THE DEVELOPMENT, OPTIMISATION AND COMPARISON OF VARIOUS VIROLOGICAL ASSAYS AND THEIR USES IN ANTIVIRAL ASSESSMENT OF COMPOUNDS WITH POTENTIAL ANTI-HIV ACTIVITY.

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Declaration

This study represents the original work of the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in the study was carried out at the Inkosi Albert Luthuli Central Hospital, Department of Virology, under the supervision of Dr. Raveen Parboosing.

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Varish Singh

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This study could not have been completed without the help of Dr. Raveen Parboosing and the rest of the staff who contributed at the Department of Virology.

Dedication

I dedicate this thesis to my parents, Mrs Shirley Singh and Mr Rajyendra Singh and to my friends and family, who have been extremely supportive.

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List of Abbreviations

AIDS – Acquired Immunodeficiency Syndome

ARV – Antiretroviral

ATP – Adenosine triphosphate

AZT - 3'-azido-2',3'-dideoxythymidine

BSL III – Bio Safety level 3

CD4 – Cluster Determinant 4

cDNA – Double stranded copy of Deoxyribonucleic acid

Cyp –Cytochrome P450

DAIDS – Division of AIDS

DMSO – dimethyl Sulphoxide

DNA – Deoxyribonucleic acid

ECLIA – Electrochemiluminecsent Immunoassay

ELISA – Enzyme linked immunosorbent assay

EMEM – Eagles Minimal Essential Medium

Env – Envelope

FBS – Foetal bovine Serum

FCS – Foetal calf Serum

FI – fusion inhibitor

Gag – Group specific antigen

Gly – Glycine

HAART- Highly Active Antiretroviral Therapy

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HR- Heptad repeat

IL2 – Interleukin 2

mRNA – messenger ribonucleic acid

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH – Nicotinamide adenine dinucleotide phosphate (reduced form)

NADPH - Nicotinamide adenine dinucleotide phosphate (oxidized form)

NASBA – Nucleic acid sequence based assay

NNRTI – non-nucleoside reverse transcriptase inhibitor

NRTI – nucleoside reverse transcriptase inhibitor

NtRTI – Nucleotide reverse transcriptase inhibitor

PBMC- Peripheral Blood Mononuclear Cell

PBS – Phosphate buffered saline

PHA – Phytohaemagglutinin

PI – protease inhibitor

PMS - Phenazine methosulfate

Pol - Polymerase

Pro – Proline

RPMI – Rosa Parks Memorial Institute

RNA – ribonucleic acid

TAT - trans-activator protein

UNAIDS – Joint United Nations Programme on HIV/AIDS

VPU – Viral Protein U

XTT - 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

◦C – degrees Celcius

% - Percent

List of figures and tables

Ethics

This research has been approved by the BioMedical Research ethics Committee, University of KwaZulu Natal.

Ref.: H027/05

Abstract

The development and optimization of anti-viral screening methods are essential to develop newer more effective, treatments against HIV.

The XTT method is a widely described method for antiviral screening. Both continuous HIV-infected cells and experimentally infected T-cells have been used in the XTT assay. We compared these methods to screen several plant-derived extracts for cytotoxicity. Several considerations were taken into account when performing these tests (effect of media, solvents and plant enymes). Experiments were performed to investigate these effects. In addition, p24 and viral load quantification were compared as antiviral screening methods.

The study showed that several modifications were necessary when performing the XTT assay on plant extracts, due to the effect of media, solvents and plant enymes. The XTT assays and p24 assays performed using experimentally infected cells are far more specific than those using chronically infected cells. The use of viral loads as an antiviral screening method consistently demonstrated the expected efficacy of AZT.

Chapter 1: Review of Literature

1.1 Comments on the HIV pandemic

The human immunodeficiency virus (HIV), the aetiological agent of the acquired immunodeficiency syndrome (AIDS), is responsible for one of the worst pandemics to affect mankind. UNAIDS has estimated that 40.3 million people globally, and 5.5 million people in South Africa were living with HIV in 2005. According to the National HIV and Syphilis Prevalence Survey (2005), approximately 39.1% of antenatal attendees in KwaZulu-Natal were HIV positive. An estimated 2.8 million people worldwide died of the disease in 2005 alone, of which 2 million deaths occurred in sub-Saharan Africa (UNAIDS).

These statistics illustrate the global burden of the HIV pandemic and in particular its devastating effects in sub-Saharan Africa. The search for a preventative or therapeutic vaccine against HIV is the focus of intense research and monetary investment. Antiretroviral therapy has transformed a life threatening illness into a chronic manageable condition for individuals who have access to treatment. However, there is at present no successful vaccine or curative therapy for HIV infection.

1.2 Viral targets

The main targets of HIV-1 are CD4+ T cells, although other cells such as monocytes and macrophages are susceptible, since these cells also possess the CD4 cell surface antigen. This was explained by the discovery that the viral-envelope glycoprotein, gp 120, has a high affinity for the CD4 receptor (Dalgleish *et al*., 1984). However, experiments using mammalian cells transfected with the human CD4 gene alone did not exhibit susceptibility to HIV infection, leading to the conclusion that CD4 was not the sole receptor responsible for HIV infection of cells. Years later, a group of cell-surface chemokine receptors was identified, two of which are significantly relevant to HIV infection, namely CXCR4, originally called Fusin, (Feng *et al*., 1996) and CCR5 (Dragic *et al*., 1996). CXCR4 is designated as the co-receptor for syncytium-inducing strains of HIV, which appear late in infection, while CCR5 acts as the co-receptor for the non-syncytium-inducing primary strains of HIV and SIV, also known as macrophage tropic viruses (Deng *et al*., 1996; Dragic *et al*., 1996). Dendritic cells are moderately susceptible to HIV since they express low levels of CD4+, CCR5 and CXCR4 (Patterson *et al*, 2001; Sol-Foulon *et al*. 2002). Stebbing *et al*., (2004) showed that macrophages, having engulfed HIV infected lymphocytes, are fairly resistant to cytolytic effects and could act as a reservoir for the virus, while viral DNA has been shown to persist in natural killer cells up to 2 years after effective HAART (Velentin et al., 2002) illustrating that natural killer cells are indeed a viral reservoir (Stebbing *et al*., 2004).

1.3 Replication

Once the virion has bound to the cell receptors, conformational change in the gp120 occurs, resulting in the fusion of the virion envelope with the cell membrane aided by the fusion protein gp41 (Haseltine *et al*., 1990). Entry is further facilitated by co-receptors CCR5 or CXCR4 chemokine receptors as a result of interaction between the viral gp120 and these receptors (Khan 1990, Lennette 1995).

Within the cell, the uncoated virion undergoes reverse transcription, resulting in the production of a double-stranded DNA copy (cDNA) of the viral RNA. This viral cDNA is transported to the nucleus where it is integrated into the host genome by the viral enzyme integrase. The viral DNA is flanked by two long terminal repeats which are sequences essential for the transcription of the provirus (Lennette *et al*., 1995; Lennette *et al*., 1999). Viral mRNA is then transported out of the nucleus into the cytoplasm where it is translated. The genomic RNA along with translated viral proteins are assembled into virions that bud at the cell membrane. HIV protease cleaves the Gag-Pol proteins into their constituents and, in doing so, completes the maturation process of budding virions. As each virion is expelled from the host cell, it takes with it a lipid bilayer envelope from the host cell membrane preventing the host immune system from immediately recognizing it as foreign (Freed and Martin, 2001).

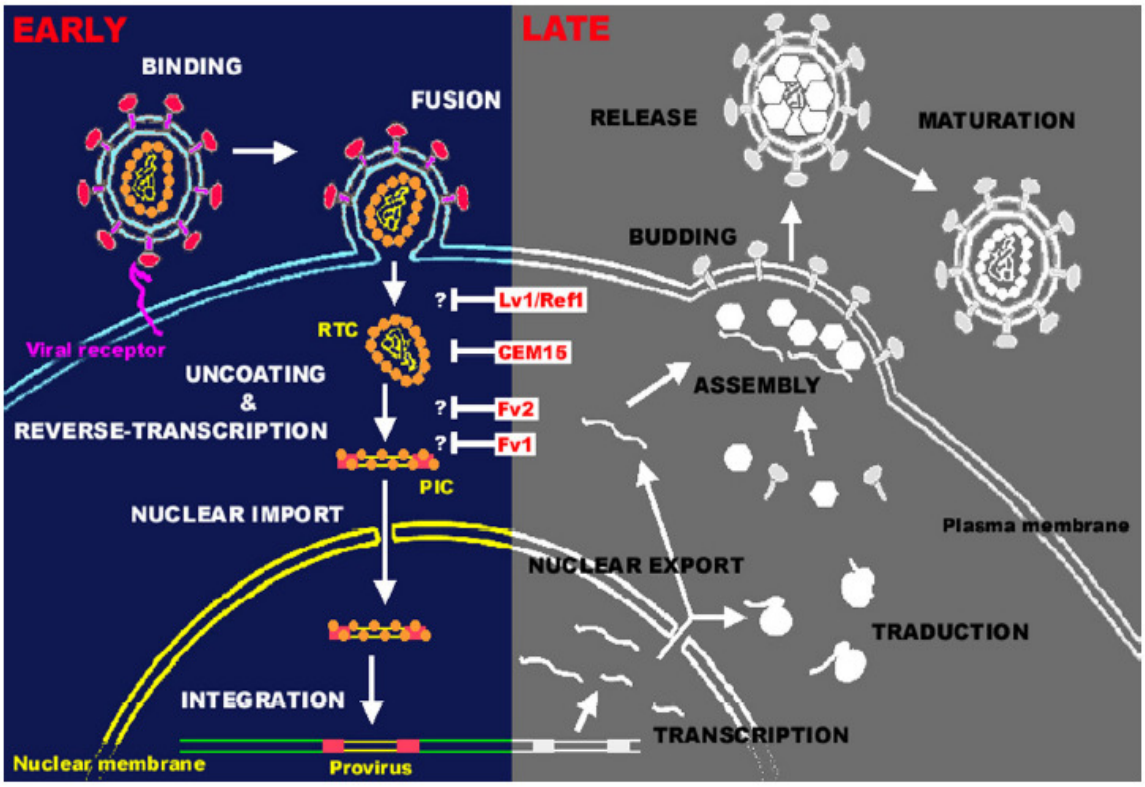

Figure 1: Early and late events in HIV-1 replication (Nisole and Saib, 2004)

1.4 Viral Phenotypes

Table 1: HIV isolate attributes

	X4	R5
Co-receptor	CXCR4	CCR5
Syncytia	SI	NSI
Replication	Rapid	Slow
Infection	Late	Early
Tropism	T-tropic	M-tropic

Isolates of HIV vary in their tropism, replication capacity, co-receptor usage, stage of disease progression and the ability to form syncytia (Regoes *et al*, 2005). These attributes need should be considered when using these isolates in cell based assays e.g. X4 HIV-1 isolates may

replicate less efficiently in leukaemic T-cell lines such as H9 (Platt *et al*, 2000; Kozak *et al,*1997).

1.5 Progression of infection

The virus reaches the regional lymph nodes where extensive replication occurs. This is followed by a rapid rise in the viral load and dissemination of virus to other parts of the body. Only about 2% of the total viral load exists in the blood while approximately 98% exists in the tissues such as those of the central nervous system and in tissue macrophages (Lennette *et al*., 1999). The initial viraemia can exceed several million copies per millilitre of blood. The immune system reduces viral replication to a steady state by initiating a virus-specific immune response. CD8 lymphocytes (cytotoxic-T cells) play a crucial role by inducing cell lysis of infected cells. A patient who has a high initial viral load is a more likely to progress rapidly to AIDS and death.

Seroconversion occurs between one week to three months (Pantaleo *et al*., 1993) after exposure and manifests as fever, diarrhoea, lymphadenopathy and a macular papular rash (Acute Seroconversion Illness). These symptoms generally last up to four weeks. This is followed by an asymptomatic phase that may persist up to 10 years or longer. Finally, the symptomatic phase results, correlating with a decline in immune competence and the appearance of secondary infections. Viral load increases, CD4 cell count drops and opportunistic infections set in. At this stage, the transition from HIV infection to AIDS is complete, and in most cases the patient will die within 2 to 10 years of the onset of infection.

The great concern with HIV infection is that, as the virus replicates, mutations are induced as well as adaptations to the environment (Douglas, 1996). This occurs because the virus reverse transcriptase lacks proofreading mechanisms and is thus more error-prone than DNA polymerases (Hinuma *et al*, 1982). However, this results in the production of at least one base change at each round of replication, and with an estimated 10^{10} virions produced each day from a genome of 10^5 bases, at least one mutation at each nucleotide is produced each day. As a result enormous amounts of continuously generated variants, each with its own selective advantage are incorporated in the infected individual. The large numbers of virions produced compensate for the proportion of non-functional virions. Thus, an HIV positive individual may be infected with a population of HIV strains, each with varying genotypic and phenotypic characteristics, known as "quasispecies".

1.6 Available Treatment

Treatment includes the use of antiretrovirals that either inhibits the viral enzymes reverse transcriptase and protease or blocks the fusion of the virion to the $CD4^+$ target cell. Treatment has been shown to reduce the amount of HIV in blood (Lennette *et al*., 1999). Several types of antiretroviral drugs are available for the treatment of HIV (Table 1). Five classes are discussed:

1. Nucleoside analogue reverse transcriptase inhibitors (NRTIs)

The NRTIs were one of the first drug types used in the treatment of HIV infection (Lennette *et al*., 1995; Potter *et al*., 2004). Their function is to intercept the synthesis of DNA by inhibiting the viral enzyme reverse transcriptase, which is responsible for copying the viral RNA into DNA in an infected cell. Since nucleoside analogues are mono-phosphorylated, two additional phosphorylations are required to achieve triphosphorylation before these analogues are able to serve their role as active, DNA synthesis inhibitors (Cohen and Fauci, 2001; De Clercq, 1987). The phosphorylated NRTIs are mistaken for natural nucleosides by the reverse transcriptase enzyme, and are incorporated into the viral cDNA chain. However, when NRTIs are incorporated into a DNA strand during synthesis, further nucleoside addition is prevented, due to the absence of a 3' OH group. This results in chain termination and the production of an incomplete copy of DNA.

2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

The NNRTIs impede viral DNA synthesis by inhibiting reverse transcriptase. Unlike the NRTIs, however, which bind to the active site of reverse transcriptase, the NNRTIs bind adjacent to the active site, and prevent its interaction with natural nucleotides by stereochemical interference. This occurs since the shape of the enzyme is changed preventing its normal activity (Lachman 1990).

3. Protease Inhibitors (PIs)

Proteases are digestive enzymes contained in living cells as well as in viruses and are responsible for cutting up and processing proteins into their functional forms. The HIV protease cleaves the *gag-pol* polyprotein into functional units. Protease inhibitors bind to the HIV protease preventing the enzyme from correctly processing the viral proteins into their functional forms (Potter *et al*., 2004). These viral protein particles are immature, and the resulting virion is therefore non-infectious. The PI drugs in use are generally peptide-mimicking compounds which greatly resemble the natural substrate of HIV protease. These drugs are also able to bind more tightly to the protease than the natural substrate.

4. Fusion Inhibitors

Drugs of this class target the HIV envelope protein gp41, which is involved in viral entry into new cells. These drugs block the interactions between regions of the gp41 molecule with the target cell membranes therefore preventing fusion (Potter *et al*., 2004). Fusion inhibitors interfere with the conformational change of the envelope molecule, required for virus entry into target cells (Lennette *et al*., 1995; Lennette *et al*., 1999).

5. Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)

The NtRTIs are a new generation of drugs that have a similar mechanism of action as NRTIs, but are monophosphorylated. The only approved NtRTI at this stage is Tenofovir, which is

metabolised intracellularly to its active form, Tenofovir diphosphate, in order to facilitate DNA chain termination. Tenofovir has a better pharmacokinetic, toxicity and resistance profile than NRTIs (Lyseng-Williamson *et al*., 2005).

1.7 AZT (3'-azido-2',3'-dideoxythymidine)

This Nucleoside Reverse Transcriptase Inhibitor was first synthesised by Horowitz *et al*. (1964) and modified by Lin and Prusoff in 1978 (Mitsuya *et al*., 1985). Ostertag *et al*. (1974) first reported the activity against the Friend retrovirus and showed that AZT in its tri-phosphorylated form results in chain termination (De Clercq *et al*., 1987).

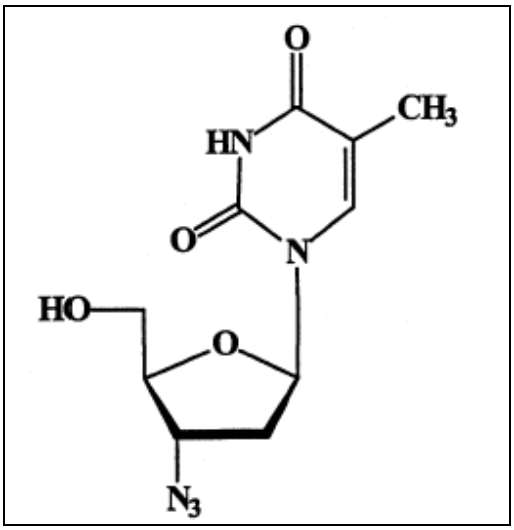

Figure 2: AZT structure (De Clercq, 2001)

The azido group contributes to the lipophillic nature of the compound, allowing it to easily diffuse across cell membranes and the blood-brain barrier (Balzarini *et al*., 1999). Cellular thymidine kinase converts AZT to the 5'-mono-, di- and tri-phosphate forms (Furman *et al*., 1986), which have the ability to inhibit HIV reverse transcriptase by competing with

endogenous nucleosides for incorporation into the DNA chain generated by the reverse transcription of HIV RNA. Chain termination is due to the absence of a 3'-hydroxyl group in AZT. The 3'-hydroxyl group allows for the attachment of nucleotides to the growing DNA chain.

Table 2: Classes of licensed HIV-1 antiretroviral drugs

Class	Drugs	Target	Mechanism of Action
Nucleoside Reverse transcriptase inhibitors (NRTIs)	Zidovudine (AZT) Didanosine (ddI) Zalcitabine (ddC) Stavudine (d4T) Lamivudine (3TC) Abacavir (ABC)	Reverse Transcriptase	• Phosphorytlated by cellular enzymes • Competitively inhibits the synthesis of viral DNA/ causes chain termination to prevent this process
Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)	Tenofovir (TFV)	Reverse Transcriptase	• As for NRTIs • Tenofovir is a nucleotide analogue
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Nevirapine (NVP) Delaviridine (DLV) Efavirenz (EFV)	Reverse Transcriptase	• Not phosphorylated • Non-competitive inhibition of viral DNA synthesis • Binds directly to the enzyme
Protease Inhibitors (PIs)	Saquinavir (SQV) Indinavir (IDV) Ritonavir (RTV) Nelfinavir (NFV) Amprenavir (APV) Lopinavir (LPV) Ataznavir (ATV) Darunavir(TMC114)	Protease	• Binds to active site on protease thereby inhibiting the enzyme
Fusion Inhibitors (FIs)	Enfuvirtide (T-20)	Envelope gr41	• Binds to HR1 of gp41

1.8 HAART

The introduction of Highly Active Antiretroviral Therapy (HAART) results in a reduction in plasma viral load to below detection limits (Potter *et al*., 2004) and has extended the asymptomatic phase of infection, thereby increasing the quality of life for many infected individuals (LaBranche *et al*., 2001). HAART, or triple therapy, which typically includes a minimum of two NRTIs and a PI or a NNRTI, has dramatically changed the prognosis of HIV-infected individuals in the developed world (Pallella *et al*.. 1998). However, given that HIV cannot be eradicated, most patients select drug resistant strains over time and need a change in their treatment combination. The accumulation of multiple resistance mutations is a major obstacle to the long-term control of viral replication (Shafer *et al*., 1998). This fact validates the continuous need for new drugs, particularly compounds belonging to new classes which target different steps of the HIV replication cycle, and lack cross-resistance with current anti-retrovirals (Poveda *et al*., 2005).

1.9 Problems with currently available drugs

Toxicity, poor adherence and development of resistance are obstacles to the success of HAART (Table 2).

1.9.1Antiretroviral Toxicity

Various factors influence the incidence of adverse effects associated with current antiretroviral drugs including the ethnic origin of the patient, nutritional status, problems with adherence, drug interactions and the use of concurrent medications. Side effects may be minor, severe or even life-threatening (Table 2) and may influence quality of life, adherence and development of resistance.

Peripheral neuropathy occurs in patients taking d4T in combination with isoniazid, an anti-TB drug. This type of drug combination is common in HIV infected patients since these individuals are susceptible to opportunistic infection such as tuberculosis. Continued treatment, in such instances, impairs the patient's ability to function, thus reducing his/her quality of life.

In some cases, side effects may escalate to life threatening conditions such as severe mucosal and skin lesions (e.g. Stevens-Johnson syndrome and toxic epidermal necrolysis) (Colebunders *et al*., 2004). These, in addition to other conditions such as severe hepatotoxicity and lactic acidosis, require treatment interruption.

In the case of pregnancy, certain drugs such as Efavirenz cannot be administered as a result of its teratogenicity (SUSTIVA Package Insert).

Mitochondrial toxicity, lipodystrophy, dyslipidaemia and hyperglycemia are adverse effects of antiretroviral therapy, collectively known as Syndrome X. NRTI inhibition of mitochondrial DNA polymerase gamma leads to mitochondrial toxicity by an impairment in the synthesis of the mitochondrial enzymes required in the generation of ATP by oxidative

phosphorylation (Moyle, 2000; Feng *et al*., 2001; Potter *et al*., 2004). Lactic acidosis results, due to elevated plasma lactate levels (Birkus *et al*., 2002). NRTI and PI use leads to lipodystrophy, characterised by peripheral lipoatrophy of the limbs, buttocks and face, accompanied by an accumulation of fat around the abdomen and trunk. Lactic acidaemia, insulin resistance, hypertriglyceridaemia and hypercholesterolaemia are the metabolic features associated with lipodystrophy (Chen *et al*., 2002).

As a result, patients on antiretroviral therapy require constant laboratory and clinical monitoring and specialised medical care to minimise the risks and adverse effects associated with treatment.

Patients also may experience side effects such as nausea or vomiting which contributes to poor adherence to drug regimens. This, compounded by issues such as poor drug penetration into certain tissues, low levels of viral replication in cellular reservoirs, high pill burden and complex regimens are also significant obstacles to successful antiretroviral treatment.

1.9.2 Development of Resistance

Suboptimal treatment with antiretroviral drugs can result in the selection of HIV variants with resistance to NRTIs, NNRTIs or PIs which may lead to treatment failure, disease progression and AIDS. The emergence of resistance can be attributed to the genetic variability of HIV arising from the high viral replication and mutation rates, viral recombination, poor adherence

and drug selective pressures (Table 2).

Genetic variability manifests in infected individuals as a complex mixture of heterogenous strains of HIV known as "quasispecies". These quasispecies compete amongst themselves to survive and propagate and this process is determined by intra-host factors. The dominant strains are those which have shown successful adaptation to this environment. However this process, while advantageous to the virus, is a key determining factor in the emergence of drug resistant virus. The number of cases involving the transmission of resistant viruses to newly infected persons is also on the increase (Blower *et al*., 2001; Hecht *et al*., 1998).

RNA viruses such as HIV have a mutation rate, due to the error-prone reverse transcriptase enzyme, which is 100 times higher than that of DNA viruses, bacteria as well as other eukaryotes (Mansky *et al*., 1995; Monno *et al*., 1999). Furthermore, HIV has a high turnover which is estimated to be 10^9 virions per day (Potter *et al*, 2004). The combination of the high mutation rate and rapid viral turnover is also a key factor in the emergence of drug-resistant variants.

Genetic recombination is a process that further contributes to resistance, particularly high-level multi drug resistance (Moutouh *et al*., 1996; Wain-Hobson *et al*., 2003; Yusa *et al*., 1997). Recombination is a strategy for viral rejuvenation and the generation of genetic diversity which may create a multiple drug resistant virus out of two single-drug resistant viruses (Moutouh *et al*., 1996; Kellam *et al*., 1995; Gu *et al*., 1995; Burke, 1997) and is likely to facilitate the evolution of viral stains displaying resistance to all anti-HIV-1 drugs.

Other factors influencing viral resistance include the selective pressures imposed by antiretroviral drugs, suboptimal pharmacokinetics, poor drug penetration, continued viral replication in cellular reservoirs and inadequate patient adherence (Larder, 2001; Potter *et al*., 2004).

Patients who fail therapy have minimal options for further clinical management. There therefore needs to be a strong focus on research into new drugs that could target other aspects of the HIV replication cycle giving rise to new classes of drugs, or new drugs in existing classes with improved pharmacological properties. This could lead to new options for HAART.

Table 3: The obstacles to successful HAART

Obstacles to effective HAART	Associated problems
Factors influencing adherence to medication	• Pill burden

	• Dietary requirements • Concurrent medication • Drug interactions and Side effects
Side Effects	Minor • Nausea, vomiting, dizziness Severe • Peripheral neuropathy • Lactic acidosis • Lipodystropy Life-threatening • Stevens-Johnson Syndrome • Hepatotoxicity Teratogenecity
Development of Drug Resistance	• High rate of viral replication • Viral genetic diversity • Viral recombination • High mutation rate • Drug selective pressures • Drug pharmacokinetics • Continued viral replication in reservoirs • Poor adherence

1.10 New drugs and drug targets

There is great urgency in the search for and development of new drugs that will be easily available to patients, well-tolerated without devastating side effects and with the potential to suppress viral strains which are already resistant to existing drugs. The challenges faced include issues of drug potency, pharmacokinetics and the ability of new drugs to "resist" resistance.

With this in mind, new viral targets have been identified, providing new opportunities for drug discovery (Table 3). Many new drugs targeting HIV reverse transcriptase and protease as

well as the fusion process are in development (Table 3), with the hope of finding new options for therapy in old classes of drugs.

1.10.1 Entry

The envelope protein complex (Env) of HIV controls the mechanism of viral entry into cells (Chan and Kim, 1998). Env is composed of the transmembrane sub-unit gp41, which is made up of heptad repeat regions known as HR1 and HR2, and the surface sub-unit gp120, which is non-covalently associated with gp-41. The process of viral entry is initiated by the binding of the virion gp120 to the CD4 molecule on target cells, which induces a conformational change resulting in the secondary binding of gp120 to the co-receptor CXCR4 or CCR5, depending on the infecting virus strain (Lenette, 1995). The result is a shift from a non-fusional state to a fusional state (Poveda *et al*., 2005).

This then triggers a conformational change in transmembrane gp41 resulting in the initiation of the fusion process. The N-terminus domain of gp41 becomes exposed and is inserted into the cellular membrane forming a fusion "pore", thus allowing the fusion of the viral membrane with the target cell membrane. This results in the entry of the viral capsid into the cytoplasm (Weiss, 2003)

In 1993 a synthetic peptide called DP-178 was designed with a similar amino acid sequence to HR2. Renamed, T-20 or Enfuvirtide (Wild *et al*., 1993) this drug mimics the HR2 region of

gp41 (Dietrich, 2001; Weiss, 2003) and became the first fusion inhibitor approved for clinical use. In addition to the fusion inhibitors, other examples of entry inhibitors include the co-receptor agonists such as bicyclams, which were initially postulated to have inhibitory effects on the viral uncoating process (De Clercq, 1995).

It is evident that HIV entry is a complex process involving an array of protein interactions, each of which may be potential targets in the search for new drugs to treat HIV infection (Cos *et al*., 2004; Kilby and Eron, 2003; Poveda *et al*., 2005).

1.10.2 Reverse Transcription

Once the viral capsid has entered the cytoplasm, enzymes within the nucleoprotein complex are activated. The viral core undergoes progressive disassembly (Nisole and Saib, 2004), known as uncoating and the pre-integration complex is formed (Krogstad, 2003). This complex includes viral RNA, reverse transcriptase, integrase and protease, and other viral proteins required for the translocation of the viral DNA complex to the nucleus where the linear viral DNA copy is inserted into the host cell genome. The RNA molecules packaged within the pre-integration complex are reverse transcribed by reverse transcriptase into a double-stranded DNA molecule or cDNA (Telesnitsky and Goff, 1997).

The HIV-1 reverse transcriptase controls three functions. These are the reverse transcription of viral RNA to DNA by the RNA-dependant DNA polymerase activity of reverse transcriptase, the degradation of the RNA template by RNAse-H activity and the duplication

of the remaining DNA strand by DNA-dependant DNA polymerase activity.

The viral reverse transcriptase is a unique target since the process of RNA-directed synthesis of DNA does not occur in uninfected cells. As a result, this enzyme has been and still is considered to be one of the foremost targets for new antiretroviral drugs in the NRTI, NNRTI and NtRTI classes.

1.10.3 Integration

On completion of the reverse transcription process, the pre-integration complex is transported through the nuclear pores into the nucleus where the double-stranded viral DNA (cDNA) is integrated into the host genome by the viral enzyme integrase (Cos *et al*., 2004; Potter *et al*., 2005; Tang *et al*., 1999). This occurs through the assembly, processing and subsequent strand transfer of the viral DNA complex (Condra *et al*., 2002). Integrase achieves these functions by first cleaving the last two nucleotides from the 3'-end of the linear viral DNA, which then covalently links to the host chromosomal DNA. Finally unfolding occurs and gaps are repaired by cellular enzymes (Krogstad, 2003; Middleton *et al*., 2004).

Studies using integrase-negative mutants of HIV show the production of non-infectious virus particles illustrating that HIV integrase would be an attractive target for inhibition.

1.10.4 Transcription and Protease Activity

The two long terminal repeats that flank the viral DNA are necessary for transcription. The trans-activator protein TAT levels increase after integration of the viral DNA into the host genome and further aids the process of transcription by stimulating the transcriptional activity of the long terminal repeats (Mann and Frankel, 1991).

The viral mRNA is transported out of the nucleus into the cytoplasm, where translation occurs. The RNA transcripts, as well as translated viral proteins are assembled into virions that bud at the cell membrane. The HIV enzyme protease then becomes active, leading to the cleavage of the Gag and Gag-Pol polyproteins and the re-organisation of the viral core (Krogstad, 2003). This process ensures the budding of mature, infectious progeny virions.

Studies using virus with mutations in the active site of HIV protease or the use of drugs which exert pharmacological inhibition of protease activity, produce immature, non-infectious progeny virions (Schatzel *et al*., 1991).

The chemistry, enzymology and pharmacology of protease inhibition in general is well studied and this information has been applied specifically to the development of new and improved protease inhibitors that can be used against HIV. HIV protease is therefore an attractive target for the pharmaceutical industry.

A novel class of drugs, the maturation inhibitors, prevents the formation of mature infectious virions and could be of great use in curbing the production of new virions.

1.10.5 Assembly inhibitors

The assembly and release of newly produced virions is mediated by the viral proteins GAG and VPU (Freed, 1998; Klimkait et al., 1990). The potential therefore exists to discover and develop drugs that are able to bind to one of these viral proteins and therefore block the process of assembly or release. According to Condra *et al*., (2002) a tripeptide, Gly-Pro-Gly-NH_2 (GPG), currently under development, binds to HIV p24 capsid protein and possibly interferes with gag oligomerisation, thereby disrupting the assembly pathway.

Once the details of such mechanisms are better understood, it would be possible to discover new drugs that work via this method and provide effective clinical results.

1.10.6 Zinc-finger inhibitors

Zinc fingers are a chain of amino acids found in the viral core nucleocapsid protein, which are involved in the packaging of viral RNA into new virions. This target could hold the potential for the discovery of a new class of antiretroviral drugs that prevent the production of mature, infectious virions.

1.11 Enhanced Pharmacokinetics

Another method of drug therapy involves combinations of drugs (Table 3). An example is

Kaletra®, which is a combination of Lopinavir® and Ritonavir®. Combining drugs reduces the pill burden, promotes adherence and improves pharmacokinetics. Protease inhibitors such as lopinavir are metabolised into their inactive states by the cytochrome P450 system, mainly by the action of cyp3A4. Frequent dosing is required to maintain these drugs at therapeutic levels. Ritonavir, a component of Kaletra® inhibits the cytochrome P450 system and thereby prevents the rapid clearance of Lopinavir, maintaining effective drug levels for a longer period of time.

1.12 Other forms of therapy

Other forms of therapy being investigated (Table 3) include immunomodulators, such as interleukin 2; therapeutic vaccines; interfering RNA (Novina et al., 2002), and gene therapy (Hirsh and D'Aquila, 1993) although none have entered the clinical arena.

Table 4: Strategies for new therapy options

	Target	Examples
New drugs in existing classes	Reverse Transcriptase	• Nucleotide analogues – tenofovir • Nucleoside analogues – eg. FTC

	Protease Fusion	and • DAPD/DXG • NNRTIs – eg. TMC-120 and DPC082 • Inhibitors – eg. Tipranavir (TPV), BMS 232632, DPC 681 and TMC 126 • Gp41 inhibitor – eg. T 1249
New drugs in new classes	Entry Integration Assembly	• Inhibitors of gp120 binding to CD4 - eg. PRO524 • Inhibitors of coreceptor CXCR4– eg. Bicyclam AMD 3100, ALX 40-4C, T22, T134 and T140 • Inhibitors of coreceptor CCR5 – eg, SCH-C, TAK 779, the anti-CCR5 monoclonal antibody PRO440 and, SCH-D • Integrase inhibitors eg. diketo acids as inhibitors of strand transfer • Inhibitors of gag oligomerisation – eg, GPG • Inhibitors of assembly and packaging of new virions – eg. Zinc-finger inhibitors
Combinations of drugs	Reduce pill burden Improve adherence Pharmacokinetic boosting	• Triomune® • Combivir® • Kaletra®
Other	New therapy strategies	• Immune therapy eg. Use of IL-2 • Therapeutic vaccine • Interfering RNA • Gene therapy

1.13 The discovery and development of new drugs

Ideal antiviral drugs should be able to achieve clinically effective levels in patients, have an acceptable therapeutic index (which is the ratio of efficiency to toxicity) as well as have adequate distribution and penetration at the tissue level (Doniger *et al*., 2004). The antiviral should also feature characteristics such as favourable dosing as well as be easily stored and stable at room temperature. In addition, the antiviral should not readily select drug-resistant variants nor should it antagonise the activity of other antiretroviral treatments. But, most importantly, an effective antiviral should be easily available and affordable**.**

Given the globally acknowledged demand for new antiretroviral drugs, sources of potential agents need to be identified and methods to optimise the testing of these agents need to be established. This process of drug discovery, from the initial preclinical testing of the potential agent to the later phases of clinical trials, is an expensive process which, on average, may take between 12 and fifteen years to bring the product to market. In order for novel drugs with potential antiretroviral activity to be investigated, these procedures need to be made more efficient and cost effective.

1.13.1 Reverse pharmacology versus conventional pharmacology

A rational or conventional approach to drug discovery is one that requires the use of screening models to test the products of drug design.

When using the empirical or reverse pharmacology approach, screening models become the primary tools for new drug discovery.

In conventional pharmacology, molecular design plays a vital role in the "creation" of potential antiviral agents. This is based on the identification of a potential target and the use of medicinal chemistry in the design of a molecule which will have specific inhibitory effects on the target of choice e.g. several protease inhibitors were developed for clinical use by rational investigation of their structure-activity relationship with the HIV protease active site (Kempf *et al.*, 1998). Various biochemical tests are used to elucidate the mechanism of action of the potential agent.

In the context of antiviral screening, reverse pharmacology, as its name suggests, involves the screening of large numbers of potential agents in order to find a lead compound with an inhibitory effect on one or more targets. The focus lies on the identification of a lead compound, using various cell-based assays, rather than on isolating the viral target that is being affected and what mechanism of action is being employed.

While this method has been criticized as being inefficient by some (Burns and Groopman, 1987), reverse pharmacology has been shown to be the most successful drug discovery approach in various areas of pharmaceuticals, particularly in the discovery of clinically useful anticancer drugs (Boyd, 1988).

The empirical screening process has a particular advantage when testing natural products for new antiviral leads. The initial testing of crude natural product extracts with indicators of potential antiviral activity generally leads to the extensive screening of the partially purified fractions of the extract.

Also, once a new lead compound of natural or synthetic origin has been identified through the empirical process, aspects of the rational method may eventually be employed to modify the compound structure in order to improve various characteristics, including solubility and stability. This process is known as "lead optimization" and occurs much later, after the empirical screening process has been successful in finding potential antiviral activity *in vitro*.

The two processes of conventional and reverse pharmacology are therefore intimately linked, and this is further demonstrated by the fact that there is a constant emergence of new ideas for the design of new molecules for drug design or rational pharmacology, which emanates from the screening of potential agents using the empirical method.

At present, a wealth of knowledge exists of the molecular biology of HIV and its effects on host cell targets. This alone provides new platforms for research into successful conventional pharmacology methods of drug design. However, reverse pharmacology has the benefit of large-scale screening of a plethora of natural and synthetic compounds, either completely novel, or previously investigated for other uses.

Boyd (1988) has documented that many of the most important clinically used drugs, across all pharmaceutical classes, have their origins as natural products that have been discovered through the empirical screening process.

Examples of commonly available drugs that have been derived from natural sources include aspirin, penicillin, digoxin and morphine. Of the world's twenty five best selling pharmaceutical agents, twelve are derived from natural products (Verpoorte, 1998).

With 21st century technology and resources at our disposal, the most prudent approach would be the complementary and synergistic use of both reverse and conventional pharmacological methods in our search for novel HIV drugs.

1.13.2 Natural products as potential anti-HIV agents

An array of natural products has been found to show inhibition of specific proteins and enzymes that are crucial to the HIV replication cycle (Notka *et al*., 2004). As reviewed by Cos *et al*., (2004), De Clercq (2000) and Vlietinck *et al*., (1998) some of these natural agents display efficient intervention with the processes of virus binding to target cells, reverse transcription, integration or protease activity.

Many natural products have been used as lead compounds because of their specific activity and low toxicity, and some have displayed mechanisms of action complementary to those of existing anti-virals (Jung *et al*., 2002; Singh *et al*., 2005).

In addition, the vast chemodiversity of plant-derived compounds and secondary metabolites have vast potential as lead compounds in the drug discovery process (Verpoorte, 1998). These secondary metabolites are bioactive compounds that are produced by plants and may, for example, act as protective barriers against disease or pests, anti-feedants or even stimulate growth and repair (Verpoorte, 1998).

There are currently no plant-derived antiviral drugs in clinical use for the treatment of HIV,

although three natural products have shown promising activity in preclinical trials. According to Singh *et al*. (2005), phase II clinical trials are being conducted by Sarawak MediChem pharmaceuticals to determine the long term anti-HIV activity of Calanolide A (a coumarin). Two other molecules, licensed to Panacos Pharmaceuticals have completed preclinical testing, with one starting phase II trials.

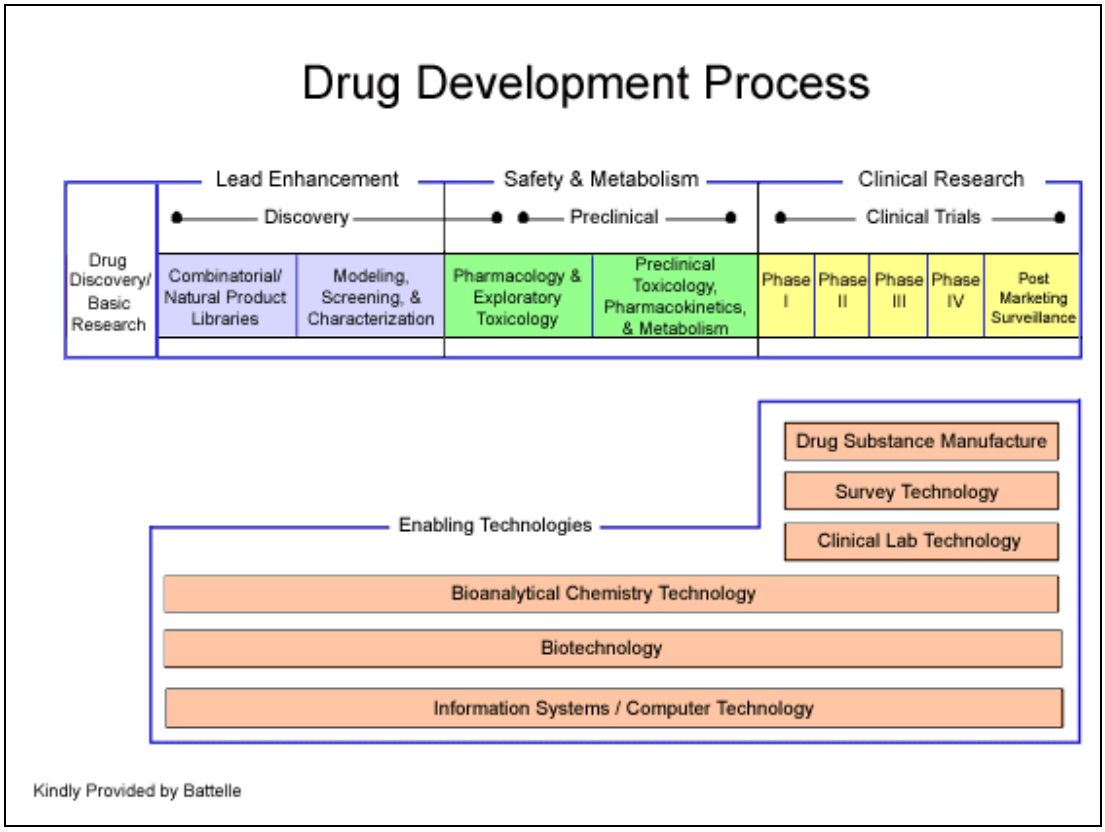

Figure 3: The Drug Development Process

1.13.3 The Drug Discovery Process

The initial steps in drug discovery are crucial in the development of an antiretroviral agent. An efficient screening process to determine the potential efficacy of an anti-HIV compound must allow for the expeditious determination of the most significant features of anti-HIV activity displayed by the agent (Buckheit, 1997). This process must also therefore, facilitate a cytotoxicity evaluation of the compound and allow the activity of the compound to be compared to known antivirals.

A variety of *in vitro* experiments can be performed to evaluate the anti-HIV efficacy and toxicity of a selected compound. Once an active compound is identified, many additional assays must be performed to determine the specific mechanisms of action which may include cellular and viral targets.

While biochemical assays may be employed to determine mechanisms of action, cell-based assays offer a holistic view of the affect of a compound on cellular activity. Both types of assays can be easily adapted to a high throughput screening approach with the use of robotics (Weislow *et al*., 1989). High-throughput screening is an invaluable tool in the drug discovery process. This method ensures that a large number of potential agents or drugs can be screened efficiently.

The benefits and limitations of each assay, the type of screening facility and the information required about the potential drug must be considered when choosing an assay type.

Biochemical assays do not require specialized bio-containment facilities and are very useful in providing a rapid method of determining the specific steps in the replication cycle. Many inhibitors of viral attachment, reverse transcription, integration and protease activity have been identified using these mechanism-based biochemical assays (Buckheit, 1993; Clancey *et al*., 1994, Hazuda *et al*., 1994; Hylans *et al*., 1990).

However, these assays do not allow for the assessment of a potential compound's ability to exert its mechanism of action in complete cells.

Cell-based assays do require specialized biosafety facilities and staff who have been trained in the implementation of specific safety practices. These assays may therefore not be an option for many institutions. The advantage of cell-based testing, however, is that the efficacy of a compound may be evaluated using intact human host cells. Furthermore, these assays can be used even before the mechanism of action of the potential compound has been determined.

There are many cell-based assays which could be used for the evaluation of compounds for anti-HIV activity, including cytopathicity, syncytial and plaque assays. These assays have been used successfully to evaluate compounds using all known mechanisms of action (Sausville and Shoemaker, 2000).

Another advantage of cell-based assays is that both cytotoxicity and antiviral activity may be screened for in parallel using the same target cell system.

A further benefit of cell-based assays is that the "therapeutic index" of the compound can be defined (Buckheit, 1997; Weislow *et al.*, 1989). This allows the comparison of the candidate compound within a class of known antivirals.

Irrespective of the initial biochemical assays used in the anti-HIV drug discovery process, cell-based assays must eventually be performed, since the main goal of the preclinical screening process is to ascertain the toxicity and antiviral efficacy of the candidate compound in a human cell line representative of HIV infected cells *in vivo*.

For the high-throughput screening process to be successful, the assays must be appropriately performed, in a rapid and cost-effective manner in order to expedite the preclinical testing of a candidate compound.

In this study a colorimetric cytotoxicity assay was optimised specifically for the testing of plant extracts. The documented methods employed in the testing of conventional compounds cannot be directly applied to plant derived products. For this reason, several modifications to conventional testing were designed and assessed in this study. An antiviral assay using a continuous HIV-infected cell line was developed to determine the anti-HIV efficacy of these extracts. Quantification of antiviral activity using established automated p24 antigen and viral load systems were compared. A new method of cell counting was designed as a safer and more efficient alterative to the well-known Trypan Blue Exclusion technique. The enhancements to the cell-based cytotoxicity and antiviral assays described in this study facilitate the adaptation of these techniques to the high-throughput screening of natural products. These enhancements have not previously been described in the literature and provide a platform for further research in this field.

The future holds a greater understanding of this complex virus and new strategies for retaliation. Undoubtedly, extensive ongoing research efforts will result in an increase in the number and quality of agents to treat HIV infection.

AIM:

To develop, optimise and compare various virological assays for use in the antiviral assessment of compounds with potential anti-HIV activity.

Objectives

- To maintain and propagate H9/HTLV III_B transfected lymphoblastoid cells
- To infect MT4 cells with HTLV III_B and quantify the viral titre using the TCID50 method
- To develop a safer method of performing cells counts on HIV-infected cells
- To compare the use of chronically infected H9/HTLV III_B transfected lymphoblastoid cells with MT4 cells infected *de novo* with HTLV $III_{B,}$ in the XTT assay
- To optimise the XTT-based cytotoxicity assay for the rapid screening of plant-derived extracts
- To compare the p24 assay and viral load assay as methods for assessing antiviral activity

Chapter 2: Cell and Virus Propagation and Quantification

The objectives of this chapter were to maintain and propagate the three lymphocyte cell lines (uninfected H9, HTLV III_B transfected H9 and MT4), describe the XTT method of cell quantification and the TCID50 method of viral quantification.

2.1 Cell lines

In adherent cell lines, cells adhere to the surface of a flask where they multiply and proliferate in a monolayer. The effect of viruses on a specific cell line can be detected without the need for an animal model. Cell lines can also be grown as suspension cultures in a flask, in which growth of the cells is maintained by RPMI-1640 rather than EMEM.

A continuous cell line is one that has been transformed to give the cells an infinite life span. Primary cell lines, on the other hand, are more sensitive and cannot be cultured over long periods since they lose their natural morphology and integrity.

Cells are generally maintained in media that has been supplemented with fetal calf or fetal bovine serum. Antibiotics such as, Penicillin, Streptomycin and Fungizone®(Aphotericin-B) are added to the media to prevent the growth of bacteria and fungi in cell cultures.

Cells require an environment conducive to growth and this is provided in the laboratory by sterile incubators, maintained at 37°C with 5% CO_2. This provides a 'natural' buffering

system where the gaseous CO_2 balances with the HCO_3 content in the culture media to maintain an optimal pH environment for cell growth. Most media, including RPMI, change colour to either yellow (acidic) or purple (alkaline) indicating the need for replenishment. The pH indicator phenol red is contained in the media for this purpose.

Cell lines can also be frozen indefinitely for use in the future, and this provides an advantage for laboratories in that a constant supply of stock cells can be made available.

2.2 Constituents of Media

Essentially, tissue culture is the propagation of animal, human or plant cells in vitro (George et al., 1996). The tissue is maintained in a specific media eg. Eagles Minimum Essential Media (EMEM) that has been fortified with serum. The basic constituents of media include inorganic salts, carbohydrates, amino acids, vitamins, fatty acids, lipids, proteins and peptides (George et al., 1996). Inorganic salts maintain cellular osmotic balance and the regulation of cell membrane potential by providing sodium, potassium and calcium ions. Carbohydrates provide the main energy source in the form of glucose, galactose, maltose or fructose. The main source of vitamins in media is derived from the addition of serum. However, some media types are commercially available enriched with vitamins. Vitamins are precursors for crucial co-factors as well as stimulators of growth and proliferation (B Vitamin family).

Fatty acids and lipids such as cholesterol and steroids are usually found in serum and need to be supplemented in serum-free media. Trace elements, such as zinc, selenium and copper are present in media. Selenium is required for the removal of oxygen free radicals.

When used for tissue culture, media is generally supplemented with fetal calf (FCS) or fetal bovine serum (FBS). In some instances, horse serum may be used. Serum is an important component of media since it contains albumin, growth factors and inhibitors and has the ability to both increase the buffering capacity of cultures and protect cells from mechanical damage. Proteins present in serum such as fibronectin and fetuin aid in cell attachment while α-2macroglobulin inhibits trypsin. Transferrin binds to iron making it less toxic and more available to the cell.

Polypeptides such as Platelet Derived Growth Factor stimulate mitogenic activity. Major Growth Factor, Fibroblast Growth Factor, Epidermal Growth Factor and Endothelial Growth Factor are also present.

The hormone insulin promotes glucose and amino acid uptake, which enhances the mitogenic effect. Hydrocortisone present in serum is needed to stimulate cell attachment and proliferation. Metabolites and nutrients in serum include glucose, amino acids and ketoacids.

Inhibitors, such as complement are also present in FCS and require removal by heat inactivation to prevent cytotoxicity.

2.3 Propagation of cell lines

For the purposes of the study, three cell lines were used:

- Uninfected H9
- HTLV III_B transfected H9
- Uninfected MT4

Source:

All cell lines were received from the National Institute of Health AIDS Research and Reference Reagent Program (Bethesda, USA).

Description:

The Human T lymphoblastoid, Hut 9 or H9 cell line is a clonal derivative of the T-cell line HUT 78 derived from the blood of a patient with Sezary syndrome. This clone was selected due to its suitability for the isolation and continuous high level production of HIV variants in patients with AIDS and pre-AIDS. Examination with DNA probes indicate identical HLA typing and DNA fingerprints to HuT-78 (Mann *et al*., 1989). Both uninfected H9 cells and HTLV IIIB transfected H9 cells were used for the purposes of this study.

The uninfected MT4 cells are human T-cells that were isolated from a patient with adult T-cell leukaemia.

Propagation:

Each cell suspension was poured into 50ml centrifuge tubes (Cellstar, Greiner BioOne, USA) containing 10mls of fresh, warm, complete RPMI-1640 and centrifuged for 10 minutes at 1500 rpm with no brake. The supernatant was poured off and the pellet reconstituted in fresh, complete RPMI-1640. Cells were grown in T25 tissue culture flasks (Corning Incorporated, USA) over a 5 day period.

Quantification and Storage:

Cell counts, using the Trypan Blue Exclusion Method (Appendix D), were performed on the uninfected H9 and uninfected MT4 cells in preparation for storing the cultures in liquid

nitrogen. This method of cell counting was not performed on the HTLV IIIB transfected H9 cells due to safety concerns since continuous cell lines may produce extremely high viral titres. Supernatant samples were therefore taken for viral load testing (NASBA NucliSens EasyQ (Biomerieux, Netherlands)) to determine viral titres prior to attempting conventional cell counts. The stored cell cultures were used for the XTT assay and subsequent antiviral assays.

2.4 Virus - HTLV III_{MN}

The $HTLVIII_{MN}$ cell-free virus was received from National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, USA). The virus was isolated from tissue culture supernatant of infected H9 cells and is free of bacteria, mycoplasma and fungi. It infects a wide variety of lymphocytes including CEM, H9 and MT4. It utilizes CXCR4 as a co-receptor and exhibits cytopathic effects.

2.5 Inoculation of MT4 cell line with HTLV III_{MN}

A sample of the MT4 cell culture was centrifuged at 1500rpm for 5 minutes. The supernatant was removed and replaced with 1 ml $HTLVIII_{MN}$ supernatant. The cells were re-suspended in the virus supernatant and incubated in a 37C, CO2 incubator for 1 hour. 10 ml PBS was added to the cells and centrifuged for 5 minutes at 1500rpm. The supernatant was discarded and the cells washed with PBS. The suspension was centrifuged at 1500rpm for 5 minutes and the wash step repeated. The supernatant was discarded and complete RPMI was added to the

cells. The culture was centrifuged at 1500rpm for 5minutes. 1ml of supernatant was retrieved for a qualitative baseline p24 test (see Appendix F). The cells were re-suspended in the media and planted in a 75cm3 flask. After incubating the flask for 3 days at 37C in a CO2 incubator, the cells were then centrifuged at 1500rpm for 5 minutes. 1000ul of virus supernatant was pipetted into labeled cryovials and stored at -800C or in liquid nitrogen for subsequent quantification by the TCID method and use in the XTT assay.

2.6 Viral Quantification using the TCID50 method

The TCID50 method is a well established and accurate technique used to quantify virus (DAIDS manual). It is based on statistical analysis of infectivity using end point dilution.

2.6.1 Materials and Methods:

150ul of complete colourless RPMI-1640 was added to rows B-H, columns 2-11 of a 96 well Microtitre plate. 50ul of $HTLVIII_{MN}$ virus culture supernatant was pipetted into each well in rows B-G, column 2. Using a multichannel pipette a serial dilution (1in 4) of virus from column 2 to column 10 was performed. Column 11 was used as the cell control. A cell count was performed on exponentially growing MT-4 cells. The cells were then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells re-suspended in colourless RPMI-1640 at $6X10^5$ cells/ml.

50ul of cells were pipetted into each well. The plate was incubated for 5 days in a 37^0C, CO_2 incubator. The plate was then read for cytopathic effect from day 1 to day 5 using phased-contrast microscopy. CPE was recorded as +.

2.6.2 Results

The $TCID_{50}$ was calculated using the Spearman-Karber method:

Dilution		0.25^1	0.25^2	0.25^3	0.25^4	0.25^5	0.25^6	0.25^7	0.25^8	0.25^9	Control	
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		+	+	+								
C		+	+	+								
D		+	+	+								
E		+	+	+								
F		+	+	+								
G		+	+	+								
H												

Figure 4: Results of $TCID_{50}$ assay depicted as per the test 96 well plate

Log TCID50 = L-d(s-0.5)

L = log of lowest dilution
D = difference between dilution steps
S = sum of proportion of positive wells
Note: For the dilutions in this protocol
L = 1
D = -1
S = 3
1-(-1)(3-0.5) =3.5
Log TCID50 =3.5

2.7 Cell quantification using the XTT method

Trypan Blue Exclusion is the conventional method of counting cells but there are safety concerns particularly when this technique is used to count continuous, HIV-infected cells producing extremely high viral titres, such as the HTLV III_B transfected H9 cells. Since the XTT tetrazolium salt can be used to determine levels of cell proliferation (Berridge *et al*., 1993), we investigated the relationship between formazan production in the XTT assay, measured by optical density, and cell number, determined by the Trypan Blue Exclusion Method.

2.7.1 Materials and Methods

Serial dilutions of a stock culture of $HTLV_{IIIB}$ transfected H9 cells were prepared.

The cell suspensions were prepared according the protocol used for XTT assay preparation (Appendix E).150µl of cell suspension was pipetted into designated wells in a 96 well plate (Costar®Corning Incorporated, USA). 20µl of XTT/PMS solution (Appendix C) were added to each well. The plate was incubated at 37°C with 5% CO_2 for 2 hours. Absorbances were read using the Tecan Sunrise™ (Tecan Trading, Switzerland) Microplate reader and analysed using Magellan™ software.

Several such experiments were performed on different stocks, using both infected and uninfected cultures. Cell counts were performed only on uninfected stock cultures using the Trypan Blue Exclusion Method (Appendix D).

The relationship between optical density (measured by the XTT assay) and cell counts in uninfected cultures (measured by Trypan Blue Exclusion) was determined by linear regression using STATA™ software.

2.7.2 Results

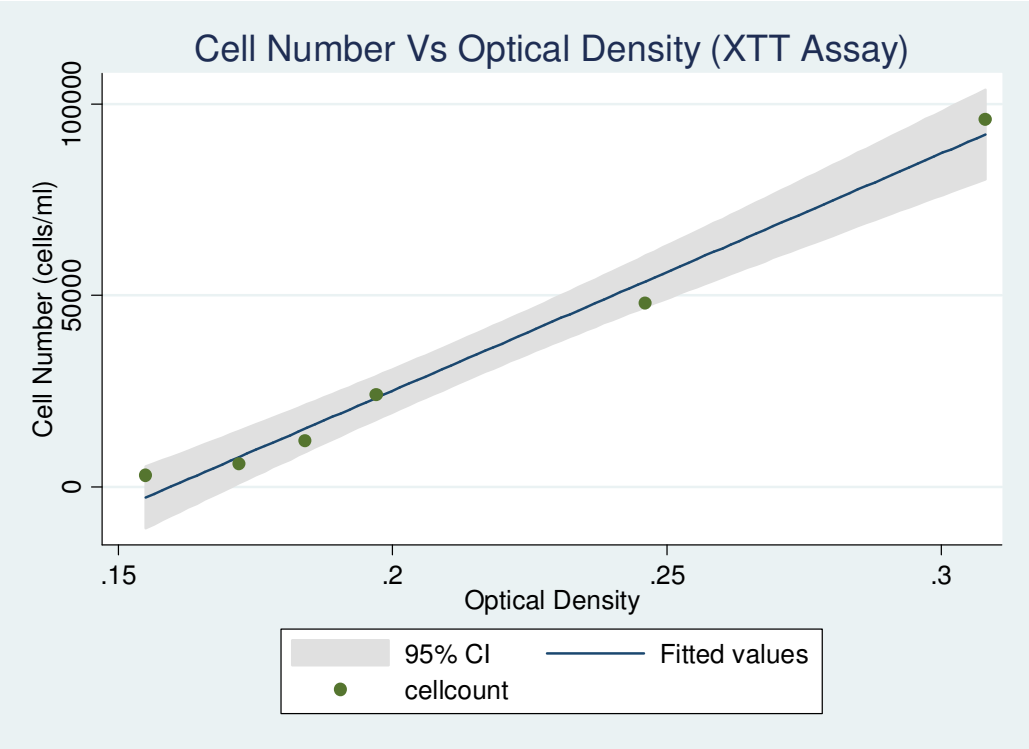

Figure 5: Linear relationship between cell number and optical density reading using a tetrazolium salt-based (XTT) cell counting assay.

Table 5: Linear regression output from STATA™

Cellcount	Coef.	Std. Err.	T	P>t	[95% Conf.	Interval]
OD	620230.3	38165.26	16.25	0.000	514266.5	726194
_cons	-98955.1	8268.954	-11.97	0.000	-121913.4	-75996.8

The result of one such experiment is shown in the above figure. The STATA™ output is shown in Table 5.

A linear relationship was found between formazan production and cell number and can be expressed in the equation:

Cell count = -98955.1 + (620230 x optical density).

A similar linear relationship was found in other experiments on uninfected cells (data not shown).

A linear relationship was found between serial dilutions and optical density in experiments with infected cells (data not shown)

2.7.3 Implications of Results

This method was applied to determine cell counts in infected and uninfected cultures for use in cell based assays.

Formazan production is a measure of cell proliferation and is proportional to cell number. Several factors such as viral infection and exposure to toxic substances may confound this relationship by affecting mitochondrial enzyme activity and hence formazan production.

The linear equation may be different depending on the cell line used and in different stocks of cells.

Nevertheless, this method is still useful. A linear equation can be derived for a stock of uninfected cells of a particular cell line and applied to infected stocks of the same cell line at the initial stages of infection.

The results obtained are approximate. Further experiments are required to refine, validate and optimize this technique.

Chapter 3: The XTT assay - Optimisation of the assay for use when testing plant extracts

This chapter documents the optimisation of the XTT-based cytotoxicity assay for the rapid screening of plant-derived extracts

3.1 Principle of the XTT assay

The use of colorimetric assays in cyotoxicity testing, particularly in the screening of antiviral compounds, is an indispensible tool. Previously, methods such as the counting of viable cells stained with a vital dye or the measurement of radioisotope incorporation as a measure of DNA synthesis were used to determine the *in vitro* cytotoxic effects of unknown compounds. There are issues of safety, test sensitivity as well as accuracy with these methods. Tetrazolium salts are commonly used in cytotoxicity, cell viability and cell proliferation assays (Berridge *et al*; 1993). Once reduced, these salts are converted into non-fluorescent formazans which precipitate out of the solution, and can either be used in the localisation of the site of reduction or solubilised and quantified (Sladowski. *et al*;1993, Specter *et al*; 2000).

Assays making use of tetrazolium salts have become the gold standard of such techniques. The description of the MTT assay in the early 80's, was followed by the synthesis of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide or XTT (Paull *et al*., 1988). The XTT assay offers a more simplified approach with its water soluble formazan product that is easily measured in cellular supernatants (Altman, 1976;

Kuhn *et al*., 2003; Roehm *et al*., 1991). This assay uses a colorimetric technique that offers rapid quantitative analysis of the inhibition of HIV-induced cell killing by anti-HIV test substances by determining cell viability.

The assay is based on the conversion of the yellow XTT tetrazolium salt into a soluble orange formazan by metabolically active cells. This process occurs as a result of mitochondrial dehydrogenases cleaving the tetrazolium ring resulting in formazan production (Kuhn *et al*., 2003). Other enzymes involved in the process include mitochondrial succinoxidase as well as cytochrome p450 systems and flavoprotein oxidases (Altman, 1976).

However, since the bioreduction of XTT is an inefficient process, an electron coupling agent called phenazine methosulfate (PMS) is added to the reaction (Berridge *et al*., 1993). The soluble formazan can be quantified using an ELISA reader.

The use of techniques such as the MTT or XTT assays when screening compounds has therefore proven to be greatly advantageous in that the salts are safe, since no radioactive isotopes are used; accurate, because tetrazolium salt conversion correlates strongly with metabolic activity and sensitive, since fairly low cell numbers can be used.

Since the assays may be carried out in microtitre plates and the quantification requires only multi-well ELISA readers, this assay also has the advantage of being both rapid and cost effective and is thus a useful tool in cytotoxic analyses of compounds when investigating antiviral susceptibility. Tetrazolium salts are also valuable tools in the determination of cell proliferation (Berridge *et al*., 1993). This study explored the use of this property in the development of an alternative cell counting assay.

3.2 Potential problems with the XTT Assay when testing plant extracts:

Plant extracts were obtained from the CSIR Bio-Prospecting Unit (Pretoria). This unit has a library of natural products, some of which have been purported to have potential antiviral activity. Both ethanolic and aqueous extracts of these natural products material were to be screened using the XTT assay, to determine cytotoxic effects as well as dose ranges.

Extracts found to have optimal selectivity indices were to be tested for antiviral effects in infected cell cultures.

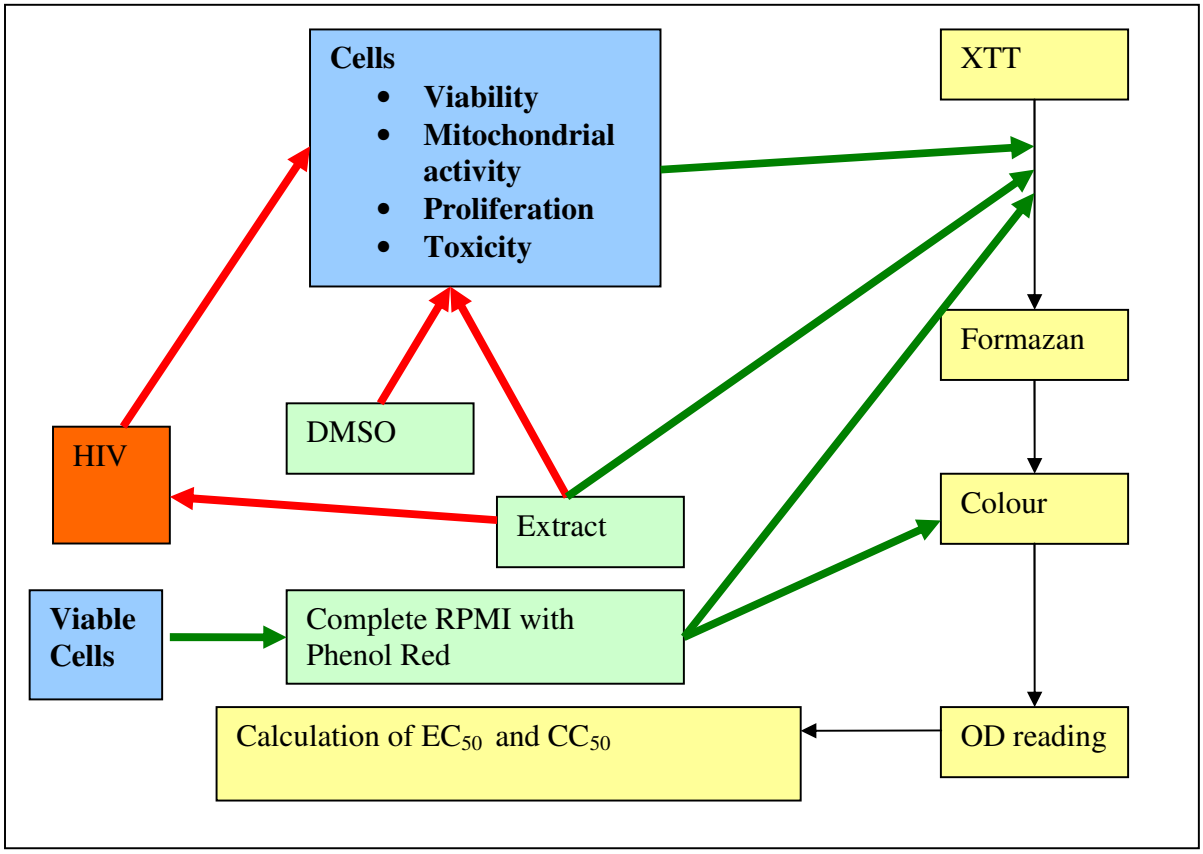

Figure 6: Factors influencing the optimisation of the XTT assay for the cytotoxic evaluation of plant extracts

The following factors were considered when testing plant extracts for cytotoxic activity (Figure 6). These factors have not been clearly addressed in the current literature.

1. RPMI containing phenol red indicator

The XTT/PMS solution is pale yellow in colour (Tox2 Kit package insert, Sigma). XTT is reduced by the activity of cellular enzymes, producing a formazan which is orange in colour. However, RPMI, containing phenol red, is metabolised by cells during assay incubation resulting in the change in the phenol red indicator to a yellow/orange colour. This yellow/orange colour of RPMI is also read at the wavelength required for formazan optical density measurement and compromises the results of the assay.

2. Toxic effect of DMSO on cells

Phenolic plant extracts may require reconstitution in either 100% or 50% DMSO to enable their complete solubility prior to testing (CSIR-BioProspecting Unit, personal communication Dr Vinesh Maharaj). DMSO is known to be toxic to cells (DMSO package insert). This would be a potentially confounding factor in interpreting the results of the cytotoxicity assay.

3. XTT conversion to soluble formazan in cell-free conditions

Plant extracts are likely to contain various plant enzymes which have the potential to reduce the tetrazolium salt to formazan. Also, since XTT requires the intermediate electron acceptor PMS, this salt can be reduced by NADH and NADPH in the absence of cells and enzymes (Berridge *et al*., 1993). It was also shown by Berridge *et al*. (1993) that superoxides play a vital role in XTT reduction in the absence of cells. The production of formazan by the activity of plant enzymes can mask decreased formazan production due to cytotoxicity.

3.3 Optimisation Experiments

1. Experiment with RPMI with and without phenol red indicator

A. The standard XTT assay method (4.2.3) was performed using RPMI with and without the phenol red indicator (RPMI-1640, and RPMI-1640 modified media respectively). No cells were used in this experiment.

150 µl of complete RPMI, with and without phenol red, was pipetted into each of 10 wells wells in a 96 well microtitre plate (Costar®Corning Incorporated, USA).

The plates were read at 450nm/620nm on TECAN Sunrise ELISA plate reader and Magellan™ software (Tecan Trading, Switzerland).

20µl XTT/PMS was added to each well.

The plate was incubated for 4 hours at 37°C with 5% CO_2.

The plates were then read at 450nm/620nm on TECAN Sunrise ELISA plate reader and Magellan™ software (Tecan Trading, Switzerland).

B. The standard XTT assay method was performed using RPMI with and without the phenol red indicator (RPMI-1640, and RPMI-1640 modified media respectively).

150 µl of uninfected H9 cell suspension, made up in complete RPMI with and without phenol red, was pipetted into each of 10 wells wells in a 96 well microtitre plate (Costar®Corning Incorporated, USA).

The plates were read at 450nm/620nm on TECAN Sunrise ELISA plate reader and Magellan™ software (Tecan Trading, Switzerland).

20µl XTT/PMS was added to each well.

The plate was incubated for 4 hours at 37°C with 5% CO_2.

The plates were then read at 450nm/620nm on TECAN Sunrise ELISA plate reader and Magellan™ software (Tecan Trading, Switzerland).

The difference in OD readings before and after incubation with XTT (Δ) was calculated and is depicted in Table 6.

Table 6: The difference in OD readings before and after incubation with XTT (Δ) and corresponding p values

	RPMI with phenol red	RPMI without phenol red	Cell suspension with phenol red	Cell suspension without phenol red
Δ	0.0623	0.0827	0.0765	0.1007
p value	0.0145		0.0182	

There was a statistically significant difference in the mean difference pre and post XTT-incubation i.e.Δ, between RPMI with and without phenol red indicator (p = 0.0182) (t-test STATA 10, College Station, Texas, USA). The mean OD values are shown in table 4.

It is not sufficient to merely subtract the baseline ODs ie before addition of XTT, from the post-incubation OD readings. The results of this experiment demonstrates that the subtraction of pre from post ODs does not cancel the effect of the phenol red indicator. The alteration in the colour of the phenol red indicator is possibly due to a pH change during the course of incubation. As a result RPMI containing indicators such as phenol red cannot be used in colorimetric cytotoxicity screens such as the XTT assay. The use of colourless RPMI is therefore essential.

2. Experiment to determine effect of DMSO on cells

Cells were exposed to varying concentrations of DMSO constituted in complete colourlesss RPMI.

The standard XTT assay was performed as per 4.2.3.

150µl of these cell suspensions were pipetted in replicates into wells of a 96 well microtitre plate. 20µl of XTT/PMS was added to each well. The plate was incubated for 4 hours at 37°C with 5% CO_2. The plate was then read at 450nm/620nm on TECAN Sunrise ELISA plate reader.

The average OD readings are tabulated below.

Table 7: The average optical density readings pre and post incubation with XTT in cell suspensions treated with varying concentrations of DMSO

Average OD	DMSO			
	0%	0.0625%	0.625%	6.25%
Before XTT Addition	0.19	0.240	0.239	0.209
After 4 hr incubation with XTT	1.587	1.526	1.505	0.285

Student's t test was performed to determine the significance of the difference between OD readings in wells with concentrations of DMSO ≤ 0.625 % DMSO and ≥ 6.25% DMSO.

A statistically significant difference was found between the 2 groups, with mean OD 1.546 and 0.285 with ≤ 0.625 % DMSO and ≥ 6.25% DMSO respectively ($p < 0.0001$).

3. Experiment to investigate XTT conversion to soluble formazan in cell-free conditions.

We conducted experiments to study the effect of cell-free plant extracts on formazan production.

Dilutions of plant extracts (0µg/ml; 0.25µg/ml; 2.5µg/ml and 25µg/ml) were prepared in colourless RPMI-1640 modified media.

The standard XTT assay was performed as per 4.2.3.

150µl of each concentration was pipetted into wells of 96 well microtitre plates (10 wells per concentration).

The plates were read at 450nm/620nm on the TECAN Sunrise Microplate Reader.

20 µl of XTT/PMS was added to each well and the plates were incubated for 4 hours at 37°C with 5%CO_2.

The plates were read at 450nm/620nm on the TECAN Sunrise Microplate Reader.

The difference, Δ, in OD reading before and after incubation with XTT was calculated. The mean baseline OD readings are shown in Table 7.

Table 8: Mean baseline OD readings of varying concentrations of extract 18 B

Concentration of Extract 18 B	0 µg/ml	0.25µg/ml	2.5µg/ml	25µg/ml
Mean baseline OD	0.0489	0.0339	0.0341	0.0475

We found no statistically significant difference between Δ of the control ie. 0µg/ml and Δ of each of the other concentrations. This suggests that there is no apparent effect of plant enzymes on formazan production.

However, there were significant differences between the baseline OD of the control and each of the concentrations of the extract ($p<0.05$). This finding suggests that the colour of the extract is a significant factor in OD calculations in the XTT assay. To account for this, pre and post XTT incubation OD readings were performed in all subsequent experiments. Pre-incubation OD readings were subtracted from post incubation OD readings. This modification is not documented in published protocols.

Chapter 4: The XTT assay - Chronically infected versus experimentally infected cells

This chapter describes the comparison between the use of two cell lines (chronically infected HTLV IIIB transfected H9 cells and MT4 cells that were experimentally infected with HTLV III_{MN}) in the XTT assay.

4.1 Overview and rationale of the experiments

- Both chronically infected and *de novo* infected cells have been used in published XTT protocols (Weislow *et al,* 1989). Since these methods may yield varying results, we compared their use in the cytotoxicity screening of plant extracts.
- Chronically infected HTLV IIIB transfected H9 cells were propagated and exposed to plant extracts and AZT
- Uninfected MT4 cells were infected *de novo* with the $HTLVIII_{MN}$ strain and exposed to the plant extracts and AZT
- The XTT assay was performed on both the XTT and MT4 cell lines.

4.2 Materials and Methods

4.2.1 Incubation of uninfected H9 and chronically infected HTLV III_B H9 cells with plant extracts and test reagents

The outer wells of a flat bottomed, 96-well plastic microtiter plate (CoStar, Corning Incorporated, USA) were filled with 200 µl of phosphate buffered saline (PBS).

500µl of HTLV III_B transfected H9 cell suspension (as prepared in 2.1.2), of the final cell concentration to be used was added to each labelled 1ml eppendorf tube. The tubes were spun at 1500 rpm for 10 minutes. The supernatant was discarded and 500µl of the appropriate concentration of the plant extract compound/AZT/test reagent/RPMI (Appendix C), made up in complete colourless RPMI, was added. The tubes were vortexed gently to reconstitute the cells. 150µl of this cell suspension was pipetted into each well, in triplicate.
This process was repeated with 500ul of uninfected H9 (as prepared in 2.1.2) cell suspension.

The infected and uninfected plates were incubated at 37^0C in a CO_2 incubator for 5 days. The cells remained in contact with the test compounds during the entire incubation period. Five days after infection the viability of the uninfected and HIV infected cells was determined spectrophotometrically by the XTT assay (see 3.3.1.3).

4.2.2 Experimental infection of MT4 cells with HTLV IIIMN MT4 cells and incubation with plant extracts and test reagent

Flat bottomed, 96-well plastic microtiter plates (CoStar, Corning Incorporated, USA) were filled with 100µl of complete medium containing dilutions of test compounds (Appendix C) using a multichannel pipette. These dilutions were performed in triplicate to allow simultaneous evalution of their effects on HIV-infected and uninfected cells. Untreated HIV-infected and uninfected cells were included as controls for each compound. AZT, DMSO and RPMI were used as controls.

A flask of exponentially growing MT-4 cells was centrifuged for 5 minutes at 1000 rpm and the supernatants discarded. The cells were re-suspended in colourless RPMI-1640 at 6 x 10^5 cells/ml. 50ul were pipetted into each well. 10ul of 100$TCID_{50}$ was used for the experiments. The outer wells of the plate were filled with 200 µl of phosphate buffered saline (PBS). 50 µl of HIV (HTLV III_{MN}) supernatant at 100 $TCID_{50}$ or RPMI was added to either infected or mock-infected wells. The plate was incubated at 37^0C in a CO_2 incubator for 5 days. The cells remained in contact with the test compounds during the entire incubation period. Five days after infection the viability of the mock- and HIV-infected cells was determined spectrophotometrically by the XTT assay.

4.2.3 Standard XTT assay

The *In Vitro* XTT based Toxicology Assay Kit (Sigma-Adrich, Missouri, USA) was used. The XTT assay was performed according to Scudiero *et al*. (1988), Weislow *et al*. (1989), and Cos *et al*., (2002) with modifications.

The kit contained XTT +1% PMS in 5mg vials.

The XTT/PMS reagent was made up according to manufacturer's instructions (Appendix C). 20µl of XTT/PMS reagent was added to each test and control well. The plates were then incubated for a further 4 hours at 37°C with 5% CO_2 before being read at 450nm, with a reference wavelength of 620 nm using the Tecan Sunrise ELISA plate reader and Magellan™ software. (Tecan Trading, Switzerland)

The percentage protection was determined by the following equations using the recorded absorbances (Cos et al., 2002):

Uninfected cells:

Optical Density reading for uninfected untreated = A = 100%

Optical Density readings at varying concentrations = Y

Formula for % of uninfected treated cells:

% of uninfected treated cells = (Y/ A) x 100

Infected:

Optical Density reading for infected untreated = B

Optical Density readings at varying concentrations = X

Formula for % protection

% Protection = [(X – B) / (A-B)] x 100

CC_{50} (50% cytotoxic concentration) is defined as the concentration of the test compound that reduces the absorbance of the uninfected control by 50%.

EC_{50} (50% effective concentration) is defined as the concentration of the test compound that achieves 50% protection in infected cultures according to the formula for % protection.

We derived an equation based on linear extrapolation to determine the EC_{50} from the values obtained in the experiment:

$$EC_{50} = V - \{[(W-50\%) / (W-U)] \times (V-T)\}$$

where,

V = is the concentration of the test compound that corresponds to the closest percentage above 50%

W= is the closest percentage above 50%

U= is the closest percentage below 50%

T= is the concentration of the test compound that corresponds to the closest percentage below 50%

SI (Selectivity Index) is defined as the ratio CC_{50}/EC_{50}.

4.2.4 Results of the XTT assay using chronically infected versus experimentally infected cell lines

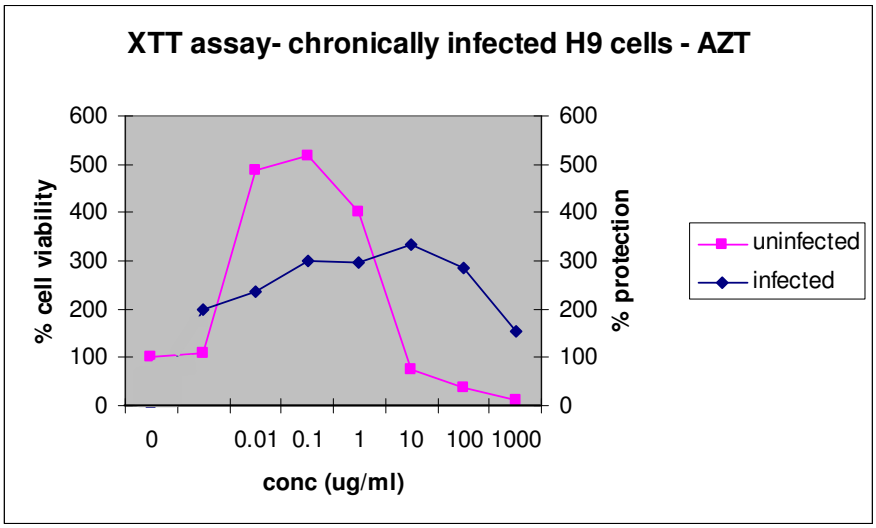

Figure 7: H9 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of AZT.

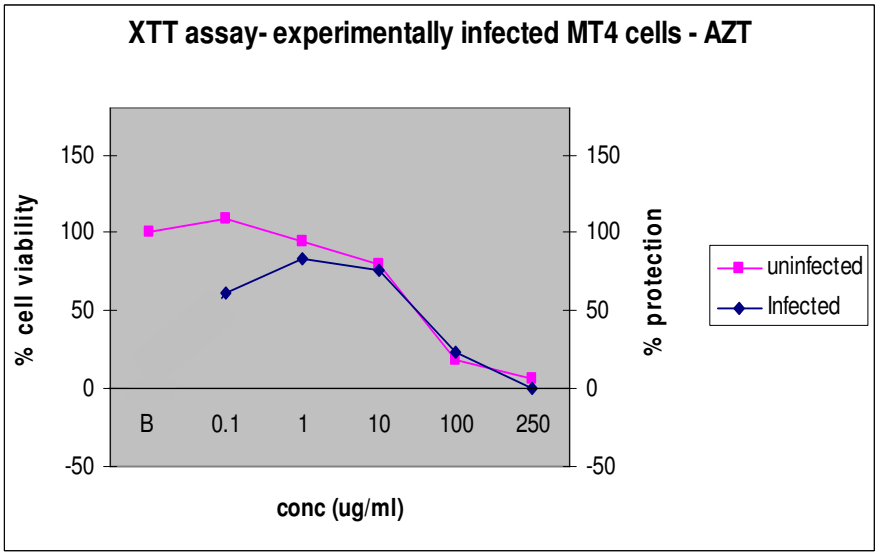

Figure 8: MT4 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of AZT.

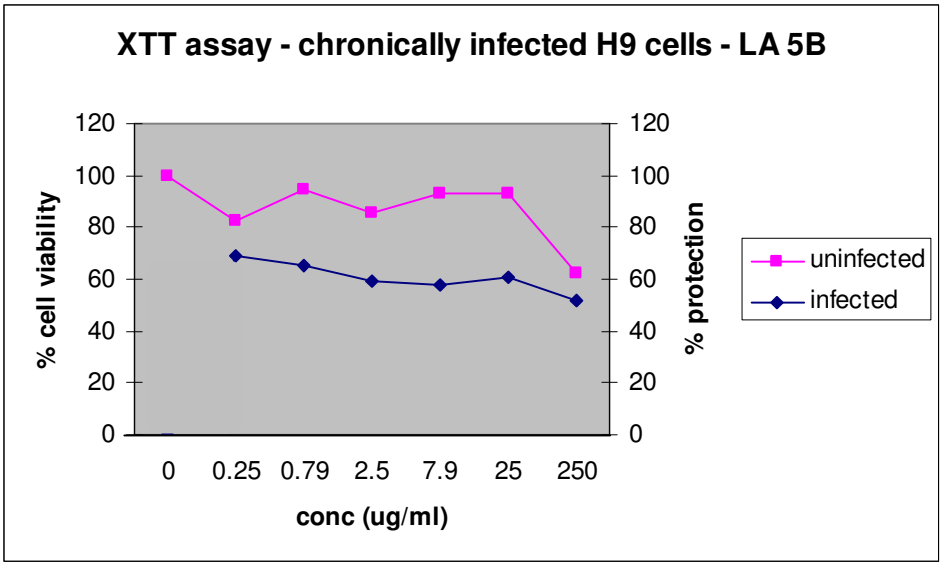

Figure 9: H9 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract LA5B.

Cell viability in uninfected cells remained above 80% up to the 25µg/ml concentration. Percentage protection in chronically infected cells increased to ~70% at 0.25µg/ml of the extract (Figure 9).

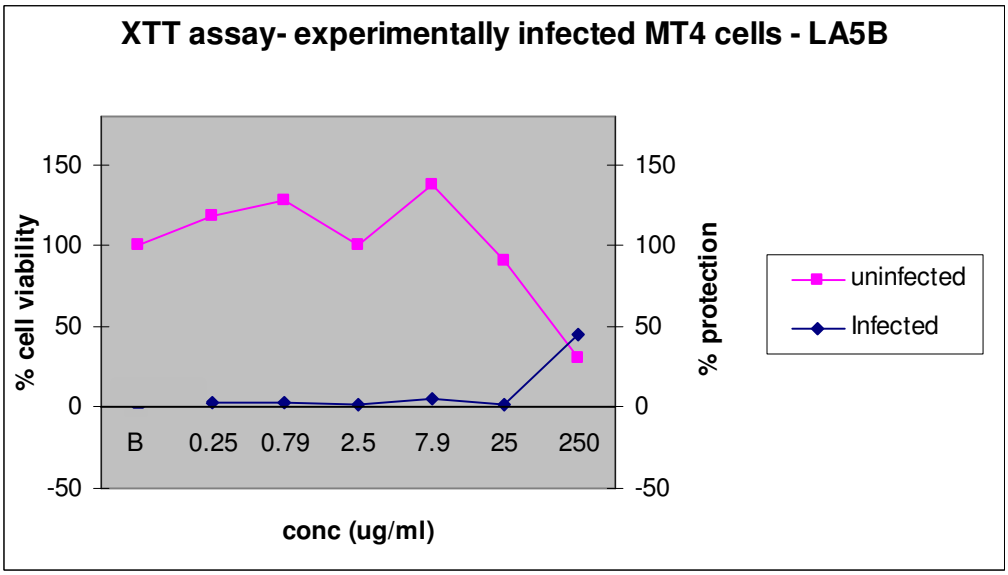

Figure 10: MT4 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract LA5B.

The sharp decline in % cell viability is due to the cytotoxic effect of the plant extract at 7.9ug/ml. The extract does not demonstrate a protective effect i.e. the % protection does not reach 50% (Figure 10).

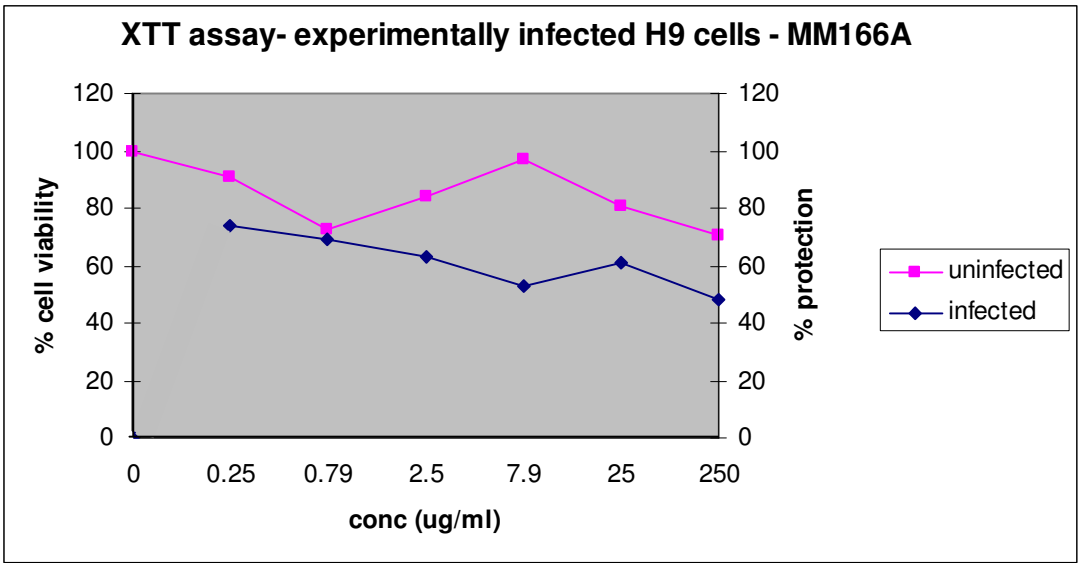

Figure 11: H9 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166A.

Cell viability in uninfected cells remained above 60% in all concentrations. Percentage protection increased to ~70% at 0.25µg/ml of the extract in infected cultures (Figure 11).

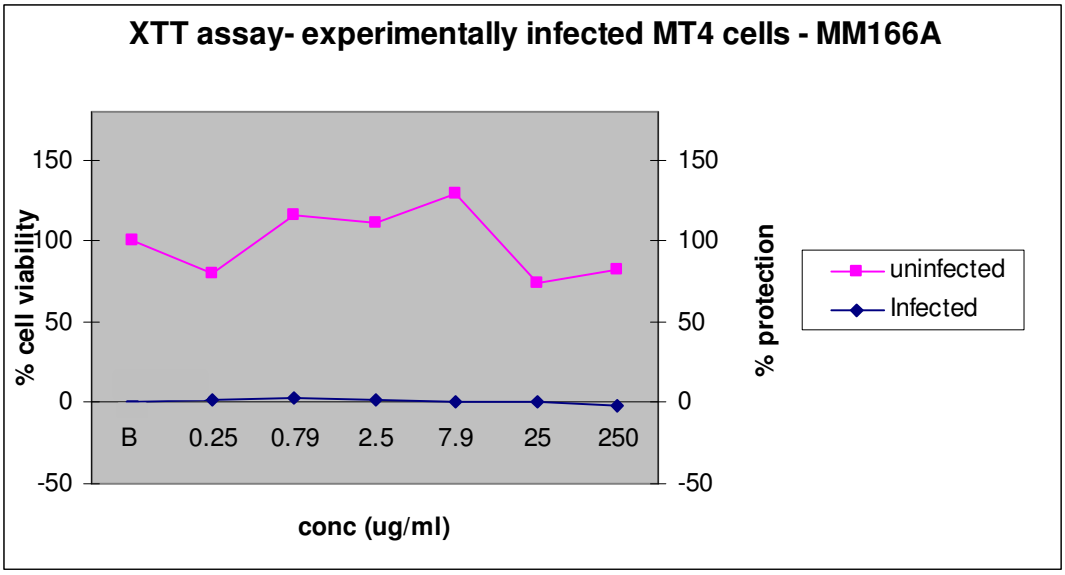

Figure 12: MT4 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166A.

The extract does not demonstrate a protective or cytotoxic effect since neither the % cell viability nor % protection reaches 50% (Figure 12).

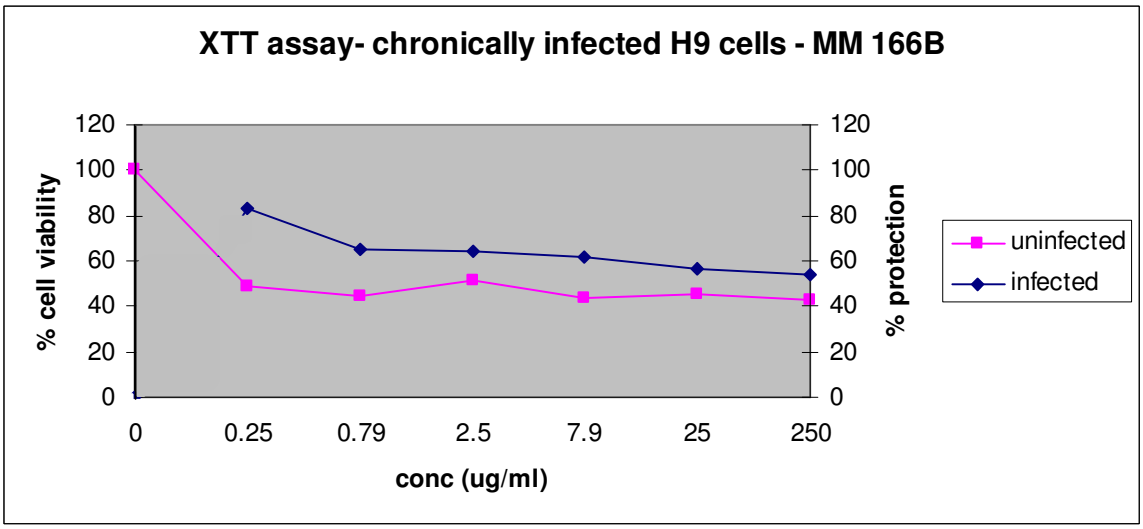

Figure 13: H9 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166B.

The extract appears to be toxic at the lowest concentration (0.25µg/ml) in uninfected cultures. Protection in infected cultures is apparent at the lowest concentration (0.25µg/ml) of the extract (Figure 13).

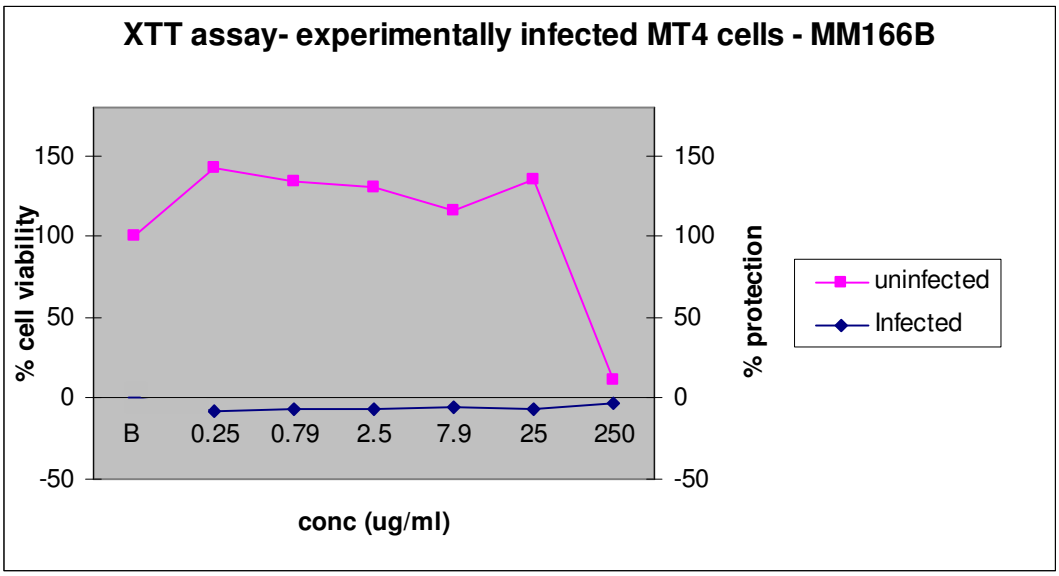

Figure 14: MT4 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166B.

The sharp decline in % cell viability is due to the cytotoxic effect of the plant extract at 25ug/ml. The extract does not demonstrate a protective effect i.e. the % protection does not reach 50% (Figure 14).

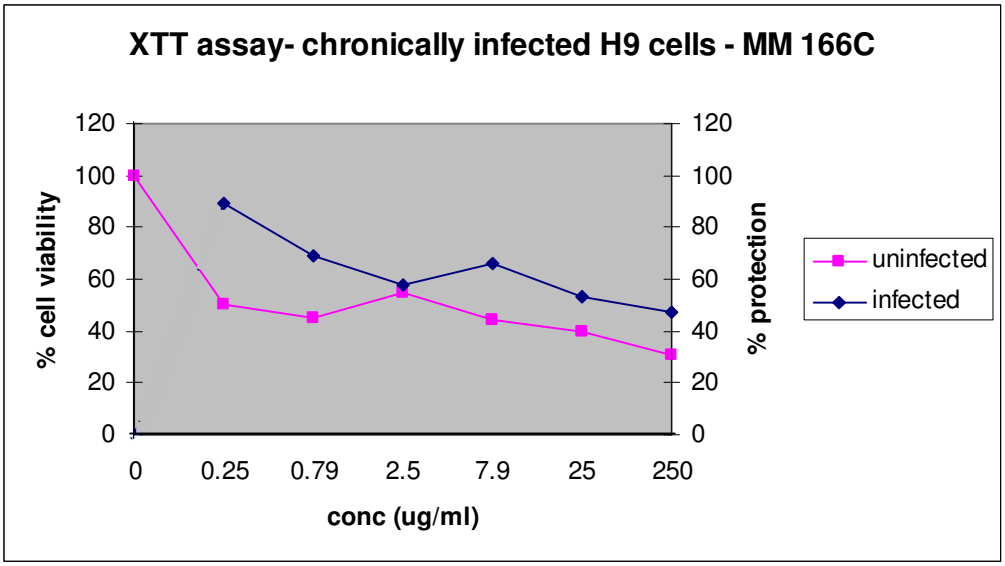

Figure 15: H9 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166C.

The extract appears to be toxic at the lowest concentration (0.25µg/ml) in uninfected cultures. ~ 100% protection in infected cultures is apparent at the lowest concentration (0.25µg/ml) of the extract (Figure 15).

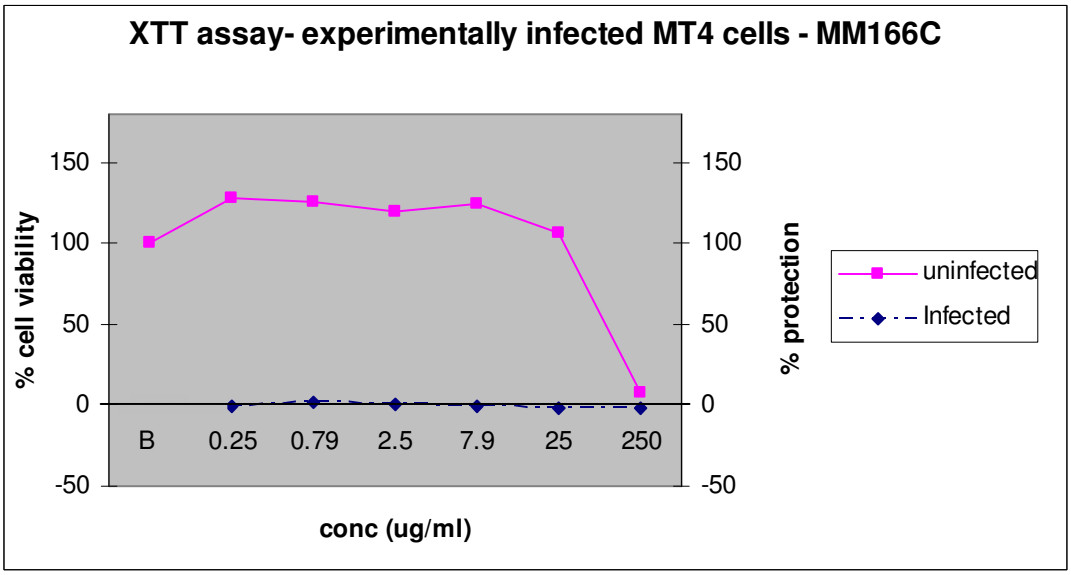

Figure 16: MT4 cell viability in uninfected cultures and cell protection in infected cultures treated with varying concentrations of extract MM166C.

The sharp decline in % cell viability is due to the cytotoxic effect of the plant extract at 25ug/ml. The extract does not demonstrate a protective effect i.e. the % protection does not reach 50% (Figure 16).

The EC_{50}, CC_{50} and Selectivity indices of the extracts are shown in Table 9.

Table 9: EC_{50}, CC_{50} and Selectivity Indicies (SI) of AZT and the four plant extracts tested

Extract	Chronically infected H9 cell line			Experimentally infected MT4 cells		
	EC_{50}	**CC_{50}**	**SI**	**EC_{50}**	**CC_{50}**	**SI**
AZT	0.08	>250	3125	0.08	53.4	650
LA 5B	0.181	>250	>1381.2	>250	179.2	<1
MM 166A	0.169	>250	>1479.3	>250	0.0037	<1
MM 166B	0.1506	~0.25	1.66	>250	179.8	<1
MM 166C	0.1405	~0.25	1.78	>250	153.3	<1

AZT demonstrates an SI of 3125 and 650. Both are in keeping with published values of the selectivity index of AZT which range from 400 to 24000 (Balzarini *et al.,*1989 ; Franchetti *et al.,* 1998; Turk *et al.,* 2002 and Weislow *et al.*, 1989). Extracts LA5B and MM166A showed favorable selectivity indices with the chronically infected H9 cells but not with the experimentally infected MT4 cells. These extracts were therefore selected for further testing

using the antiviral assay. Extract MM166B and MM166C demonstrated little or no antiviral activity in both the chronically and experimentally infected cells.

Chapter 5: A Cell-based Antiviral Assay using p24 and Viral Load Quantification Methods

The objective of this chapter is to compare the p24 assay and viral load assay as methods for assessing antiviral activity

5.1 Antiviral testing

Antiviral testing is a laborious and time-consuming process that does not guarantee the discovery of a lead compound. This is related to the fact that most antiviral drugs have been discovered in specialised commercial chemistry laboratories and not in prominent pharmaceutical industries (Oxford *et al*., 2000).

However, in the search for novel agents with anti-HIV properties, new and improved *in vitro* screening methods have become a fundamental tool in improving the antiviral screening process.

HIV susceptibility testing uses PBMC co-cultivation in order to assess the susceptibility of clinical HIV isolates to known or potential antiviral agents. This is done by comparing the concentration of the drug needed to inhibit the clinical isolate with that of a wild type reference strain (Haubrich, 2004).

Cell culture, particularly novel methods of growing suspension cultures of human T cells, plays an important role in screening of agents with antiretroviral properties. T-cells that have

been transfected to permit a continuous life span are therefore an invaluable tool for use in antiviral testing (Oxford *et al*., 2000). In addition, 96-well plates that have been manufactured with a special coating to permit cell culture have the added advantage of suitable optical characteristics. The latter allows for the use of colorimetric antiviral-assays which require plates to be read by an ELISA reader, an advantage that has enabled high-throughput antiviral drug-screening (Oxford et al., 2000).

The main purpose of the testing method described in this chapter was to develop a cell-based assay that could be used efficiently in the high throughput screening of agents with antiretroviral potential.

Al-Jabri *et al*. (1996) and Oxford *et al*. (2000) describe a method for *in vitro* antiviral assays where a selected cell line, known to be susceptible to infection by the particular virus, is incubated with the test compound. Thereafter, cell-free virus stock is added to the cells, and the culture is grown over a fixed time-period. An appropriate drug control, which is known to reduce the activity of the virus, must be used. If the test compound has any antiviral effects, the ability of the virus to infect cells or replicate will be reduced. Virus replication is usually determined by quantification of serial samples taken from cell systems incubated for 2 to 7 days.

5.2 Viral quantification methods

For the purposes of this study, viral quantification was performed using a quantitative p24 antigen electrochemiluminescent assay (ECLIA) as well as a viral load assay.

The Roche Cobas p24 kit and Roche Modular analyser (Roche Cobas, Elecsys and Modular analytics, Germany) was used to determine the amount of p24 in each sample. This type of p24 assay requires the interaction of the sample with biotinylated monoclonal HIV p24-specific antibodies as well as Ruthenium complex labelled monoclonal HIV p24 specific antibodies to form a sandwich complex in an initial incubation step. Streptavadin-coated microparticles are then added, following a second incubation which allows for the interaction between the strepavidin and biotin. The reaction mixture is then transferred to a measuring cell where the microparticles are magnetically captured onto an electrode. Unbound reagent and sample are removed by washing, after which a voltage is applied to induce a chemiluminecsent emission. This signal is measured by a photomultiplier and the results are determined automatically by comparing the electrochemiluminescent signal from the sample with the cutoff value obtained by previous calibration

The NASBA NucliSens EasyQ (Biomerieux, Netherlands) was used to measure viral RNA levels in the samples.

HIV-1 Viral loads have become an indispensable tool in the initiation and monitoring of antiretroviral therapy. Viral loads may also predict efficacy of treatment and be of prognostic significance (Wuesten *et al*., 2002).

The NucliSens EasyQ system, employed for the purposes of this study, combines a technique known as NASBA RNA amplification (Van Gemen *et al*., 1993; Lin *et al*., 1998; Yen-Lieberman, 1998; Biomerieux, 2002) with a real-time detection method that used molecular beacons.

The initial step involves 'Boom' extraction, which is a method incorporating solid phase based nucleic acid release and isolation. The advantage of this method is that nucleic acids can be isolated from various sources including whole blood, serum, cerebrospinal fluid, semen, urine, vaginal lavage, saliva and most importantly for the purposes of this study, cell culture fluid (Bartlett, 1999). Also, the 'Boom' method is compatible with all anticoagulants and has a broad range of input volumes.

There are essentially 4 stages in the NucliSens HIV-1 EasyQ assay:

Nucleic acid release is accomplished by adding the sample to the Lysis buffer, containing guanidine thiocyanate and Triton X-100, which degrades debris and cells and inactivates RNases and DNases.

Nucleic acid isolation: In addition to the nucleic acid from the sample, synthetic RNA that is used as an internal control and calibrator, is added to the Lysis buffer. These RNA constructs are required to produce a calibration curve required for the quantification process. The calibrator RNA differs from the sample RNA by only a small sequence. Under high salt conditions all the nucleic acid in the lysis buffer becomes bound to silica dioxide particles,

which act as the solid phase. The silica-bound nucleic acids are washed several times in wash buffer to remove all lipids and proteins and finally, purified nucleic acid is eluted.

Amplification of nucleic acid is based on primer extension and repeated transcription of both sample RNA and calibrator RNA from intermediate DNA molecules produced by T7-RNA polymerase (Weusten *et al*., 2002). Three important enzymes, avian myeloblastosis virus reverse transcriptase, RNase H and, as mentioned earlier, T 7 RNA polymerase are required for the process, in addition to two primers, which are short single-stranded DNA fragments, one of which contains a T 7 RNA polymerase promoter which binds to HIV RNA. The amplification is isothermal and takes place at 41°C. During the reaction, reverse transcriptase generates a single DNA copy of the viral RNA sample (sense strands) once the first primer has bound. This results in the production of an RNA:DNA hybrid. RNase H hydrolyses the RNA strand and the second primer binds to the remaining DNA strand. The DNA polymerase activity of reverse transcriptase then extends the second primer to produce a double stranded copy of the original sense RNA with an intact T 7 RNA polymerase promoter. T 7 RNA polymerase recognises the promoter and generates transcription of antisense RNA. From the antisense strands, new DNA copies are generated in a process similar to that using the sense RNA strands, except that the second primer binds first, and the final product is double stranded DNA with the T7 promoter so that new RNA may be produced once again.

Nucleic acid detection: Since the anti-sense RNA produced cannot be detected on its own, molecular beacons are added to the sample which bind to the RNA. The total fluorescence signal generated by this complex is used to determine the concentration of the RNA (Biomerieux Training Manual).

5.3 Materials and Methods:

5.3.1 Incubation of chronically infected HTLV III_B H9 cells with plant extracts and test reagents

Concentrations of AZT and plant extracts to be tested were made up in complete colourless RPMI (Appendix C).

Cell counts were performed on infected H9 cells according to the XTT method described in Chapter 3.

100µl of the HIV-infected H9 cell suspension and 400µl uninfected H9 cell suspension were pipetted into 1ml Eppendorf tubes. The tubes were spun at 1500rpm for 5 minutes. The supernatant was discarded and the pellets were washed twice in 10ml warm PBS by resuspending the pellets in the volume of PBS in 50ml tubes (Cellstar® Greiner BioOne, USA), centrifuging at 1500rpm for 5 minutes, discarding the supernatant and replacing with fresh warm PBS. The process was repeated and the final pellet was resuspended in 3ml of concentration of AZT or test extract. A drug/extract free control was included in each cell system.

The cell suspensions were pipetted into wells in 6-well plates (Sterilin, Great Britain) and the plates were incubated at 37°C with 5% CO_2 for 6 days. A supernatant sample was taken from the drug/extract free control well on Day 1 after plating. Wells were sampled at Days 3 and 6.

100ul of each supernatant sample was pipetted into tubes and tested using the Roche p24 ECLIA (Appendix G). 500µl of each supernatant sample was taken for viral load testing. The viral load assay was performed on the supernatant samples according to package insert and accompanying protocol (Appendix H).

5.3.2 Experimental infection of MT4 cells with HTLV III_{MN} MT4 cells and incubation with plant extracts and test reagent

Concentrations of AZT and plant extracts to be tested were made up in complete colourless RPMI (Appendix C).

Cell counts were performed on uninfected MT4 cells according to the Trypan Blue Exclusion Method (Appendix D).

500µl of the uninfected MT4 cell suspension was pipetted into 1ml Eppendorf tubes. The tubes were spun at 1500rpm for 5 minutes. The supernatant was discarded and the pellets were resuspended in 3ml of concentration of AZT or test extract. A drug/extract free control was included in each cell system. The cells were incubated with the AZT or test extract for 30 minutes at 37°C to allow for drug/cell equilibrium to be reached. 50 µl of HIV (HTLV III_{MN}) supernatant at 100-300 $TCID_{50}$ was added to each cell suspension containing the different dilutions of AZT or test extract. The cell suspensions were incubated for 3 hours at 37°C with 5% CO_2. The cells were washed twice in 20ml RPMI by resuspending the pellets in the volume of PBS in 50ml tubes (Cellstar® Greiner BioOne, USA), centrifuging at 1500rpm for 5 minutes, discarding the supernatant and replacing with fresh warm RPMI. The process was repeated and the final pellet was resuspended in 3ml of concentration of AZT or test extract. A drug/extract free control was included in each cell system.

The cell suspensions were pipetted into wells in 6-well plates (Sterilin, Great Britain) and the plates were incubated at 37°C with 5% CO_2 for 6 days. A supernatant sample was taken from the drug/extract free control well on Day 1 after plating. Wells were sampled at Days 3 and 6.

100ul of each supernatant sample was pipetted into tubes and tested using the Roche p24 ECLIA (Appendix G). 500µl of each supernatant sample was taken for viral load testing. (Al-Jabri *et al*.,1996).

The viral load assay was performed on the supernatant samples according to package insert and accompanying protocol (Appendix H).

5.4 Results:

5.4.1 Antiviral assay performed on chronically infected HTLV III_B H9 cells

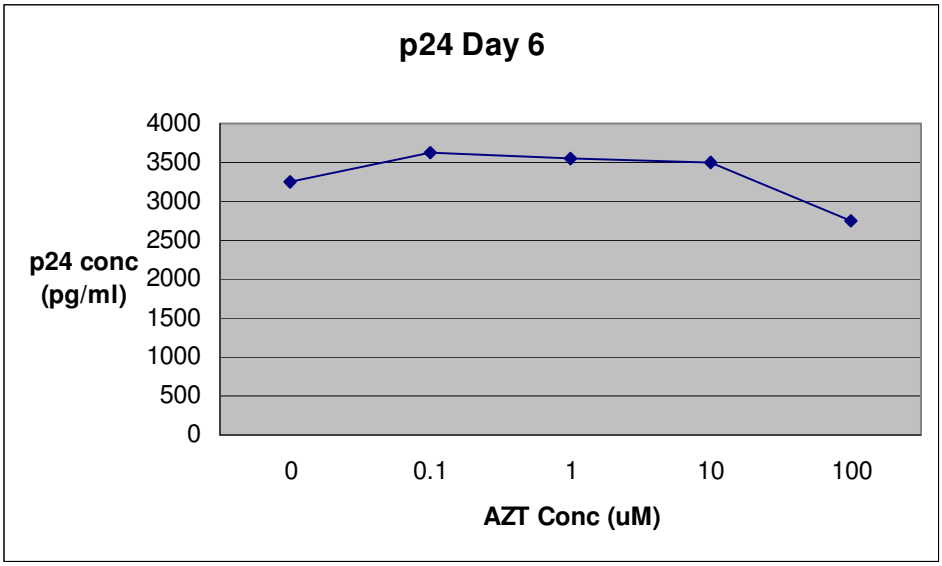

Figure 17: p24 concentrations at Day 6 of the antiviral assay performed on chronically HIV-infected H9 cell cultures treated with varying concentrations of AZT

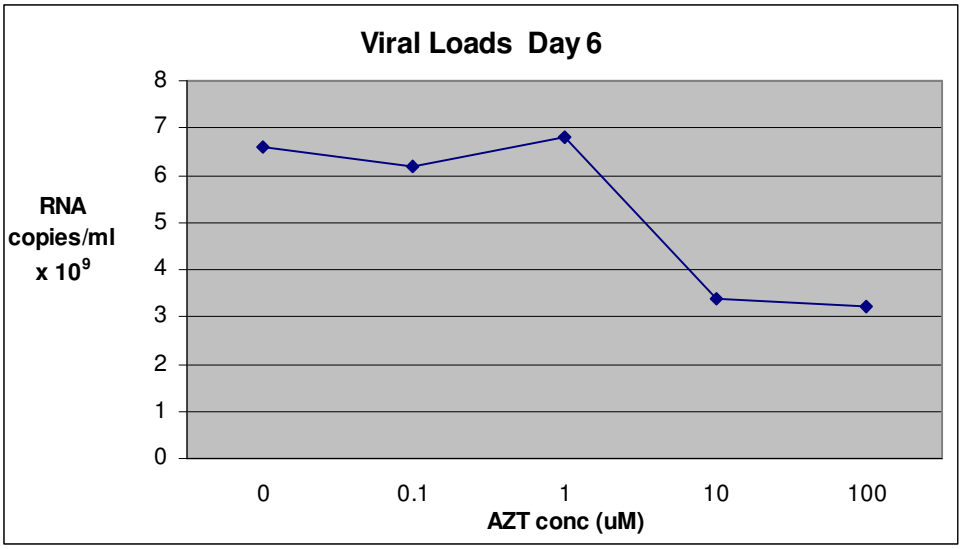

Figure 18: Viral load measurements at Day 6 of the antiviral assay performed on chronically HIV-infected H9 cell cultures treated with concentrations of AZT

The experiments were repeated several times and showed consistent results.

The p24 results fail to demonstrate the efficacy of AZT even at the highest concentration of the drug.

The viral load measurements show the reduction of viral replication at 10 μM of AZT.

In order to establish the accuracy of the p24 assay, we performed p24 measurements of 10 fold serial dilutions of virus stock. The concentration of p24 as measured by the ECLIA method, were plotted against the respective dilutions.

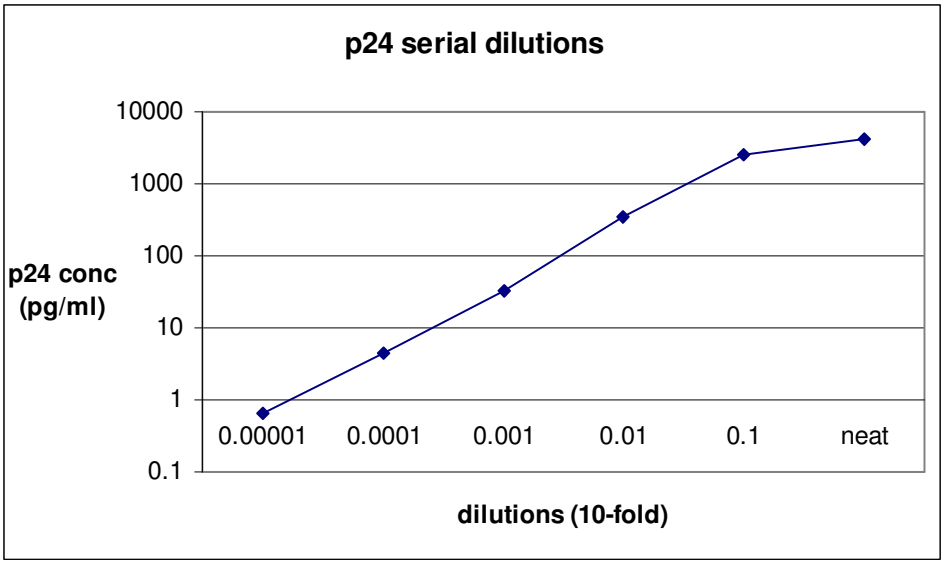

Figure 19: p24 concentrations of serial dilutions of viral stock as measured using the p24 ECLIA

The results are shown in Figure 19. This demonstrates the linear range of the assay and confirms its accuracy.

We also found that extremely large amounts of cell and virus free p24 antigen were produced in the robust HIV infected continuous cell cultures used in the study. Several thousand-fold dilution of the supernatants were necessary to allow p24 antigen concentrations to fall within the linear range of the assay.

It was also found that even after several washes of infected cell pellets and reconstitution in fresh RPMI, inconsistent baseline readings were still obtained and p24 concentrations were very high. Such high p24 concentrations, even in freshly seeded cultures of HTLV transfected H9 cells could be attributed to residual cell and virus free p24 antigen that remained on cell surfaces and the long half-life of p24 antigen.

Table 10: Viral load measurements on supernatant samples from chronically infected H9 cultures (Baseline and Day 6). Values are expressed as copies per ml x 10^9.

	Day 0 copies per ml x 10^9	Day 6 copies per ml x 10^9	Log Difference
Control for plant extracts	3.7	31	0.92
LA5B 250µg/ml	3.7	12	0.51
MM166A 250µg/ml	3.7	11	0.51
Control for AZT	5.1	6.8	0.12
AZT 0.1µM	5.1	6.2	0.08
AZT 10µM	5.1	3.4	-0.18
AZT 100µM	5.1	3.2	-0.2

We exposed chronically HIV-infected H9 cultures to various concentrations of AZT and the test compounds LA5B and MM166A and measured the viral load at baseline and at day 6 post exposure. Table 10 shows the results for a range of concentrations of AZT, and the optimal concentration of the extracts as calculated by the XTT assay. The log difference between baseline and Day 6 viral loads is also shown.

The log differences in the VL experiments clearly illustrate the efficacy of AZT in reducing viral replication at concentrations above 10µM.

The log differences for extracts LA5B and MM166A is less than that of the control.

The increase in viral load in the control exceeds the increase in viral load in experiments using the extracts. It is therefore possible that these extracts decrease viral replication in vitro at these concentrations.

The experiments were repeated and the results yielded the same conclusion (results not shown)

The p24 assay did not demonstrate the reduction of viral titres by AZT in repeated experiments. In contrast, the viral load did show a reduction of viral replication with 10µM AZT.

5.4.2 Antiviral assay performed on experimentally infected HTLV III_{MN} MT4 cells

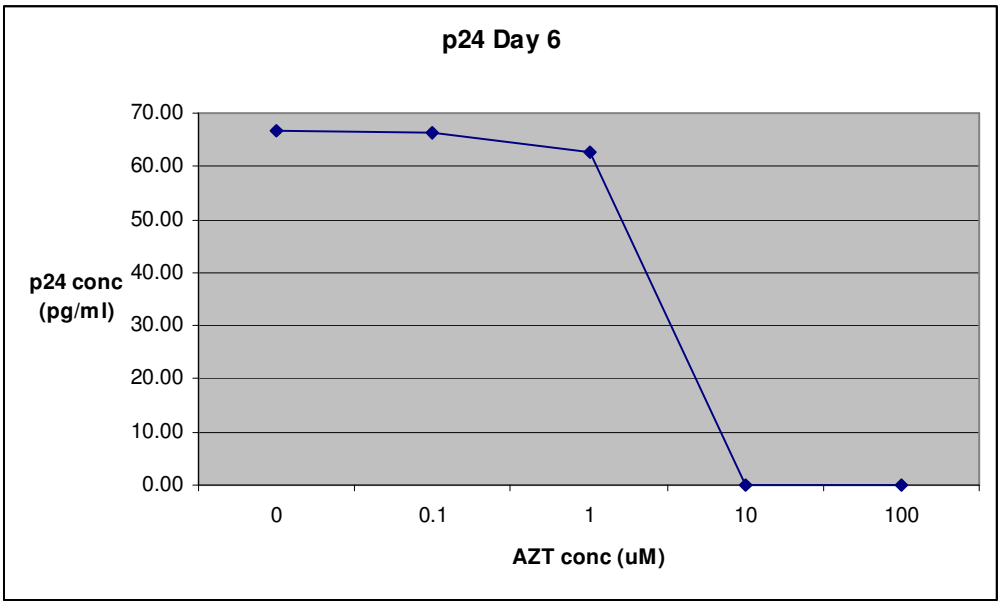

Figure 20: p24 concentrations at Day 6 of the antiviral assay performed on experimentally HIV-infected MT4 cell cultures treated with varying concentrations of AZT

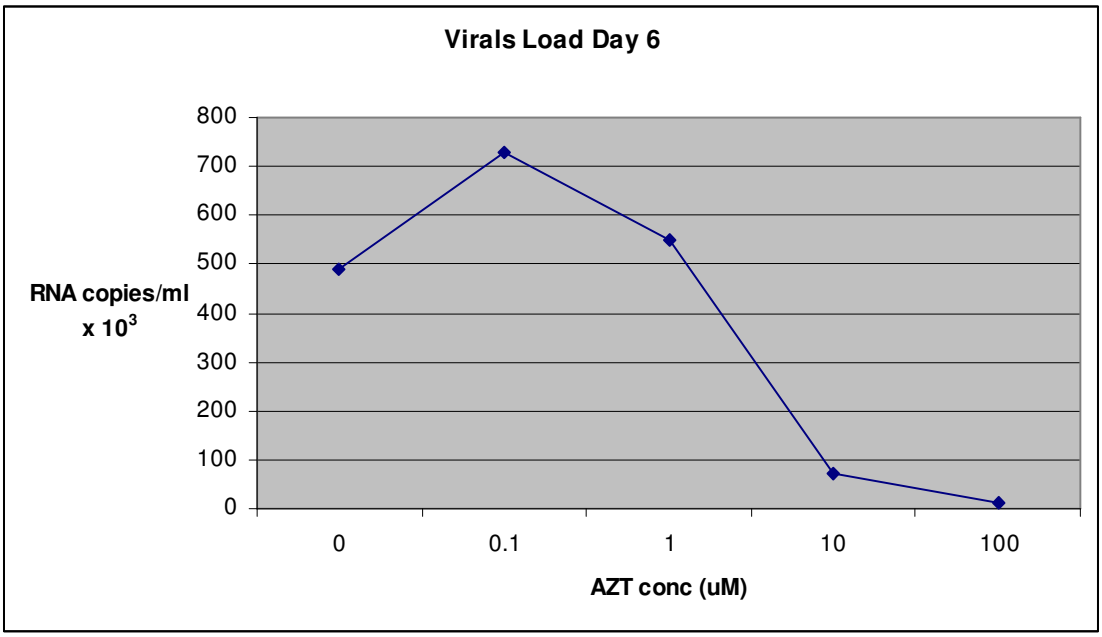

Figure 21: Viral load measurements at Day 6 of the antiviral assay performed on experimentally HIV-infected MT4 cell cultures treated with concentrations of AZT

The experiments were repeated several times and showed consistent results.

The p24 results clearly demonstrate the efficacy of AZT. The viral load measurements show the reduction of viral replication at 0.1 µM of AZT.

Table 11: Viral load measurements on supernatant samples from experimentally infected MT4 cultures (Baseline and Day 6). Values are expressed as copies per ml x 10^3

	Day 0 Copies per ml x 10^3	Day 6 Copies per ml x 10^3	Log Difference
Control for plant extracts	28	1500	1.7
LA5B 250µg/ml	22	970	1.6
MM166A 250µg/ml	17	2200	2.1
Control for AZT	46	490	1.0
AZT 0.1µM	23	730	1.5
AZT 1µM	14	550	1.6
AZT 10µM	10	73	0.9
AZT 100µM	9.6	11	0.059

Experimentally HIV-infected MT4 cells were exposed to various concentrations of AZT and the test compounds LA5B and MM166A. The viral loads were measured at baseline and day 6 post exposure. Table 11 shows the results for a range of concentrations of AZT, and the optimal concentration of the extracts as calculated by the XTT assay. The log difference between baseline and Day 6 viral loads is also shown.

Chapter 6: Discussion

The AIDS pandemic is considered to be one of the most devastating scourges of all time. UNAIDS has estimated that 40.3 million people globally, and 5.5 million people in South Africa were living with HIV in 2005. An estimated 2.8 million people worldwide died of the disease in 2005 alone, of which 2 million deaths occurred in Sub-Saharan Africa.

A large arsenal of drugs is available that can be used to improve the quality of life of infected individuals. However, this form of treatment does not achieve viral clearance and requires lifelong therapy. Furthermore poor adherence and the development of resistance are implicated in the failure of antiretroviral therapy. Options are limited by overlapping toxicities and cross-resistance between classes of anti-retrovirals.

As a result, a need exists for new drugs to help in the treatment of HIV infection. These drugs may either be a modification of existing drugs, or completely novel agents. Such novel drugs may either have similar mechanisms of action as current classes of drugs or have completely new targets.

However, the process of new drug discovery is time-consuming and resource intensive. It generally takes years to see the development of a new antiviral drug to completion, from discovery to preclinical testing, all the way through to clinical trials. The initial screening process is probably the most critical step in the new drug discovery process because it is here that the cytotoxic and antiviral properties of a potential agent are first identified.

The purpose of this study was the development of an initial screening process that could be adapted to a high-throughput environment for the testing of agents with potential antiretroviral activity.

All methods were compared using a chronically infected HTLV III_B H9 cell line and MT4 cells that were newly infected with HTLV III_{MN} prior to use in the experiments. The established $TCID_{50}$ technique was used in order to quantify the virus used to infect the MT4 cells. The results were calculated using the Spearman-Karber method.

A safer method of performing cell counts on HIV infected cultures was explored since viral titres in HIV-transfected H9 continuous cell line were found to be extremely high rendering the usual methods of cell counting unsafe. This method, described in Chapter 2, adapted the use of XTT as an indicator of cell proliferation (Berridge *et al*., 1993). A regression equation was derived which illustrated the linear relationship that was found between formazan production and cell number. This technique was used as an alternative to the Trypan Blue Exclusion Method to determine cell numbers in both uninfected and HIV-infected cultures.

The next objective of the study focused on the optimization of the XTT tetrazolium-salt based cytotoxicity assay for the testing of plant extracts. Several factors were considered that were unique to the testing of these extracts, details of which are not available in the current published literature. As part of the optimization of the XTT-assay we investigated the effects of the phenol red indicator in RPMI, the use of DMSO as a solvent, and the effects of the plant extracts on absorbance measurements.

The phenol red indicator contained in RPMI may compromise the optical density measurement of the XTT assay. We conducted experiments to determine the effects of both colourless RPMI and RPMI containing phenol red indicator on cell suspensions, and the overall effects on OD readings.

There was a statistically significant difference in the mean difference pre and post XTT-incubation i.e.Δ, between RPMI with and without phenol red indicator. This is possibly a result of the alteration in the colour of the phenol red indicator due to a pH change during the course of incubation. Therefore, RPMI containing indicators such as phenol red cannot be used in colorimetric cytotoxicity screens and the use of colourless RPMI is essential.

Phenolic plant extracts may require reconstitution in DMSO. Due to potential toxicity of DMSO to cells (Weislow *et al*., 1989), we conducted experiments to determine a concentration range of DMSO which would be suitable for both the solubilisation of the plant extracts and cell-based cytotoxcity testing. All aqueous plant extracts were reconstituted in 100% H_2O. Phenolic extracts, which required reconstitution in 50-100% DMSO, would be diluted in complete RPMI to achieve concentrations of 0.625% DMSO or less prior to use in cell based tests.

Plant extracts are likely to contain various plant enzymes that have the potential to reduce the tetrazolium salt to formazan. Berridge *et al*. (1993) have shown that XTT may also be reduced by NADH and NADPH in the absence of cells and enzymes and that superoxides

play a vital role in XTT reduction in the absence of cells. The production of formazan by the activity of plant enzymes would mask decreased formazan production due to cytotoxicity and provide erroneous results.

We conducted experiments to investigate this hypothesis and found no statistically significant effect of plant enzymes on formazan production.

However, there were significant differences between the baseline OD of the control and each of the concentrations of the extract ($p<0.05$). This finding suggested that the colour of the extract is a significant factor in OD calculations in the XTT assay. To account for this, a modification was made in the analysis step of the assay. Both pre and post XTT incubation OD readings were taken in all subsequent experiments so that pre-incubation OD readings could be subtracted from post incubation OD readings. This provided OD readings that solely indicated formazan production.

The modified XTT assay protocol was used for the testing of plant extracts, provided by the CSIR, and was used to determine EC_{50} and CC_{50} values of these extracts prior to further investigation of their potential antiviral properties. The percentage protection of each extract was calculated at a range of concentrations (Appendix C) and the selectivity indices established. AZT was used as a control in all experiments.

Two established and published XTT assay methods were compared, one using a continuous HIV-infected cell line and the other an experimentally infected T-cell line. Both methods yielded acceptable selectivity indices for AZT, however, the results were not comparable for the plant extracts tested. Furthermore, the absolute percent cell viability and percent protection were markedly higher for the chronically infected cells. There are several possible explanations for this. The use of different cell lines in the assays is known to yield differing selectivity indices (Turk *et* al, 2002). The experimental infection of the MT4 cells allowed for the exact quantification of the number of cells and infectious virions. This would ensure that the multiplicity of infection and proportion of infected cells per well were constant. In contrast, the chronically infected cells may produce a variable and unknown number of virions and the proportion of infected cells per well could not be determined. Experimental infection using 100 $TCID_{50}$ virus ensures that at least 50% of lymphocytes are infected. The percent of infected cells in chronically infected lymphocytes cannot be determined and is usually low since uninfected cells outgrow the infected cell population. In the XTT experiments using chronically infected cells, the uninfected cell population may greatly exceed the number of infected cells. Furthermore, healthy uninfected cells may survive preferentially compared to infected cells. The uninfected cells will proliferate and be measured as viable cells which may be spuriously interpreted as protection of infected cells by the test substance. XTT assays performed using experimentally infected cells are far more specific than those using chronically infected cells. This needs to be taken into consideration when interpreting and comparing selectivity indices reported in the literature.

Of the 4 extracts tested, we found two with favorable selectivity indices. These two extracts LA5B and MM166A were selected for further testing.

A viral susceptibility assay was developed in order to investigate several plant-derived extracts for potential anti-HIV activity. Continuous HIV-infected H9 cells and experimentally infected MT4 cells were cultured and exposed to varying concentrations of AZT (used as a control) and those plant extracts that demonstrated suitable selectivity indices in XTT assay. Cell culture supernatant samples were taken at 3 day intervals for quantification of viral replication using both quantitative p24 tests and viral loads.

The p24 results were inconsistent in chronically infected cells and did not demonstrate the expected antiviral efficacy of AZT. The inconsistency of the p24 measurements could possibly be due to the residual cell and virus free p24 antigen that remained on cell surfaces in the continuous infected cell line that was used in this study as well as the long half-life of p24. In contrast, the tests using experimentally infected cells clearly demonstrated the antiviral activity of AZT. The accurate quantification of virus used to infect the cells ensured that the virus did not replicate to immeasurable titres and hence avoided the problems seen in the use of the chronically infected cells.

Compared to the p24 tests, the viral load results were consistent for both experimentally infected cells and chronically infected cells in demonstrating the expected efficacy of AZT.

This is possibly due to the great linear range of the viral load assay and thus its ability to measure extremely high virus concentrations, even in the chronically infected cells where it was initially found that viral loads in the culture supernatant greatly exceeded the upper limit

of detection of the NASBA viral load techniques (10 x 10^6 copies per ml). Measurements on the NucliSens instrument which exceed the upper limit are indicated by error code "E10" – no calibrator. Repeated experiments were required to determine the optimal dilution of the supernatant which would permit measurements to be within the dynamic range of the assay. The typical dilution factor was 1 000 to 10 000 fold and lysis Buffer was used as a diluent when testing the supernatant samples from the chronically infected H9 cultures.

In order to ensure that the infected cells were exposed to the plant extracts through the various stages of the virus replication cycle, we used both chronically infected cultures and newly infected cultures for all cytotoxicity and antiviral assays.

We do however acknowledge several limitations to our study.

The plant extracts used in this study have not been resolved to compounds that can be chemically defined. They may contain toxins, inhibitors and stimulators of cell growth that could affect the results. Our tests will not yield a positive hit if the extract contains compounds that antagonize each other. In this case compounds within the extract that have potential anti-HIV activity will be missed. This requires fractionation and "dereplication" and further testing of the extract resolved to its individual components. On the other hand, screening of crude extracts is still useful and necessary because these extracts may contain compounds with synergistic effects which may be lost when they are tested as individual compounds.

Only one control (AZT) was used. However, it was not an objective of this study to compare compounds with different mechanisms of action. The control was used only to validate the experiments and not to compare the efficacy of compounds.

A limitation of the application of the methods described in this study is that all experiments using HIV infected cells must be carried out in specialised BSL III safety facilities.

Only individuals with skills and expertise in HIV culture and who have been trained to work in BSL III facilities may perform such experiments. This limits their capacity for use in high throughput screening in laboratories without the appropriate personnel and facilities.

The XTT-based cytotoxicity assay is a rapid, sensitive and cost-effective method of determining $EC_{50,}$ CC_{50} and SI. This test may be adapted for use in a high-throughput environment for the screening of potential antiviral compounds. Although 6-day incubations provide a sufficient time for the interaction of the test compound with cell cultures, this test may be optimised for more rapid cytotoxicity testing. The HIV-infected continuous H9 cells and MT4 cells may also be cultured in wells in 96 well plates so that cytotoxicity and antiviral testing may be performed concurrently.

Blair et al. (2004) describe a novel antiviral high throughput screening approach called the HIV-1 dual reporter assay for the discovery of HIV-1 inhibitors. This assay allows for the simultaneous evaluation of cytotoxicities and the potential antiviral activities of compounds in a high throughput screening format using gene reporters. This assay can also be used to distinguish specific antiviral compounds from non-specific inhibitors or cytotoxic compounds.

Antiviral high throughput screens are a valuable option for identifying novel anti-HIV agents but few are used in practice on an industrial scale due to the labor-intensive process and extensive costs. Novel approaches to antiviral screening provide a platform for newer, more improved techniques that would enable the effective execution of industrial scale high throughput screening. This would allow for greater returns on resource investment when compared to previous methods and potentially lead to the discovery of novel agents against HIV infection.

APPENDICES:

Appendix A. Laboratory Safety

HIV CULTURE LABORATORY

1. All procedures involving the culture of HIV were carried out in Biosafety Level III conditions as specified in the *Laboratory Biosafety Manual* (published by the World Health Organisation) and *Biosafety in Microbiological and Biomedical Laboratories* (published by the Centres for Disease Prevention and Control).

2. These guidelines included, but were not restricted to, the following
    a. Standard Microbiological Practices
    b. Special Practices
        i. The use of needles and sharps was prohibited
        ii. Use of a biological safety cabinet when working with any hazardous materials.
        iii. Regular decontamination was performed
    c. Primary Barriers
        i. Gowns made of resistant material were worn when perfoming procedures in the HIV culture lab
        ii. Two pairs of long-cuff gloves were worn at all times.
        iii. Plastic face shields were worn at all times
        iv. Surgical caps and shoes were worn at all times

d. Secondary Barriers: Laboratory features including

   i. restricted access

   ii. sink for handwashing and an eyewash station

   iii. design of floors, windows and benchtops

   iv. ventilation system

   v. on-site autoclave

   vi. biosafety cabinets

3. All procedures involving the culture of HIV were carried out only in designated areas. These areas had access which was restricted to authorized personnel only.

4. All procedures involving the culture of HIV were carried out under the supervision of experienced personnel as determined by the Head of Department of Virology.

5. All procedures and protocols relating to accidental exposure to infectious materials were made available in the laboratory.

6. All procedures and protocols relating to accidental spillage of infectious or toxic materials were made available in the laboratory.

7. All safety equipment, devices and materials were regularly maintained and monitored for efficacy.

Appendix B. Cell Culture

1. Complete RPMI:

- RPMI-1640 media supplemented with L-glutamine containing phenol red indicator (Sigma)
- RPMI-1640, modified media supplemented with L-glutamine without phenol red indicator (Sigma)
- Foetal Calf Serum (Sigma)
- Antibiotics (Penicillin, Amikacin, Fungizone) (Sigma)

Complete RPMI (with phenol red) containing 10% foetal calf serum and 0.1% antibiotics was used to maintain cell cultures

Complete RPMI (without phenol red indicator) containing 10% foetal calf serum and 0.1% antibiotics was used to make up plant extract, AZT and DMSO dilutions.

Complete, colourless RPMI was also used for the XTT cytotoxicity assays.

Appendix C. Preparation of Plant Extracts, Drugs, Test Reagents:

All compounds to be tested were received from CSIR BioProspecting unit in Pretoria

1. HIV-1-18 A
2. HIV-1-18 B
3. HIV-1-18 C
4. HIV-1-18 D
5. HIV-1-19A
6. HIV-1-20A
7. HIV-1-21A
8. WG-1-94
9. WG-1-94B
10. EK-III-68
11. LA-13-5B
12. MM-11-166A
13. MM-II-166B
14. MM-II-166C

Compounds **18-A, 18-B, 18-B** and **18-C** were reconstituted in 1:1 DMSO and distilled water.

Compounds **WG-1-94** and **WG-1-94B** were reconstituted in 100% DMSO.

All other compounds were reconstituted in 100% sterile, deionised water.

Compounds were diluted in complete colourless RPMI to yield the following concentrations (µg/ml): 0.25, 0.79, 2.5, 7.9, 25, 250

AZT:

Dilutions of AZT were made up to the following concentrations (μM) in complete RPMI-1640 modified media without phenol red:

0.0001, 0.001, 0.01, 0.1, 1, 10, 100, 1000

DMSO:

DMSO, 99.5% GC (Sigma, USA) was used.

Dilutions of DMSO were made up to the following concentrations in complete RPMI-1640 modified media without phenol red :

6.25%, 0.625% and 0.0625%

XTT

XTT/PMS solution was made up according to instructions on the package insert.

5ml RPMI-1640 modified media without phenol red was added to 5mg XTT/PMS.

The solution was vortexed to mix.

Appendix D. The Trypan Blue Exclusion Method of Cell Counting

20µl of cell suspension and 20µl of 0.1% Trypan blue solution was pipetted into a 500µl Eppendorf tube.

A 1:1 ratio of cell suspension to Trypan blue solution was used, resulting in a dilution factor of 2.

The cell suspension and Trypan blue solution were mixed thoroughly by pipetting up and down 10 times.

The Eppendorf tube was incubated at room temperature for five minutes.

The Neubauer Haemocytometer was wiped down with 70% Ethanol solution and dried to remove any debris.

10µl of the cell suspension-Trypan blue solution was pipetted into each chamber in the haemocytometer by pipetting the volume between the counting slide and the glass coverslip.

The cytometer was place on the stage of the light microscope and the counting grid viewed using the appropriate objective.

Unstained (clear, refractile) cells in all four outer quadrants of both grids were counted.

This number was divided by eight to determine the average number of cells in one quadrant.

The following equation was used to calculate the number of cells per ml of suspension:

Average number of cells per quadrant x Dilution factor x 10 000 = Number of cells/ml

The number of cells in a volume of cell suspension was calculated by multiplying the number of cells per ml by the volume of cell suspension.

Appendix E. Preparation of cell suspension for the XTT assay:

500µl of cell suspension, of the final cell concentration to be used was added to each labelled 1ml eppendorf tube.

The tubes were spun at 1500 rpm for 10 minutes.

The supernatant was discarded and 500µl of the appropriate concentration of compound, made up in complete colourless RPMI, was added.

The tubes were vortexed gently to reconstitute the cells.

150µl of this cell suspension was added to each well, in triplicate.

Appendix F. Qualitative p24 ELISA

The Murex HIV Antigen mAb is suitable for testing cell culture supernatants (Abbott Murex package insert).

List of Reagents:

1. Wash Fluid - Concentrated wash fluid contains 45ml concentrated phosphate buffer and 0.15% Proclin® as a preservative.
2. NP40 – contains 10 ml NP40, bovine casein as a stabiliser and bovine aprotinin as a protease inhibitor, and 0.05% Proclin®.
3. Conjugate 1 – contains 0.4ml concentrated biotinylated murine anti-p24 monoclonal antibodies, bovine albumin as a stabiliser, heat inactivated mouse serum, and 0.05% Proclin®.
4. Conjugate Diluent 1 – contains 4ml aggregated human IgG in phosphate buffer, heat inactivated mouse serum, and 0.05% Proclin®.
5. Conjugate 2 – contains 0.3ml peroxidase conjugated steptavidin.
6. Conjugate Diluent 2 – contains 30ml phosphate buffer, bovine casein as a stabiliser and bovine aprotinin as a protease inhibitor, and 0.05% Proclin®.
7. Chromogen solution – contains 0.3ml concentrated tetramethylbenzidine (TMB) dissolved in dimethylsulphoxide (DMSO), and 0.02% Thiomersal as preservative.
8. Substrate buffer – contains 30 ml phosphate citrate buffer and 0.006% hydrogen peroxide.
9. Negative control – contains 2.5ml human serum negative for HIV antigens and 0.1% sodium azide as a preservative.

10. Matrix – contains 15ml phosphate buffer, bovine casein as a stabiliser and bovine aprotinin as a protease inhibitor, and 0.05% Proclin®.
11. Standard solution – contains 2.5ml recombinant p24 diluted in matrix, bovine casein as a stabiliser and bovine aprotinin as a protease inhibitor, and 0.05% Proclin®.
12. One plate of 96 wells, coated with human anti-HIV polyclonal antibodies with silica gel as a drying agent.

Reagent preparation for one 8 well strip:

All reagents were brought to room temperature and prepared as required.

1. Wash buffer was prepared by adding 2ml concentrated wash fluid to 48ml distilled, deionised, sterile water.
2. Conjugate working solution 1 was prepared by making up 500µl of Diluted Conjugate 1, (25µl of Conjugate 1 to 475µl of Diluent 1) and adding this to 500µl NP40.
3. Positive control was made up by adding 60µl standard solution to 60µl matrix.
4. Conjugate working solution 2 was prepared by diluting Conjugate 2, 100 times, with Conjugate diluent 2 by adding 20µl Conjugate 2 to 1.98ml Diluent 2.
5. Substrate solution was made up by adding 20µl Chromogen solution to 1.98ml substrate buffer.

Manual mixing and washing procedures:

1. Mixing using pipette:

   The pipette to be used was set at the appropriate volume so that all liquid could be pipetted.

   The entire contents of the well was pipetted up and down for the number of times required.

   Pipette tips were changed after each mixing.

2. Wash procedure

   The first row of wells was aspirated and the contents discarded.

   The entire row of wells was filled with working strength wash fluid.

   This procedure was repeated for every row of wells in turn.

   Each row of wells was left to soak for at least 30 seconds before the wash fluid was aspirated and the process repeated 5 times.

   The wells were inverted and tapped dry on blotting paper after each wash.

Method:

Wash buffer, positive control and conjugate working solutions were prepared as described above.

100µl of conjugate working solution 1 was added to each well, including wells designated for positive and negative controls. 100µl of the appropriate cell culture supernatant (sample) was added to each well. 100µl of positive control and 100µl of negative control was added to each designated control well.

The samples/controls were mixed thoroughly with the conjugate working solution 1 in each well 5 times, according to the manual mixing method described.

The strip/s were covered using the provided adhesive plate sealers and incubated for 60 minutes at 37◦C (Incubation 1).

At the end of Incubation 1, conjugate working solution 2 was prepared as described.

Each well was then washed 5 times with wash fluid, according to the wash procedure described.

200µl of Conjugate working solution 2 was added to each well. The strips were covered with an adhesive plate sealer and incubated for 30 minutes at 37◦C (Incubation 2).

Substrate solution was prepared at the end of incubation 2.

Each well was washed 5 times according to the wash procedure.

200µl of substrate solution was added to each well and the strips were incubated for 30 minutes at room temperature (15◦C to 30◦C).

The reaction was stopped by adding 50µl 1.5M sulphuric acid to each well. The strips were tapped gently to ensure mixing.

The absorbances in the wells were read at 450nm, reference wavelength 620nm, using the Tecan Sunrise Microplate Reader. This was done after 5 minutes but within 15 minutes of stopping the reactions.

Calculation of Results:

N = mean absorbance of Negative controls

P = mean absorbance of positive controls

S = absorbance of the sample

The cut-off value was defined as N + 0.050.

Interpretation of results:

The sample was considered non-reactive if $S < N + 0.050$

The sample was considered reactive if $S \geq N + 0.050$.

Appendix G. Quantitative p24 ECLIA

The Roche Cobas HIV antigen kit was used and analysis was performed using the automated Roche MODULAR ANALYTICS E170 analyzer.

Calibration:

Calibration was performed once per reagent lot, using the Elecsys HIV Ag Negative calibrator (Cal 1) and positive calibrator (Cal 2), and fresh reagents opened within 24 hours prior to calibration.

p24 Antigen test principle

Incubation 1:

- 50µl of sample, a biotinylated monoclonal HIV p24-specific antibody and the monoclonal HIV p24 specific antibody labeled with a Ruthenium complex were reacted to form a sandwich complex.

Incubation 2:

- Streptavadin-coated microparticles were added allowing the complex to be bound to the solid phase as a result of interaction between steptavidin and biotin (second incubation)

(Avidin has an extraordinarily high affinity for biotin, 1million times greater then Antigen-Anitbody complexes, rendering the binding irreversible.)

Washing:

The reaction mixture was aspirated into the measuring cell and the microparticles were magnetically captured onto the surface of the electrode. Any unbound reagent and sample was removed by washing with the system buffer (ProCell).

Detection:

A voltage was applied which induced chemiluminescent emission that was measured by a photomuliplier.

Results:

The results were determined automatically, using Roche ELECSYS software, by comparing the electrochemiluminescent signal from the sample with the cutoff value obtained by previous HIV antigen calibration. The evaluation and calculation of concentration of the antigen was carried out by means of a calibration curve that was established using standards of known antigen concentration.

Appendix H. Viral Load Assay:

The NASBA NucliSens EasyQ (Biomerieux) was used.

Lysis:

The lysis buffer was centrifuged for 10 seconds at 1500g. 1000µl of the test sample supernatant was added to the buffer. The tube was vortexed and incubated at room temperature for 10 minutes

Binding

After incubation, the lysis buffer and sample were centrifuged for 10 seconds at 1500g. 20µl of calibrator (made up in elution buffer) was added to the tube. 50µl of vortexed magnetic silica solution was added to the tube. The tube was closed and vortexed immediately after addition of silica and incubated for 10 minutes at room temperature

Washing

The tubes were centrifuged for 2 minutes at 1500g. The supernatant was aspirated and discarded. 400µl of wash buffer 1 was added to the silica. 450µl of the silica-wash buffer solution was transferred to 1.5ml NucliSens Mini Mag tubes and the silica was washed for 30

seconds at “STEP 1” on Mini Mag with the Magnet on. With the magnet on, all liquid was aspirated and discarded, without removing silica. With the magnet off, 400µl wash buffer 1 was added and the wash step was repeated. 500µl of Wash buffer 2 was added and the wash step repeated. 500µl of wash buffer 3 was added and the solution washed at “STEP 1” for 15 seconds (Magnet on). All liquid was removed and discarded without disturbing the silica.

Elution

25µl of Elution buffer was added to each tube. Tubes were incubated for 5 minutes at 60°C in thermoshaker at 1400 rpm.Tubes were placed on a magnetic rack and 20µl of sample was transferred to clean storage tubes. 5µl of this eluate were transferred to 0.2ml 8-strip tubes

Enzymes

45µl enzyme diluent was added to the lyophilized enzymes. The enzyme tube was tapped gently and allowed to stand for 15 minutes

Primers

90µl primer diluent was added to the lyophilized primers and vortexed until the solutions were clear. 10µl primer mix was added to the bottom of each tube containing the eluate. The tube strip was placed in a strip holder in the EasyQ incubator and the program initiated. The tube caps were placed upside down on the strip holder and 5µl enzyme solution was added to

each cap. After the last incubation step, the tubes were closed and the strip centrifuged for 2 seconds in the Mini-Strip centrifuge. The tubes were transferred to the NucliSens EasyQ analyzer for analysis.

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