reactions with proteins on the surface of the virion rendering it non infectious (Shaw, 1989). This is another structure function (activity) relationship.

It was also generally observed that in both the RT and the PR assays, none of the ligands (TTL3, TTL10, TTL17 and TTL24) tested had inhibitory effects on these enzymes. Similar observations have been made by other authors (Chircorian and Barrios, 2004, Sutton *et al*., 1972) who found that it was the gold in the gold complex itself and not the ligand that was necessary for therapeutic activity.

It is worth noting that the concentration (100 µM) at which these complexes inhibited HIV-1 protease were not clinically relevant concentrations (Georgiou, 2002, Traber *et al*., 1999, Yoshida *et al.,* 1998). However, because this was a cell free assay, it is possible that administration of these gold complexes at this concentration could allow for attainment of the relevant concentration in a whole cell or host situation such that their potential as anti-HIV protease inhibitors should be exploited.

4.4 CELL BASED ANALYSIS

4.4.1 ICP-AES

The use of an ICP source for quantification of metal ions in tissue culture specimens provides information about the disease state in the case were accumulation is due to illness (Stadler *et al*., 2004). ICP has also been applied in disciplines like environmental monitoring, geochemistry, material sciences, semiconductor fabrication, nuclear technology and biomedicine (Houk, 1994). The use of ICP for the measurement of uptake of drugs and gold phosphine complexes has been reported by McKeage *et al* (2000). It has been proposed that gold compounds may enter cells by a sulfhydryl shuttle (Snyder *et al.*, 1986). In this model, gold complexes undergo ligand exchange to bind to sulfhydryl groups in or on the cell membrane.

Knowledge of the amount of drug taken up allows for comparison of *in vitro* and *in vivo* activity such as toxicity and efficacy. It has been shown that some gold compounds that inhibited HIV-1 RT in direct enzyme assays *in vitro* could not be taken up by cells such that bioactivity was impossible (Zhang *et al*., 1995). Successful uptake allows for bioavailability and distribution and thus activity. We assessed the uptake of our compounds using ICP-AES for both the continuous cell line CEM.NKR-CCR5 and

PBMCs. At 25 and 50 uM, the uptake concentration in ppm/uM for the continuous cell line CEM.NKR-CCR5 is shown to drop compared to that observed in the PBMCs (Table 3.4). This could be attributed to the fact that the gold complexes were cytotoxic to this cell line (Figure 3.9 and 3.15) and hence a reduction in the number of cells available for uptake. The compounds however were not toxic to PBMCs (Figure 3.11 and 3.17) hence the higher uptake concentrations seen in these cells compared to the CEM.NKR-CCR5 cell line. On the other hand, because PBMCs are a mixture of cells (monocytes, T lymphocytes, natural killer cells etc) while CEM.NKR-CCR5 is a T cell line, the T lymphocytes present in the PBMCs could also be undergoing an unnoticeable cell death just as the CEM.NKR-CCR5. But because of the mixture of cells that make up PBMCs that may resist death, the cell death pattern observed with CEM.NKR-CCR5 cell line was not observed with the PBMCs.

It should however be noted that the concentration reported for uptake was not that of the gold complexes as a whole but that of gold metal as this was used to represent uptake of the gold complexes. Since the concentrations of the complexes tested were in the range of those tested for cytotoxicity and other bioassays, a conclusion could be made on the amount of gold taken up in comparison with bioactivity. Gold uptake of EK231 by CEM.NKR-CCR5 cells was the highest (0.77 µM- Table 3.4) and this compound was shown to reduce p24 secretion in HIV-1 infected CEM.NKR-CCR5 cells the most (Figure 3.18). Increased uptake and reduced cytoxocity therefore all contributed to the increased inhibition as seen for this compound in pathogen/host interactions.

4.4.2 Infection Confirmation

Bioassays were performed on both infected and uninfected cells. Infected primary cells were obtained from HIV-1 positive patients attending the Helen Joseph Hospital (Johannesburg, South Africa) and the cell lines were infected *in vitro* by the use of polybrene and spinoculation which both enhance the infection process (Le Doux *et al*., 2001, O'Doherty *et al*., 2000, Greeff, 2005). Once the cells had been infected, RT-PCR and DNA gel electrophoresis were used for confirmation of infection. Even though *in vivo* infection with HIV occurs relatively easy, the difficulties associated with obtaining successful *in vitro* infections necessitates confirmation of successful infection. The most accurate means of confirming infection by HIV-1 *in vitro* is by amplifying a portion of the viral genome from DNA isolated from the experimentally infected cells in this case the *gag* gene. According to our assay conditions, HIV-1 DNA peaked at 81.5 ºC (Figure 3.7) while the HIV-1 specific amplicon was detected at 142 bp (as expected based on positive primers used) (Figure 3.8). Both confirmation methods correspond to findings by other authors such as Gibellini *et al* (2006) who used SYBR green to detect HIV-1 in the genome of plasma samples.

4.4.3 Cytotoxicity Studies

Cell viability was assessed using the viability dyes MTT and XTT and an LDH cytotoxicity detection kit. Confirmatory assays were performed using flow cytometry. The complexes were screened over a concentration range of 1-200 µM.

Apart from the ligands and gold complexes EK231 and KFK154b, all the other gold complexes had an overall toxic effect on both the CEM.NKR-CCR5 (Figure 3.9) and PM1 (Figure 3.10) cells lines on which they were tested. These toxic compounds were

the gold (I) phosphine complexes (TTC3, TTC10, TTC17, TTC24, EK207, EK208, EK219, MCZS1, MCZS2 and MCZS3). The cytotoxicity observed may imply that the complexes either remained as gold(I) species and were quickly taken up as such or that one of their metabolites was highly cytotoxic (Bruni *et al*., 1999). EK231 is a gold (I) phosphine complex and did not appear to dissolve completely in DMSO. This might be a possible reason for its non toxicity. This cytotoxicity or lack of it was observed for both the uninfected and infected cell lines. Gold (I) phosphine complexes have been reported to be toxic to other cell types (Rackham *et al*., 2007, Mirabelli *et al*., 1986). Phosphines are reducing agents and it is possible that reduction of essential cellular components might result in cytotoxicity observed in these cells (Bernners-Price *et al*; 1986). Another possible cause of toxicity could be the oxidation of gold (I) to gold (III) (Goebel *et al.*, 1995, Shaw *et al*., 1994) which is a more cytotoxic form of gold (Parish and Cortrill, 1987). A proposed mechanism for this cell death caused by gold (III) could be by mitochondria dysfunction (Rackham *et al*., 2007). This was however not the case for KFK154b which is a gold (III) complex. There is a possibility that KFK154b was rapidly taken up as a gold (III) complex and was converted to cellular products that were non toxic to the cells as the trivalent form of gold has been reported to be more cytotoxic (Parish and Cottrill, 1987). Alternatively, this compound (KFK154b) might be tolerated more by the cell types that we employed here i.e. PBMCs, CEM.NKR-CCR5 and PM1

The compounds were non toxic to infected and uninfected PBMCs as viabilities were maintained to > 50% (Figure 3.11) when screened for cytotoxicity using MTT.

Using the LDH cytotoxicity detection kit, LDH release was lowest for the ligands, EK231 and KFK154b but not for the rest of the complexes. Increased LDH release (as

measured in the culture supernatant) is indicative of cell death or plasma membrane damage as LDH is a stable enzyme found in the cytoplasm of all cells. LDH release was prominent for both the CEM.NKR-CCR5 and PM1 (Figure 3.12 A and B) cell lines but the percentage of LDH released was least for the PBMCs. These findings confirm observations that were made when both the cells lines and the PBMCs were tested for cytotoxicity with XTT and MTT i.e. compounds more toxic to the cell lines than to PMBCs. However, TTC10, EK208, EK219 and EK231 appeared to be cytotoxic to the PBMCs at the 3 concentrations tested (Figure 3.13) contrary to findings in the MTT assay (Figure 3.11). A direct comparison can however not be made between the two assays as the compounds were incubated for different time periods (7 days in the XTT and MTT assays and 24 hrs in the LDH assay) with the cells. There is a possibility that in the PBMC MTT assay, cellular mechanisms to accommodate the compounds as the time of exposure increased were in play leading to increased proliferation over time (up to 7 days-Figure 3.11) than in the 24 hrs LDH assay. This concept is supported by reports that gold compounds activate the Nrf2 antioxidant pathway (Kataoka *et al*., 2001), a key cellular response pathway to accommodate electrophillic stressors over time (something not observed with the cell lines).

The fact that PBMCs are a mixture of cells types while the cell lines consist of just one cell type might be a contributing factor to the non toxicity observed for PBMCs and toxicity seen in the cell lines. The probability of the cells (in PBMCs) withstanding stressors is increased unlike in the cell lines where just one cell type was involved. Alternatively, the fact that cells lines were propagated continuously prior to use

(following freeze thaw cycles) while PBMCs were isolated and used for bioassays immediately might also play a role in the toxicity observed in the cell lines.

Flow cytometry was therefore a better comparative assay to the XTT and MTT experiments as the incubations times were the same.

4.4.4 Flow Cytometry

Confirmatory viability assays using flow cytometry were performed for uninfected and infected CEM.NKR-CCR5, PM1 and the PBMCs. A confirmatory assay was necessary as cells shown to be viable by tetrazolium dyes may already be undergoing some form of death (Traore and Meyer 2002). It was assumed that viral replication leads to decrease cellular viability while inhibition of viral replication would be reflected by increases in cellular viability for infected cells treated with compounds. As expected, the ligands, EK231 and KFK154b were the least toxic with viabilities well above 50% for uninfected and infected CEM.NKR-CCR5 cell line (Figure 3.15). In the case of the PM1 cell line, untreated cells had a viability of 50% while EK231 and KFK154b were below the 50% mark but with no significant difference in their percentage viability compared to the untreated control sample at 50 % (Figure 3.16). According to Dillon *et al*., (2001) rapidly dividing cells may have unstable membranes and therefore bind annexin V- a possible reason why the viability in these three samples were low i.e. the untreated control, EK231 and KFK154b.

The infected and uninfected PBMCs all had viabilities above 50% except for MCZS1 and MCZS3 (47 and 43% respectively) in the infected cell population (Figure 3.17 B). This is contrary to data obtained in the MTT assay where these gold complexes were non toxic at this concentration (25 µM - Figure 3.11 B). The observation by Traore and Meyer (2002)

that cells shown to be viable by tetrazolium dyes may already be undergoing some form of death may apply here.

In both the infected CEM.NKR-CCR5 and PBMC populations, higher concentrations of cells were dying by apoptosis which could be attributed to viral and immunological mechanisms (Roshal *et al.*, 2001). Viral mechanisms include toxicity caused by the accumulation of unintergrated viral DNA (Shaw *et al.*, 1984), membrane permeability changes resulting from viral particles budding at the surface of the infected cells (Fauci, 1988), and terminal differentiation causing a shortened life span of the CD4+ T-lymphocytes (Zagury *et al*., 1986) which all led to programmed cell death. Some of the immunological mechanisms that may contribute to death of CD4+ lymphocytes during HIV-1 infection are killing by specific CTLs and signaling through the CD4 molecule, leading to apoptosis (Kroemer, 2003, Finkel and Banda., 1994, Ameisen *et al*., 1991). Various groups have suggested that deleterious immunological interactions lead to the loss of immune cells by apoptosis (Ameisen *et al*., 1991, Laurent *et al*., 1991, Terai *et al*., 1991). Generally, fresh peripheral blood lymphocytes (PBL) from HIV-1 infected patients have an increased propensity to undergo apoptosis following stimulation *in vitro* compared to those from healthy individuals (Groux *et al*., 1992, Meyaard, 1992) which is similar to what we observed in our case (Figure 3.16). Terai and his colleagues (1991) also observed that acute HIV infection of a cell line (MT2 lymphoblasts) also led to increased susceptibility to apoptosis similar to what we observed with the CEM.NKR-CCR5 lymphoblastic cell line (Figure 3.15).

In general, in both cell types (continuous and primary), there was a higher percentage of cell death in infected cells than uninfected. The uninfected cells were killed only by the compounds while infected cells were killed by both the cytotoxic effect of the compounds and the cythopathic effect of the virus. Infection with HIV-1 *in vitro* also leads to enhanced expression of Fas ligand (Badley *et al*., 1996, Westendorp *et al*., 1995), such that apoptosis may also have been caused by inappropriate ligation of over expressed Fas Ligand with its receptor, Fas, on the surface of CD4+ cell lines. The uninfected cells however had more necrotic cells compared to infected cells. This is possibly because the virus continues to replicate in the infected population and finally overrides any necrotic mechanism while in the uninfected population the compound concentration stays constant hence the necrosis pattern observed.

A comparison of the data obtained using MTT, XTT and flow cytometry gave a similar pattern of death for both the cell lines and the PBMCs. This comparison was possible since both bioassays were performed over the same time period. Only one concentration of the complexes were tested for flow cytometry because flow cytometry is more sensitive, the CV values obtained indicates a lower variation between duplicate repeats (appendix section B Table 4).

4.4.5 Antiviral p24 Antigen Assay

The secretion of p24 was measured after pre-treating infected PBMCs from seropositive HIV patients and acutely infected CEM.NKR-CCR5 cells with gold compounds for 7 days. Compounds that were active against RT and PR at non cytotoxic concentrations to either the cell lines or PBMCs were assayed for p24. The concentration of p24 using the Coulter p24 kit allows for the generation of a standard curve that has a detection limit of

7.8-125 pg/ml. The p24 secretion in asymptomatic patients usually diminishes to undetectable levels following seroconversion as a result of the formation of immune complexes with antibodies (Rodriguez-Iglesias *et al*., 1992). The concentration of p24 released could therefore not be determined from the standard curve obtained (in the case of chronically infected PBMCs-Figure 3.19) due to its very low concentration. The secretion of this protein was therefore expressed as a percentage of the absorbance obtained relative to an untreated control sample with cells only (Traber *et al.,* 1999). With the acutely infected cells (CEM.NKR-CCR5), detectable p24 concentrations (Figure 3.18) were noted and fell within the range of detection for the standard curve obtained for the Beckman Coulter (California USA) p24 ELISA kit. EK231 significantly inhibited HIV-1 replication and as mentioned earlier (section 4.4.1), this compound was taken up the most by the CEM.NKR-CCR5 cell line (Table 3.4). A possible reason for its increased activity as it was more biologically available.

There was an observed difference in response of gold compounds during acute infection (Figure 3.18) and chronic infection (Figure 3.19) suggesting that the compounds as treatment may proof more successful early on in infection (acute infection) rather than at the chronic stages.

The production of p24 in acute infection (*in vitro* infection in this case) indirectly serves to confirm successful *in vitro* infection.

4.4.6 Hypothesis Revisited

According to the *in vitro* results obtained where 8 of the gold complexes were shown to inhibit HIV-RT and 4 inhibiting HIV-1 PR at concentrations within the clinically relevant range of concentrations of gold compounds notably used in chrysotherapeutically treated

patients, these gold compounds therefore warrant futher evaluation as lead compounds for anti-HIV drug development. Gold containing complexes can inhibit HIV replication *in vitro* and can thus serve as drug leads as was hypothesized. This inhibition of HIV *in vitro* is further worth evaluating considering that the gold compounds were not toxic to primary cells (both HIV uninfected and infected – Figure 3.11) and maintained their stability for at least a week in DMSO (solvent required for dissolving the compounds) solution. Although none of the gold compounds inhibited p24 (Figure 3.19) at 25 µM in PBMCs, the suggested reasons (i.e. the formation of immune complexes by p24 with antibodies as suggested by Rodriguez-Iglesias and his colleagues 1992) for this lack of inhibition warrants further evaluation of the compounds as anti-HIV agents following dissociating these complexes using acid (Pokriefka *et al.*, 1993).

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 CONCLUSIONS

Potential drug candidates must be tested in *vitro* prior to animal studies using different bioanalysis techniques. Most of the compounds tested here remained generally stable in DMSO after a 1 week storage period at 4 ºC (determined via UV-visible spectroscopy) and 37 ºC (confirmed via ^{31}P and ^{1}H NMR). The compounds were not stable in a physiological buffer. Because the *in vivo* environment is a physiological one, instability in such a medium could mean loss of bioactivity. Since gold drugs have been shown to be prodrugs, instability in a physiological media is not so serious because for bioactivity, the prodrug must still be converted to the active form.

The gold complexes were shown to be taken up by both the continuous cell line CEM.NKR-CCR5 and the primary cells PBMCs. These gold compounds can therefore have an intracellular effect. It has been reported that without uptake into cells, some gold compounds that had been shown to have activity in cell free assays lost their activity as reactions necessary for this activity normally occur within the cell.

Inhibition of vital viral enzymes such as RT (by eight gold complexes) and PR (by four gold complexes) indicates the potential of these compounds as RT and PR inhibitors. Gold compounds have been shown to generally inhibit HIV by inhibition of RT, this observation therefore further confirms the pool of information that has been gathered before. The mechanism of inhibition of our gold complexes is therefore by inhibition of

reverse transcriptase and protease activity. This is notwithstanding that other mechanisms of inhibition might be possible but which have not been exploited.

The complexes were shown to be taken up by both continuous cell lines and primary cells and are therefore capable of biological activity. This property thus allowed for further cell based assays.

The gold complexes were generally toxic to cell lines (CEM.NKR-CCR5 and PM1) and not to the primary cells (PBMCs). As mentioned earlier (section 4.4.3), the probability of the cells (lymphocytes, monocytes, natural killer cells etc) in PBMCs withstanding stressors is increased unlike in the cell lines where just one cell type (T cell line for both CEM.NKR-CCR5 and PM1) is present. Isolation of the T cell population from PBMCs for toxicity studies will therefore give a better idea of the toxicity observed in the CEM.NKR-CCR5 cell line. It is also possible that the toxicity exhibited by the gold complexes on the continuous cells lines is associated with the fact that these cells are immortalized cells (Trkola *et al*., 1999) and have cancerous properties i.e. both CEM.NKR-CCR5 (Howell *et al*., 1985) and PM1 (Lusso *et al*., 1995). Gold compounds have been reported to have anti-tumour activity e.g. auranofin was found to be toxic to some tumour cell lines (Bruni *et al*., 1999) and it and its analogues have shown both *in vitro* cytotoxic potency and *in vivo* anti-tumour activity against P388 leukamia cells in mice (Mirabelli *et al*., 1986). The observed death of the continuous cell lines after treatment with gold compounds and not primary cells (which are non cancerous cells) might be attributed to the anti-tumour activity of these compounds responding to the cancerous properties of these specific cell lines.

Since the PBMCs are immune system cells and were used to mimic the *in vivo* environment of HIV infection, non toxicity of the compounds to these cells is therefore advantageous in fostering them as potential anti-HIV drug candidates.

In the whole virus/cell scenario, EK231 significantly inhibited p24 expression at a non cytotoxic concentration in acutely infected CEM.NKR-CCR5 cells but not in chronically infected PBMCs. The compounds as drugs should therefore be considered for the treatment of acute infection and may need modification(s) in synthesis before they can be used in chronic infection.

The potential of these novel compounds as anti-HIV agents with respect to inhibition of RT and PR therefore warrants further investigation. The hypothesis that gold containing complexes inhibit HIV replication *in vitro* was thus verified.

5.2 FUTURE STUDIES

In order for pre-clinical and eventual clinical trials of these novel gold compounds to be suggested several preliminary analysis need to be completed.

5.2.1 Shelf Life Determination

The shelf life of these compounds at room temperature, 4 ºC and at physiological temperature will allow for knowledge on when and where to store compounds as well as when biological activity is lost as the exact duration after which RT activity was lost is not yet known.

5.2.2 Reactions with Proteins

Gold compounds are known to undergo ligand exchange reactions with sulfhydryl groups present on proteins (Berners-Price *et al*., 1986, Allaudeen *et al*., 1985). These are very

important reactions since gold drugs are prodrugs and the active component is not necessarily the administered compound (Shaw, 1999). To gain an insight into the potential role of sulfhydryl interactions on the *in vitro* and *in vivo* pharmacology of the compounds, it would be necessary to treat them with albumin and transferrin which are both metal transport proteins in blood (Berners-Price *et al*., 1996). The resulting structural switches induced by gold compounds on the proteins can be monitored by either ^{31}P NMR or ^{1}H NMR (Berners-Price *et al.,* 1996) ultimately giving an idea into their mechanism of action.

5.2.3 Test Lower Non Toxic Concentrations

Lower concentrations (< 1 µM) of the complexes should be tested for toxicity on the cell lines in particular followed by antiviral activity determination. In the current study, it was difficult to determine the effect of the complexes on p24 secretion in acutely infected CEM.NKR-CCR5 and PM1 cell lines as the complexes were generally toxic to these cell lines. Lower non toxic concentrations could thus be used to ascertain the effect of HIV-1 p24 secretion following treatment with the compounds. The assessment of these lower concentrations should be done with some caution as there are reports on some gold compounds having been shown to loose their activity below 1 µM and moreover, this concentration is below the clinically relevant concentration (Traber *et al.,* 1999). Alternatively, the antiviral effects of the compounds could be assessed in cell lines other than the two that were used here as different isolates can differ in their tropism and replication rates in different cell lines (Reeves and Doms 2002) thus resulting in different responses such as p24 secretion.

5.2.4 Use of Acutely Infected PBMCs for Determination of p24 Secretion.

Acutely infected PBMCs should be used in determining antiviral p24 secretion following treatment with the compounds. The compounds were not toxic to these cells so it will be easier to determine p24 on the acutely infected cells as opposed to the chronically infected cells. Also, p24 secretion was below the detection limit in chronically infected PBMCs due to bound antibody to this protein in asymptomatic patients (Rodriguez-Iglesias *et al*., 1992) such that acute infection *in vitro* will give a better picture. Since acute infection would be done *in vitro,* the concentration of virus used would be higher limiting interactions that could occur with any antibodies present. On the other hand, methods to dissociate the immune complexes that are formed by the virus and antibodies (Rodriguez-Iglesias *et al*., 1992) could be used. Pokriefka and his colleagues (1993) proposed the use of 0.5 N HCI or 1.5 M glycine buffer to achieve this dissociation.

5.2.5 Effect of the Compounds on other Viral Enzymes such as Integrase

The gold compounds could be assessed for inhibition of HIV-1 integrase. Integrase is also of vital importance in the HIV life cycle just as RT and PR. It has been targeted for therapeutic development with a recent anti-HIV drug (raltegravir) approved in October 2007 being an integrase inhibitor (Cahn and Sued, 2007, AIDSINFOnet).

5.2.6 Check if Compounds can be Viral Entry Inhibitors

Gold (I) compounds have been reported to protect MT-4 and CEM cells against HIV-1_{NL4-3} induced cytopathogenicity through the modification of a surface component of the virus (Okada *et al*., 1993). This applies to complexes having a thiol ligand such as MCZS1, MCZS2 and MCZS3. Ligand exchange reactions between the thiol ligand and

proteins on the surface of the virion render it non infectious (Shaw, 1989). The effect of the aurothiolates (MCZS1, MCZS2 and MCZS3) in modifying the surface proteins on HIV virion could thus be investigated.

5.2.7 Effect of the Compounds on Cytokine Secretion e.g. IL-2

Gold compounds have been shown to act by reducing the production of cytokines such as IL-6 and IL-8. These cytokines and others such as TNF-α are under the control of NF-κB. In cells latently infected with HIV, activation of this transcription factor leads to the triggering of viral genes resulting in an explosive increase in HIV replication (Traber *et al*., 1999). By monitoring the release of IL-2 (a cytokine that normally decreases in HIV infection - Lee *et al* 1996), the effect of the compounds on latently infected HIV- cells can be determined. Latent forms of HIV are the main deterrent for the complete eradication of the virus following HAART (Furtado *et al*., 1999) such that the potential of these compounds to eradicate latent HIV would be invaluable in the search for a cure for HIV. TNF-α (elevated in HIV infection – Rizzardi *et al*., 1998) and other cytokine levels known to be altered in HIV disease can also be monitored following treatment with the compounds to ascertain the effect of these compounds on cytokine production.

5.2.8 *In vivo* Evaluation for Antiviral Activity in a Mouse AIDS Model.

In vivo studies using mice to check cytotoxicity and the effect of the compounds on a mouse AIDS (MAIDS) model would be beneficial as preclinical studies. Many candidate drugs for the treatment of HIV have been evaluated using the MAIDS system as a small animal model (Yamaguchi *et al*., 2001). The complexes that were shown to have anti-RT and anti-PR activity could be tested in this model.

CHAPTER 6

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The following are additional websites that were all accessed and active between November and December 2007.

1) http://msl.cs.uiuc.edu/~yershova/bcb495/bcbProject-3_files/image015.gif.
2) http://tutor.lscf.ucsb.edu/instdev/sears/immunology/images/figure19-08a.jpg
3) http://www.engenderhealth.org/res/onc/hiv/understanding/images/hiv2d.gif.
4) http://www.new-science-press.com/info/illustration_files/nsp-immunity-10-4-10_12.jpg
5) http://www.research.philips.com/technologies/misc/matanalysis/downloads/icp-aes.pdf .
6) https://www.rocheappliedscience.com/servlet/RCConfigureUser?URL=StoreFramesetView&storeId=10151&catalogId=10151&langId=-1&countryId=za.

APPENDIX - SECTION A

UV-VISIBLE SPECTRA

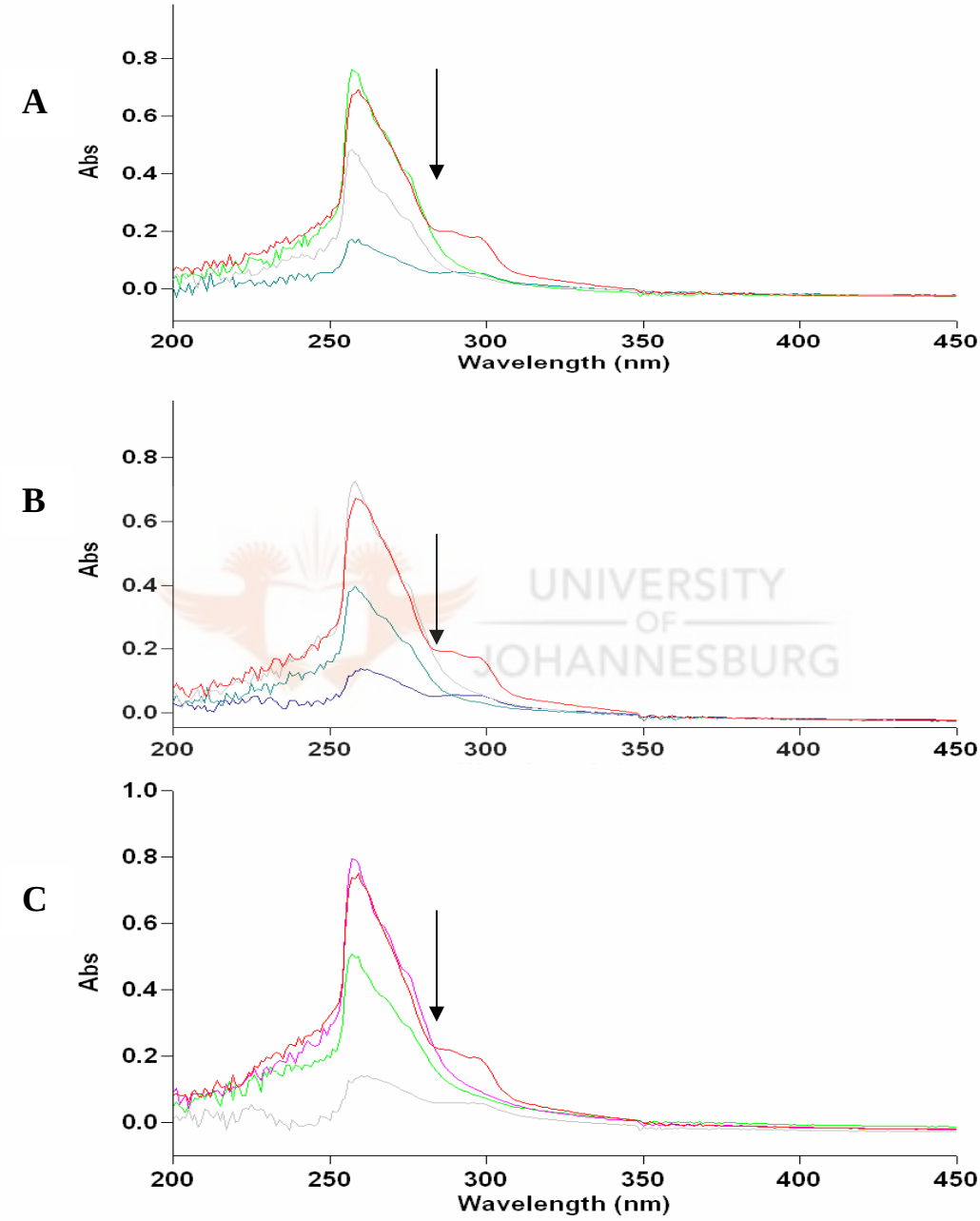

Figure 1: The electronic spectra of the gold (I) phosphine gold complexes. The complexes were dissolved in DMSO and the spectra taken immediately (A), after 24 hrs (B) and after 1 week (C) with intermittent storage at 4 ºC. In the direction of the arrow we have from TTC24, TTC3, and TTC17 and TTC10. There was no change in the electronic spectra (maximum absorbance is at 259nm) of the complexes overtime indicating that the complexes are stable over this time period in DMSO.

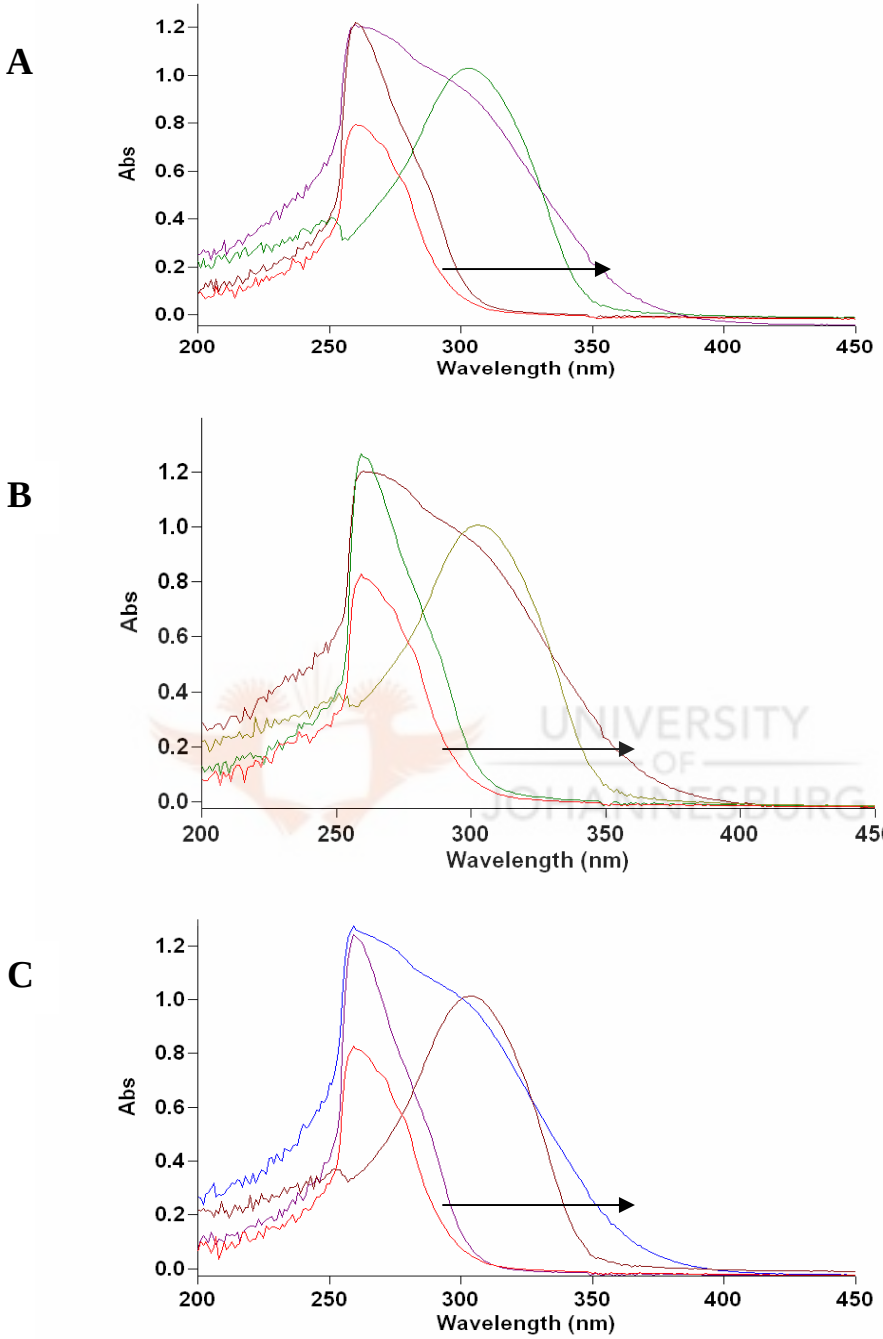

Figure 2: Absorption spectra of the gold DPPE group of gold complexes.
The gold complexes were dissolved in DMSO and spectra over UV-Visible obtained immediately (A), 24 hrs (B) and 1 week (C) after dissolution with intermittent storage at 4 ºC. In the direction of the arrow from left to right are EK207, EK219, EK231 and EK208.

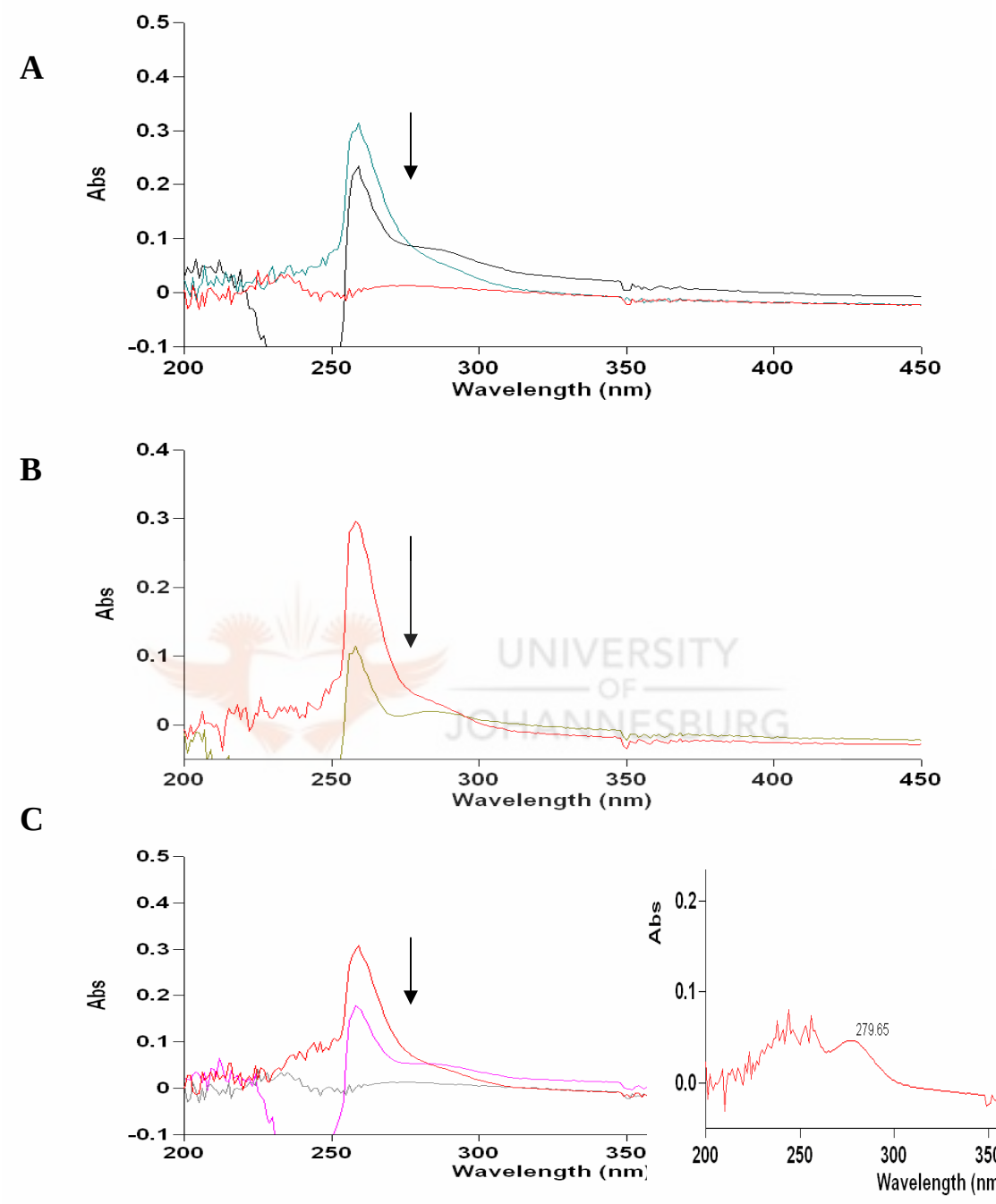

Figure 3: UV-Visible spectrum of auranofin (MCZS2) and analogues taken on immediate dissolution in DMSO (A), 24 hrs later (B) and after 1 week (C). In the direction of the arrow we have MCZS1, MCZS3 and MCZS2). Maximum absorbance for all three complexes is at 258 except for MCZS1 (279 nm - insert)

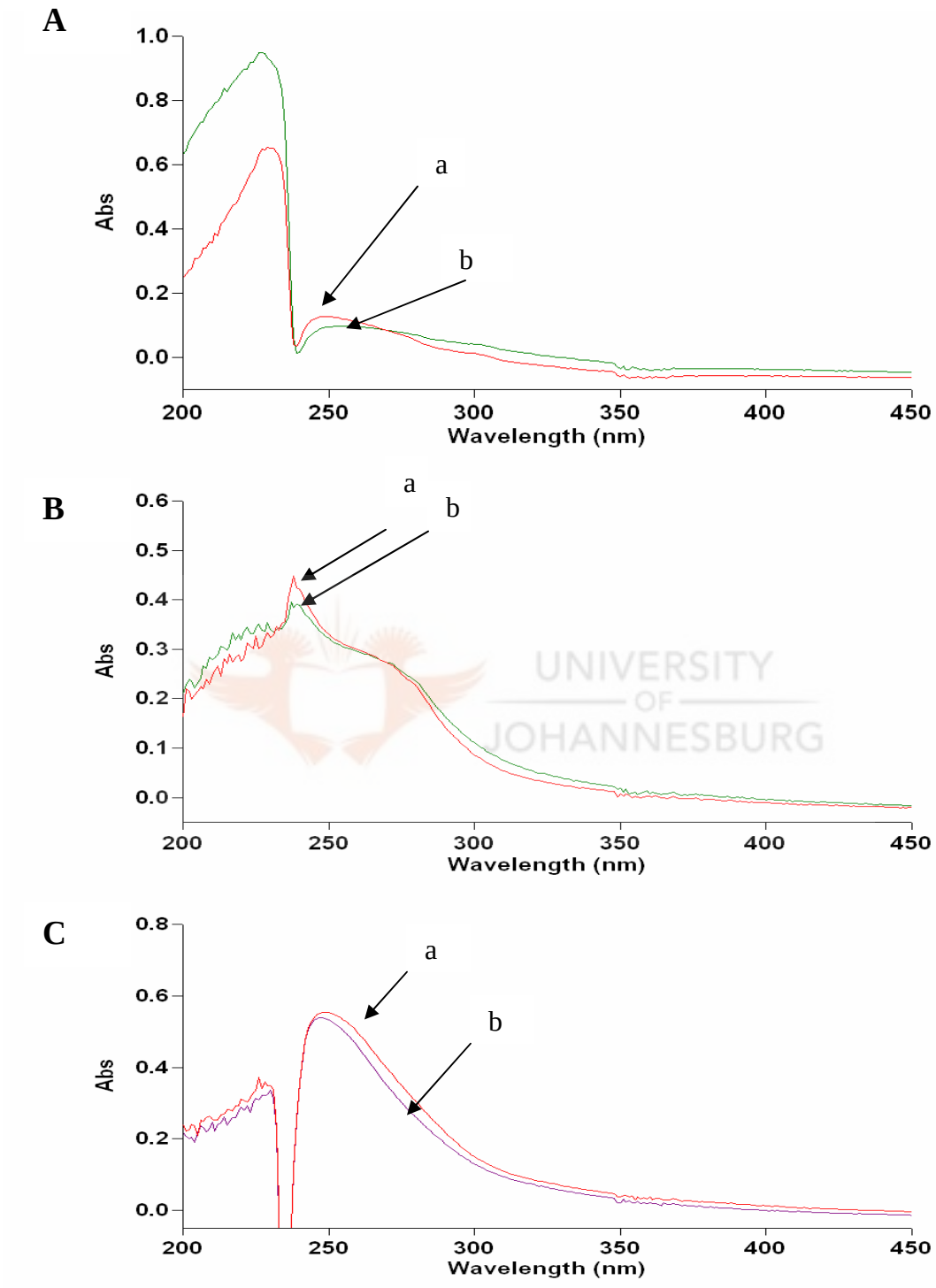

Figure 4A: Electronic spectra showing hydrolysis of TTC3 (A), EK207 (B), and KFK154b(C) under physiological conditions (PBS, pH=7.4).
The spectra were recorded at time intervals of 1 min (trace a) and 40 mins (trace b)

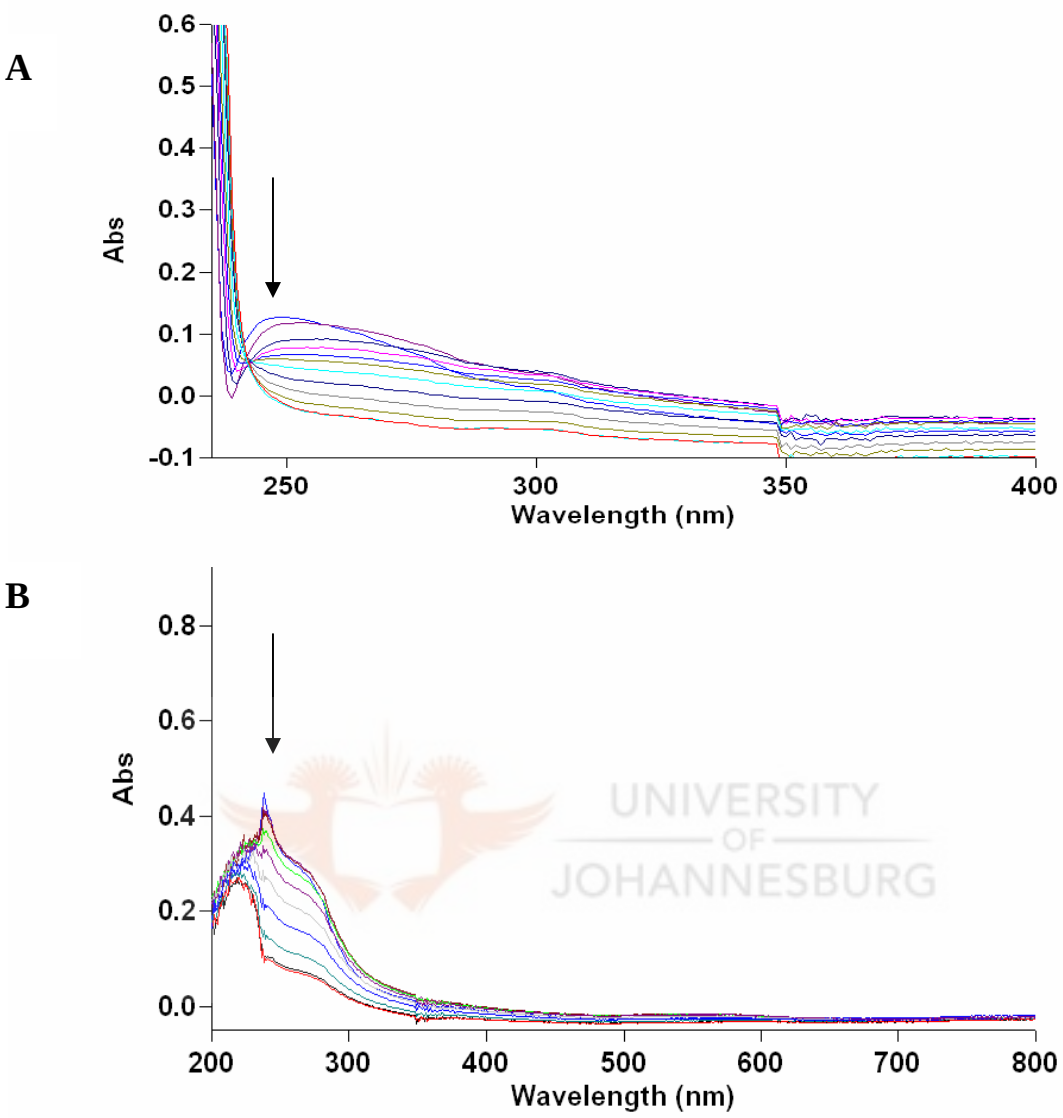

Figure 4B: Complete hydrolysis of TTC3 and EK207 over time (in a physiological buffer).
Complete hydrolysis of both gold complexes occurred after 14 hrs. The arrows indicate the direction of hydrolysis over time with intervals of 2 hrs.

NMR SPECTRA

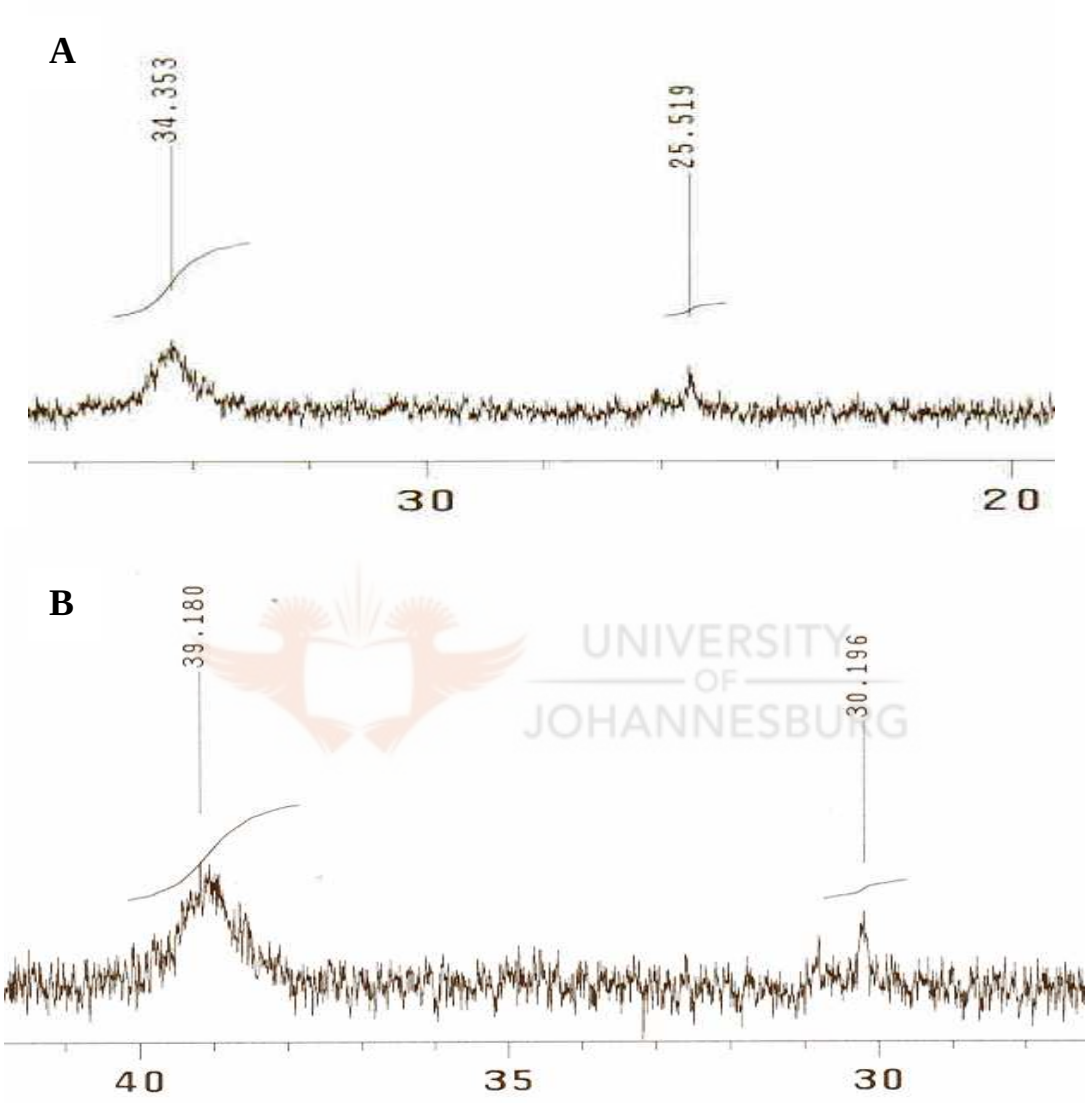

Figure 5: ^{31}P NMR of TTC10 on immediate dissolution (A) and after 24 hrs (B) in DMSO solution.

The observed chemical shift from 34.4 to 39.1 after 24 hrs is not relevant as there is a corresponding shift in the peak down stream from 25.5 to 30.2 (the change observed was therefore just a shift in the scale and not decomposition of the gold complex.

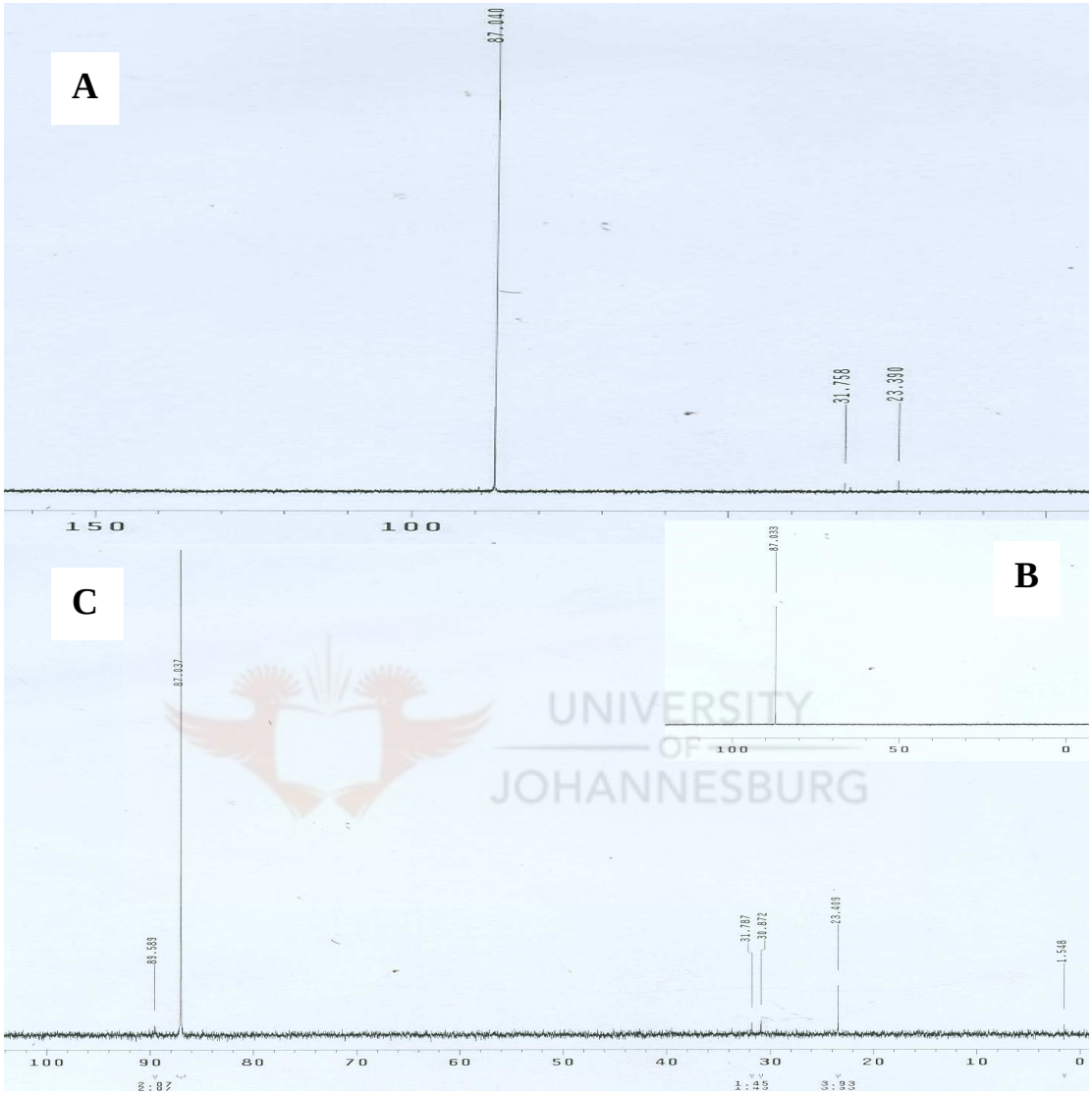

Figure 6: ^{31} P NMR of EK207 on immediate dissolution (A), 24 hrs later (B) and after 1 week in DMSO (C).

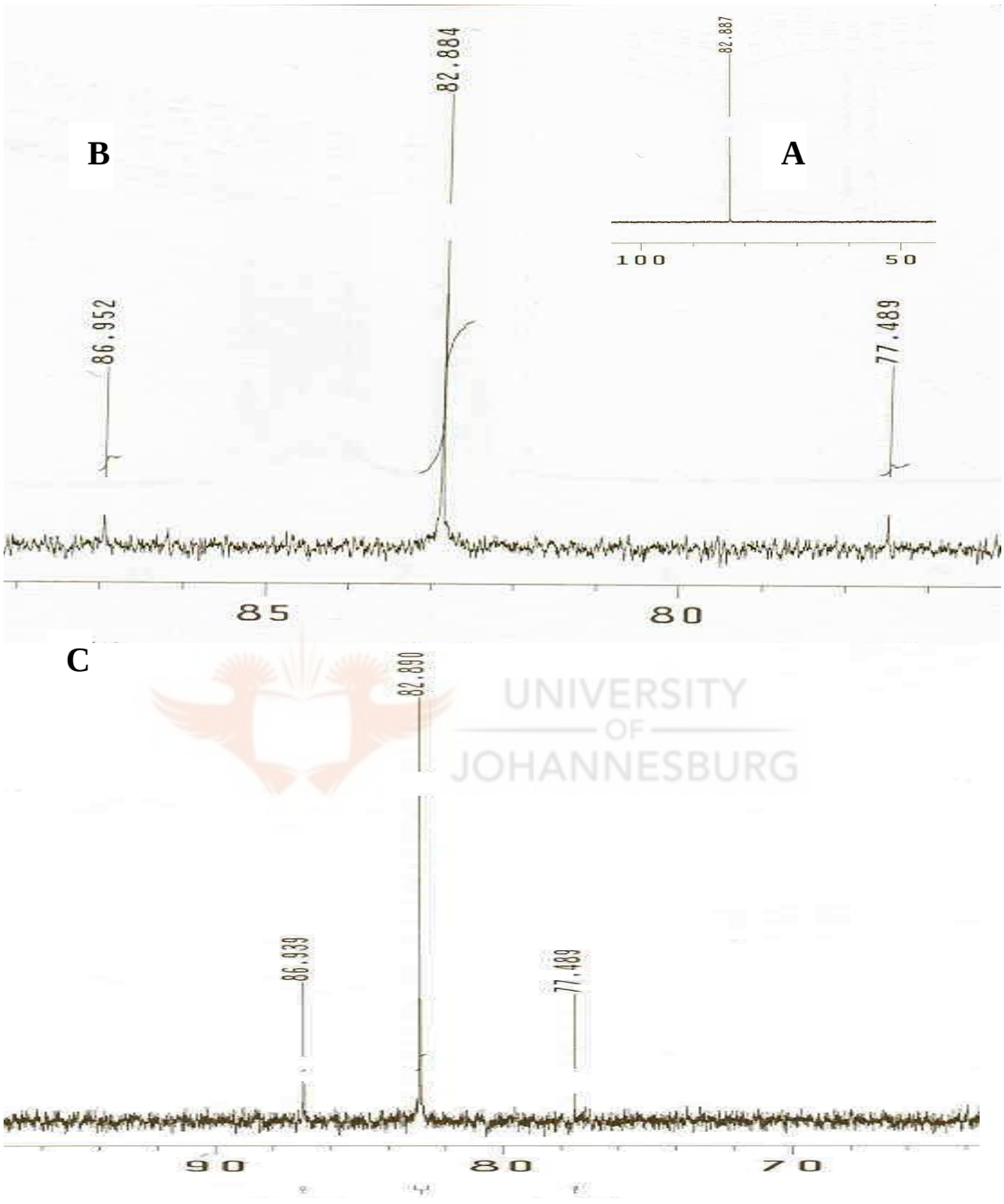

Figure 7: ^{31}PNMR spectrum of EK231 after 24 hrs (B) and 1 week (C) in DMSO solution.
The spectrum on immediate dissolution had a single peak at 82.8 ppm (A-insert)

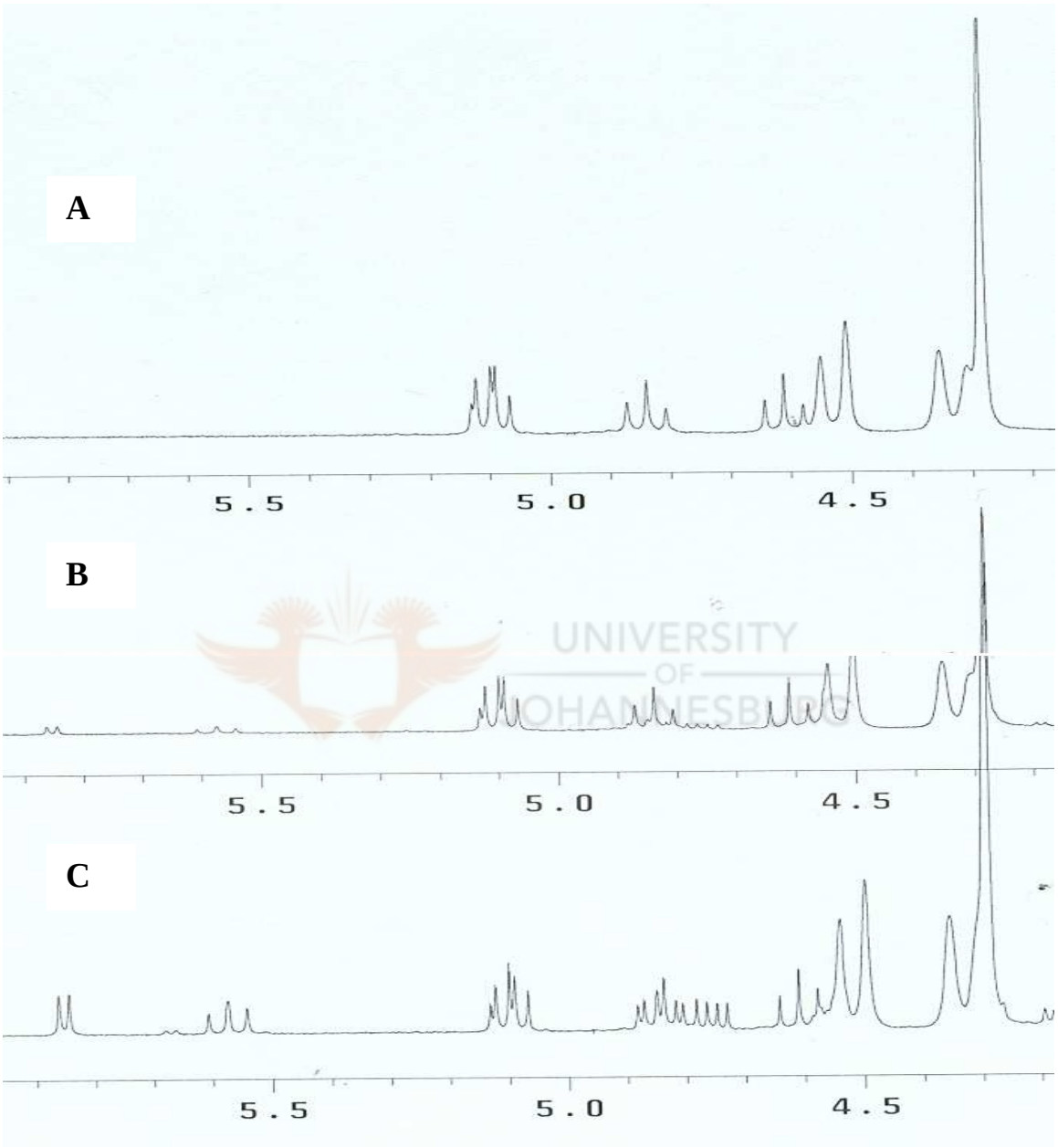

Figure 8: ^{1}H NMR of MCZS1 on immediate dissolution (A), 24 hrs (B) and 1 week (C) in DMSO solution.
A triplet (between 5.5.and 5.6 ppm) and a duplet peak (5.5 and 5.9 ppm) start appearing after 24hrs (B) and are even more visible by 1 week (C).

APPENDIX- SECTION B

% CV VALUES

Table 1: % CV values for infected and uninfected CEM.NKR-CCR5 cells using XTT.

Compounds	Uninfected CEM.NKR-CCR5			Infected CEM.NKR-CCR5		
	6.25 µM	25 µM	100 µM	6.25 µM	25 µM	100 µM
TTL3	32.76	34.33	69.55	9.01	4.07	4.79
TTC3	40.42	74.29	65.43	12.40	1.24	2.65
TTL10	26.73	46.31	51.83	2.99	11.66	37.95
TTC10	68.89	69.61	69.32	42.56	1.18	3.55
TTL17	33.72	51.50	74.10	16.77	19.65	3.01
TTC17	84.81	68.97	80.46	2.53	0.61	2.13
TTL24	22.52	18.46	62.21	3.74	4.58	4.19
TTC24	88.32	105.39	97.49	21.00	42.65	38.68
EK207	104.49	105.48	44.88	101.14	2.40	4.29
EK208	113.97	101.98	85.01	0.63	2.44	2.61
EK219	108.56	109.22	113.09	107.47	1.70	1.29
EK231	18.14	63.00	87.50	9.76	10.38	5.35
MCZS1	108.91	105.82	101.51	123.53	1.68	0.00
MCZS2	72.80	107.08	120.99	12.43	2.44	0.60
MCZS3	103.25	81.17	44.25	44.99	5.62	7.69
KFK154b	29.15	26.32	57.56	18.80	27.09	6.67

Table 2: % CV values for uninfected and infected PM1 cells using XTT and MTT

Compounds	Uninfected PM1 - XTT			Infected PM1
	6.25 µM	25 µM	100 µM	25 µM
TTL3	13.24	21.81	21.94	7.67
TTC3	21.79	17.07	41.39	5.46
TTL10	19.90	20.42	26.03	
TTC10	29.10	32.99	28.44	2.86
TTL17	13.72	12.44	56.92	1.63
TTC17	20.84	29.85	62.61	8.79
TTL24	17.68	13.45	3.00	
TTC24	11.51	5.37	75.08	6.93
EK207	63.94	216.85	56.08	5.74
EK208	29.12	11.88	9.80	15.96
EK219	5.38	22.74	19.00	11.93
EK231	7.44	7.49	9.06	1.16
MCZS1	6.61	0.00	15.11	20.00
MCZS2	26.46	35.77	43.04	1.63
MCZS3	26.89	38.44	7.03	0.16
KFK154b	29.29	26.81	26.21	6.65

Table 3: % CV values for uninfected and infected PBMCs using MTT

Compounds	Uninfected PBMCs					Infected PBMCs			
	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	6.25 µM	12.5 µM	25 µM	50 µM
TTL3	20.43	22.54	110.34	4.43	0.29	11.33	9.90	11.30	10.90
TTC3	16.27	22.51	138.32	17.00	5.57	3.86	10.79	5.85	6.75
TTL10	23.78	26.62	111.95	13.02	4.58	17.01	15.84	9.59	10.99
TTC10	19.72	23.28	118.10	22.20	10.54	4.58	8.02	2.68	6.58
TTL17	21.65	23.57	108.89	14.06	6.57	8.77	9.78	8.49	6.65
TTC17	1.35	2.55	189.30	3.60	4.84	6.20	4.65	4.90	4.95
TTL24	4.24	0.83	19.59	7.64	3.31	7.15	4.97	5.31	4.01
TTC24	7.16	5.67	79.14	3.05	9.03	6.12	2.84	1.96	3.88
EK207	5.92	8.54	144.22	5.38	13.29	6.66	3.37	6.77	5.88
EK208	3.88	7.39	190.49	1.68	1.81	26.39	5.79	2.13	3.33
EK219	26.77	7.70	28.77	13.33	9.10	1.47	19.89	5.25	10.75
EK231	27.06	1.85	6.85	13.13	4.49	3.82	17.90	10.77	6.98
MCZS1	25.56	3.08	12.06	11.76	10.21	6.72	19.44	8.56	5.63
MCZS2	15.98	2.84	17.77	16.20	8.03	5.46	8.03	5.19	4.75
MCZS3	15.59	5.21	33.43	5.69	3.64	8.50	8.64	9.61	16.70
KFK154b	3.02	3.46	114.56	11.82	10.93	11.33	9.90	11.30	10.90

Table 4: % CV values for uninfected and infected CEM.NKR-CCR5 (abbreviated CEM), PM1 and PBMCs using flow cytometry

Compounds	25 µM				
	Uninfected CEM.	infected CEM	Uninfected PM1	infected PBMCs	Infected PBMCs
TTL3	9.96	0.79		1.97	7.39
TTC3	7.09	32.45	10.87	6.25	0.11
TTL10	7.78	3.12		3.05	17.64
TTC10	11.56	57.33	7.16	15.57	9.78
TTL17	9.42	44.36		3.53	8.82
TTC17	6.90	27.25	5.94	13.78	19.00
TTL24	0.55	9.98		6.80	7.05
TTC24	8.31	15.79	14.58	7.34	12.83
EK207	5.41	0.00	8.71	17.42	0.82
EK208	16.73	15.02	0.90	11.89	7.40
EK219	4.44	6.78	27.16	21.34	3.34
EK231	0.62	8.66	12.87	14.41	1.79
MCZS1	0.69	6.78	8.47	12.70	11.31
MCZS2	15.89	8.66	8.24	3.19	1.60
MCZS3	3.24	22.53	6.41	1.97	16.53
KFK154b	0.55	6.99	1.63	6.25	10.84

Table 5: Comparative characteristics of viability assays used.

Viability/ cytotoxicity assay	Type and incubation time*	Parameters analysed	Detection Method	Advantages	Disadvantages	Wavelength
MTT	Tetrazolium (7 days)	Reduced metabolic activity	Spectroscopy	- Rapid - Automated - Suitable for large number of samples - Short incubation time (LDH)	- Needs solubilisatioin step. - Reduced by some component present in cell culture media e.g. glucose - MTT toxic to cells. - Generally longer incubation times with cells	540-690 nm
XTT		Reduced metabolic activity			ND	450-690 nm
LDH	Enzyme (24 hrs)	Released lactate dehydrogenase from damaged cells			ND	490-690 nm
Flow cytometry	Fluorochromes (7 days)	Apoptosis, necrosis and viability	Fluorescence	- Sensitive fluorochromes which target specific cell components such as PS and DNA in our case.	- Non specific binding of annexin in rapidly dividing cells might occur.	488-520 nm

ND = not determined, * This incubation time is the time during which the compounds were incubated with the cells prior to the addition of the dyes, antibodies or LDH working solution.

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