CHAPTER 1
INTRODUCTION

Infection with HIV and the subsequent acquired syndrome continue to be global health and socio-economic concerns. Although there has been declining trends with respect to new infections and the number of deaths from HIV/AIDS related illnesses over recent years (UNAIDS, 2010), HIV remains a major health threat. Sub-Saharan Africa is the hardest hit area having up to 68% of the world's total number of infected people (UNAIDS, 2010). HIV affects the immune system depleting it of crucial T helper cells (CD4+ lymphocytes) needed by both the humoral and cellular arms of the immune system, thereby rendering the individual immunocompromised. This depletion leads to an AIDS state characterised by wasting, morbidity, a host of opportunistic infections and ultimately mortality if not treated. To combat HIV/AIDS, therapies that increase the lifespan of infected individuals have been developed. The strategy of these therapies is reduction of viral load and the prevention of further loss of CD4+ cells (Lori *et al*., 2007). The use of highly active antiretroviral therapy (HAART) which is the combination of a PR inhibitor or a non nucleoside RT inhibitor (NNRTI) and two nucleos(t)ide RT inhibitors (NRTIs, Pommier *et al*., 2005) has been paramount in this approach of lowering viral load and preventing CD4+ cell loss. More recently drugs targeting the viral IN and antagonists to the CCR5 receptor of host cells were approved as first line treatment or salvage therapy for the treatment-experienced patients (McColl and Chen, 2010) increasing the range of available drugs. While these developments have greatly improved the standard of living of HIV/AIDS patients by decreasing morbidity and mortality (Hogg *et al.*, 1999, Palella *et al*., 1998), these drugs have limitations which include problems of toxicity, uncomfortable side effects (such as nausea, vomiting and diarrhoea, Montessori *et al*., 2004) and the development of drug resistance by the virus (Chen *et al.,* 2004, Skillmann *et al*., 2002). Other limitations are accessibility to treatment and the lack of infrastructure to monitor treatment especially in resource limited settings (developing countries, Lever, 2005b).

On the other hand, the development of a viable cure has not been achieved (Heagarty, 2003) but encouraging advances have been made. For example an HIV infected man who received stem cell transplantation from a donor homozygous for the *chemokine receptor 5* (*CCR5*) delta32 gene as treatment for acute myeloid leukemia was cleared of the virus (Hutter *et al*., 2009). A mutation in the *CCR5* gene is known to confer resistance against infections caused by HIV since the CCR5 co-receptor is involved in viral entry into cells. Another recent development towards a cure was the report from a group of Australian scientists who successfully cleared an HIV-like virus from mice by boosting the function of cells vital to immune responses (Pellegrini *et al*., 2011). The authors showed that interleukin-7 (IL-7) which is important for immune activation and homeostasis could lower the expression of the

suppressor of cytokine signalling 3 (*Socs3)* gene leading to increased cytokine production and T cell function. This, the authors suggested, may have enhanced innate anti-viral mechanisms.

In the field of prevention, an encouraging advancement was the recent report on an antiretroviral (ARV) vaginal gel containing the anti-viral drug, tenofovir, which resulted in moderate protection against HIV infection. A 39% lower risk of acquisition and up to 54% reduction of infection in women who achieved the best adherence was observed while blocking infection from herpes at the same time (Abdool Karim *et al*., 2010). This was a significant breakthrough especially because women are the most susceptible group in contracting new HIV infections (Quinn and Overbaugh, 2005). A recent breakthrough in prevention were the findings from the HIV Prevention Trial Networks (www.htpn.org, accessed 5/06/2011) in the study known as HPTN 052 which reported that the early administration of ARVs to infected men and women reduced the risk of HIV transmission to their partners by 96%. Other measures to curb infection have been intensive education and campaigns which have shown benefits in countries such as Uganda (Lever, 2005b).

Vaccine development has also been slow but has gained renewed interest after findings of limited protection from HIV infection were reported in the RV144 trial in Thailand (Rerks-Ngarm *et al*., 2009).

While HAART has been a success story, the mentioned limitations such as toxicity and the development of resistant viral strains and the transmission thereof, are amongst the drawbacks which could eventually render this therapy ineffective. In addition, the identification of latent reservoirs of HIV-1 in patients on HAART (Finzi *et al*., 1997) was one of the earliest limitations observed during therapy such that treatment has to be life-long. Because of the toxicity problems that are also associated with HAART, patients find it difficult to comply to prescriptions by physician (Ren and Stammers, 2005, Chen *et al.,* 2004). As a result, suboptimal doses of the drugs are taken, further enhancing resistance problems since optimal viral suppression is not attained. Identifying potential drug candidates that can be used in combination with or to supplement HAART with the goal of finding those with tolerable side effects that can also work against resistant strains is crucial. In this study, the possibility of using gold-based compounds as anti-HIV agents was investigated.

The focus here was mainly on bioactivity testing of gold-based compounds synthesized and provided by chemists from the Project AuTEK Biomed Consortium (Mintek and Harmony Gold, South Africa). The compounds were tested on the HIV-1 subtype C strain because it is the most prevalent subtype in Southern Africa (HIV clades are geographically distributed, Schiavone *et al*., 2008). Currently, subtype C infected patients are administered subtype B specific drugs. This leads to the emergence of resistant viral forms similar to those seen for subtype B as well as others not seen for the B subtype strain (Kantor and Katzenstein, 2004), which further complicates treatment and treatment options. By testing the compounds against

subtype C strains, the likelihood that more specific inhibitors could be identified which would eventually reduce the resistance burden seen when drugs designed for the subtype B strain are used was increased.

Gold compounds have medicinal properties that have mainly been exploited for the treatment of rheumatoid arthritis (Ahmad, 2004, Best and Sadler, 1996, Sutton, 1986). These compounds also show activity against cancers and microorganisms including the malaria parasite (Khanye *et al.*, 2010, Gabbiani *et al*., 2009, Sanella *et al*., 2008, Navarro *et al*., 2004, Navarro *et al*., 1997) and HIV (reviewed by Fonteh *et al*., 2010). This laboratory previously contributed evidence in a proof of concept study on the effect of gold compounds against HIV enzymes (RT and PR, Fonteh *et al*., 2009, Fonteh and Meyer, 2009).

The scope of this research was further extended here by determining how comparable the gold compounds were to known drugs with regards to functional groups and physical properties (drug-likeness), effects on host cells in cell-based assays (to determine compound effects on host cells and whole virus), and on viral enzymes (direct enzyme bioassays and *in silico* to determine binding modes). In addition to the sixteen compounds previously tested in the proof of concept studies (Fonteh and Meyer 2008), eleven new ones were included in this study resulting in twenty-seven compounds that span five different chemical classes based on synthetic precursors used.

Binding mode interactions of the compounds with the RNase H site of RT and the IN cofactor site showed favourable enthalpic contributions but require structural modifications of the compounds to enhance activity. An outstanding novel observation of this study was the identification of the mechanism by which three compounds (designated PFK7, PFK8 and EK207) inhibited cellular infectivity by a dual subtype C strain of HIV-1. Inhibition of infectivity was not as a result of the compounds' interaction with viral surface components but rather was as a result of the compounds' cytostatic effect which was observed using the dye dilution technology of carboxylyfluorescein succinimidyl ester (CFSE) and the impedence (resistance) technology of a real time cell electronic sensing (RT-CES) device. The cytostatic mechanism (anti-proliferative effect on the cell rather than on the virus) was further confirmed for complexes PFK7 and EK207 using multi-parametric flow cytometry where the frequency of CD4+ cells from peripheral blood mononuclear cells of HIV infected individuals was significantly reduced ($p = 0.0049$ and 0.027 respectively). The ability of these compounds (PFK7 and EK207) to reduce T cell numbers could be interpreted to mean that the compounds were capable of blocking viral replication as a result of the ability to prevent antigen presenting cells from activating T cells, a finding which has been shown for gold and other metal compounds (De Wall *et al.*, 2006). Compounds which have a cytostatic mechanism of action and which lower CD4+ cell numbers (such as hydroxyurea) have demonstrated a significant role in HIV research both *in vitro* (Clouser *et al*., 2010, Lori *et al*., 2005, Mayhew *et al*., 2005) and in clinical trials (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al*.,

1998). The cytostatic effect of hydroxyurea is also known to be as a result of the inhibition of ribonucleotide reductase (RNR) which is an enzyme involved in converting ribonucleotides to dNTPs (DNA building blocks, Lori, 1999). At 10 µM PFK7 also inhibited RNR significantly ($p = 0.003$). Combining these agents with compounds that directly target the virus is known to result in the overall restoration of immune parameters in infected patients and to a better resistance profile compared to HAART (Lori *et al.*, 2007). Three of the twenty seven compounds (PFK7, PFK8 and to a lesser extent EK207) have the potential of being combined with compounds that directly target the virus and function in the new emerging class of combination therapy known as virostatics. Such a combination stands a better chance in managing HIV since drug resistance (which this combination minimises) has become the greatest threat to HAART. In addition, the drug-like properties that were seen for complexes PFK7 and PFK8 (drug score of 6 out of 7) makes these cytostatic agents highly favoured as potential components of virostatic cocktails.

In chapter 2, general background and literature review of topics relevant to this study is provided. This is followed by three chapters that provide detailed information on each of the main research aims which were (1) determining the drug-likeness of the compounds (chapter 3), (2) the effect of the compounds on immune system cells and whole virus (chapter 4) and (3) effect on viral enzymes (chapter 5). An overall conclusion on the study is then provided (chapter 6), followed by a comprehensive list of references (chapter 7). Supplementary data is provided in the appendix (chapter 8) followed by a glossary with definitions for uncommon words. Finally, copies of two published manuscripts containing information obtained during this study are provided.

CHAPTER 2
LITERATURE REVIEW AND BACKGROUND

2.1 HIV AND AIDS

HIV is a primate lentivirus belonging to the *Retroviridae* family and affects cells of the immune system ultimately leading to AIDS (Gonzalez *et al*., 2009, Campbell and Hope, 2008). Two viral types exist, HIV-1 and HIV-2 both being enveloped retroviruses (Campbell and Hope, 2008, Lever, 2005). HIV-1 found worldwide is more pathogenic than HIV-2 but both cause similar illnesses (Lewthwaite and Wilkins, 2005). Patients with HIV-2 have lower viral loads, slower CD4 decline and lower rates of vertical transmission (Lewthwaite and Wilkins, 2005), not seen in HIV-1 infection. The nucleic acid sequences of the two types are only 40% similar (Lever, 2005b). HIV is closely related to another primate lentivirus, the simian immunodeficiency virus (SIV). HIV-1 is similar to SIV found in a group of chimpanzees while HIV-2 is more closely related to SIV found in sooty mangabey monkeys (Lemey *et al.*, 2003, Sharp *et al*., 1995). The former virus (HIV-1, which is the focus of this study) has a high genetic variability and has been classified into major (M), outlier (O), and non-M/O (N) groups (Sanabani *et al*., 2006). Group M has nine subtypes ranging from A-J which include subtypes A, B, C, D, F, G, H, J and K as well as circulating recombinant forms (CRFs), Carr *et al*., (1998). The most prevalent strain worldwide and in Southern Africa is the subtype C strain (Nkolola and Essex, 2006, Wouter *et al*., 1997).

Infection with HIV if not treated culminates in death from infections that lead to AIDS defining diseases like candidiasis, cryptosporidiosis, cytomegalovirus, *Pneumocystis carinii* pneumonia, toxoplasmosis and tuberculosis (Pozio and Morales, 2005). The AIDS state stems from the depletion of CD4+ T helper lymphocytes (Rambaut *et al*., 2004) which are critical for effective immune function. AIDS, which is usually the last battle between HIV and the body's immune system, occurs when there is a drop of the total CD4+ T cell count to approximately 200 cells/µL of blood (World Bank, 1997). In addition to CD4 T cells, the virus also infects other cells that express cell surface receptors that allow for viral entry such as the CD4 and chemokine co-receptors consisting of CCR5 or CXC chemokine receptor 4 (CXCR4), Dragic *et al*., (1996), Choe *et al*., (1995).

In the next subsections, the epidemiology, transmission modes, structure, life cycle and the course of HIV-1 infection will be provided. This will be followed by information on vaccine development, HIV's effect on the immune system, available therapy and information on the need for new drug development. Finally, the research hypothesis will be stated and the main research questions introduced. HIV will be used throughout this document to refer to HIV-1.

2.1.1 Epidemiology

The global view of HIV infections in 2009 according to the UNAIDS report of 2010 has not changed significantly compared to the previous year where an average of 33.4 million people were living with the virus worldwide as detailed in Figure 2.1. A revision of the 2008 statistics showed that 32.8 million people were living with the virus, which is within the uncertainty range of the previous estimate. According to these statistics, Sub-Saharan Africa still bears the greatest burden with regards to the number of infected people (with 22.4 of the total 33.4 million worldwide estimate in 2008) and new infections.

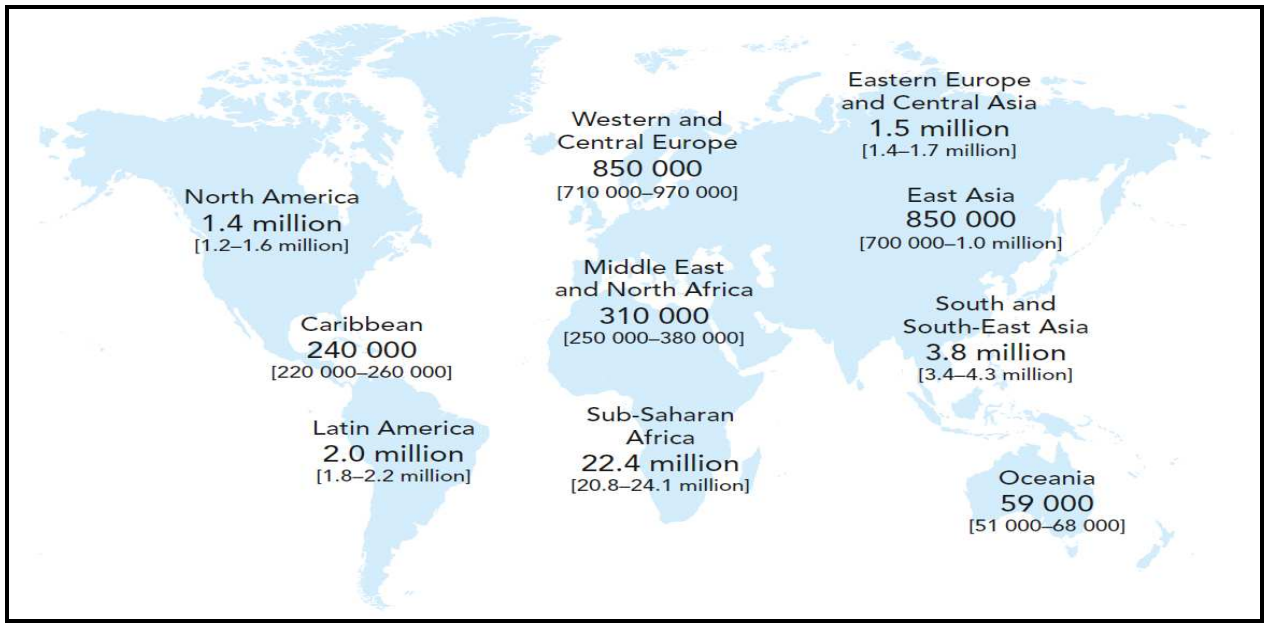

Figure 2.1: Global view of HIV infections (2008). An average of 33.4 million adults and children were living with HIV (UNAIDS report, 2009).

The good news according to the 2010 report is that although up to 32.8 million people are infected globally, there were declining trends in new infections in 2009 where only 2.1 million were noted compared to 2001 where 3.1 million people were newly infected. Figure 2.2 shows the changes in the incidences of new infections over 2001 to 2009. In 33 countries including South Africa there has been decreasing incidence of newly infected people by >25% from 2001 to 2009 (Figure 2.2). Not only are new infections decreasing but the death rate from HIV is also decreasing across the spectrum due to increased access to antiretroviral agents. The decrease in new infections has been attributed to an overall combination of factors including the impact of prevention efforts (UNAIDS report, 2010).

The lack of survey data in some instances and the absence of diagnostic test for very early detection of HIV are factors that limit the determination of the actual infection rate such that only estimates are obtained. In addition, while the rate of infection is declining in some regions, statistics show that the reverse is true for others such as parts of Asia (Figure 2.2) with an increasing rate of >25%. These findings suggest that the battle is still on and more effort than ever has to be directed to researching solutions for managing and preventing HIV infection.

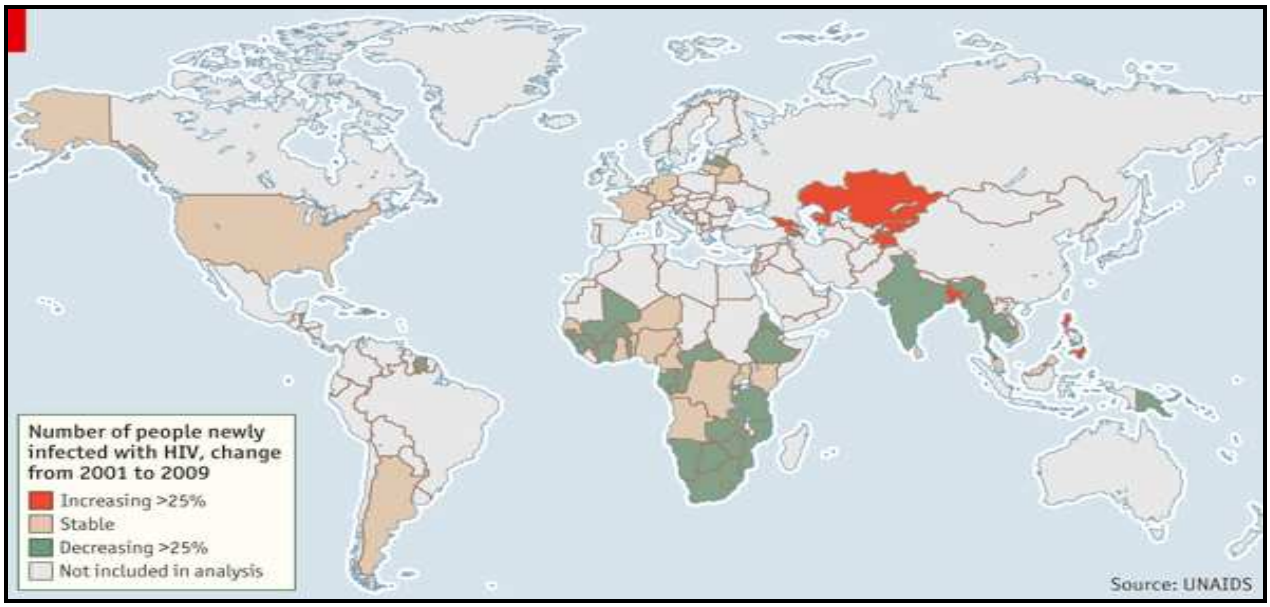

Figure 2.2: Changes in the incidence rate of HIV infections from 2001 to 2009 for selected countries (http://media.economist.com/images/images-magazine/2010/11/27/st/20101127_stm958.gif, accessed 02/02/2011).

2.1.2 Mode of Transmission

HIV is transmitted through bodily fluids and mostly sexually (homosexual or heterosexual), but also occurs vertically from mother to child, through blood transfusion and sometimes through unidentified means (Monavi, 2006). Sexual transmission is the most important route since it is the most common means of transmission of HIV (Walker *et al*., 2003). Vertical transmission is common in developing countries in pregnancy and at birth or during breast-feeding (Lewthwaite and Wilkins, 2005). Injection drug use is also one of the ways by which HIV is transmitted and although it is relatively low in countries such as the United Kingdom, its prevalence can be up to 50% in others such as Eastern Europe, Vietnam, India and China (Lewthwaite and Wilkins, 2005).

2.1.3 HIV Genome Organisation and Structure

The HIV genome consists of nine genes that encode 15 viral proteins (Gotte *et al*., 1999). These include the group-associated antigen (*gag*) encoding structural core proteins, a polymerase (*pol*) portion encoding the enzymatic proteins PR, RT, IN, and an envelope (*env*) frame encoding the receptor binding protein. The genome codes for two regulatory proteins (Tat and Rev) and four accessory proteins (Vif, Vpr, Vpu and Nef) required for proper virion replication. A schematic representation of the viral genome is shown in Figure 2.3. Two long terminal repeats (LTRs) flank both the 5' and 3' ends of the proviral DNA genome. The 5' LTR includes the HIV promoter and enhancer sequences that regulate viral gene expression. The genome constantly undergoes variation as a result of mutational and evolutionary pressures and pressure from the immune system such as those exerted by viral specific CD8+ T lymphocytes, which also leads to escape mutants (Sanabani *et al*., 2006, Karlsson *et al*., 2003).

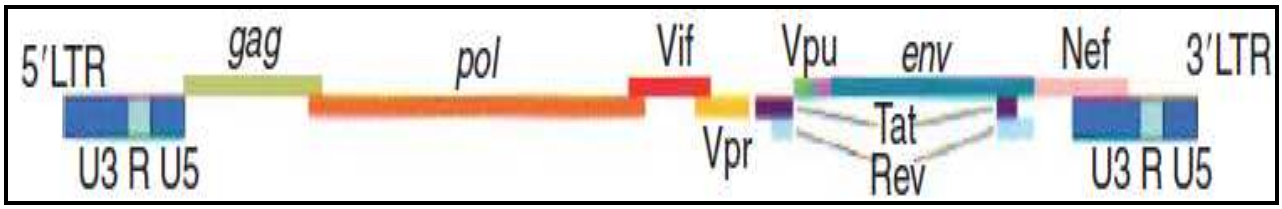

Figure 2.3: Schematic representation of HIV genome. Replication genes (*pol*, *vif*, *nef*, *tat*, *rev*, *vpu*, *vpr*) and assembly genes (*gag* and *env*) are represented. The figure was taken from Schiavone *et al*., (2008).

The structure of HIV (shown in Figure 2.4) described by Turner and Summers (1999) consist of an enveloped lipid bilayer derived from the host membrane, contains exposed surface glycoproteins (gp120) and is anchored to the virus by the transmembrane protein (gp41). A matrix shell comprising approximately 2000 copies of the matrix protein (MA, p17) lines the inner surface of the viral membrane, and a conical capsid core shell comprising ± 2000 copies of the capsid protein (CA, p24) is located in the centre of the virus. The capsid particle encloses two copies of the unspliced viral genome, which is stabilized as a ribonucleoprotein complex with approximately 2000 copies of the nucleocapsid protein (NC, p7), and also contains the three essential virally encoded enzymes namely: RT (p66/p51), PR (p11) and IN (p31). The unspliced viral genome consists of two similar RNA molecules approximately 10 kb in length (Coffin *et al*., 1997).

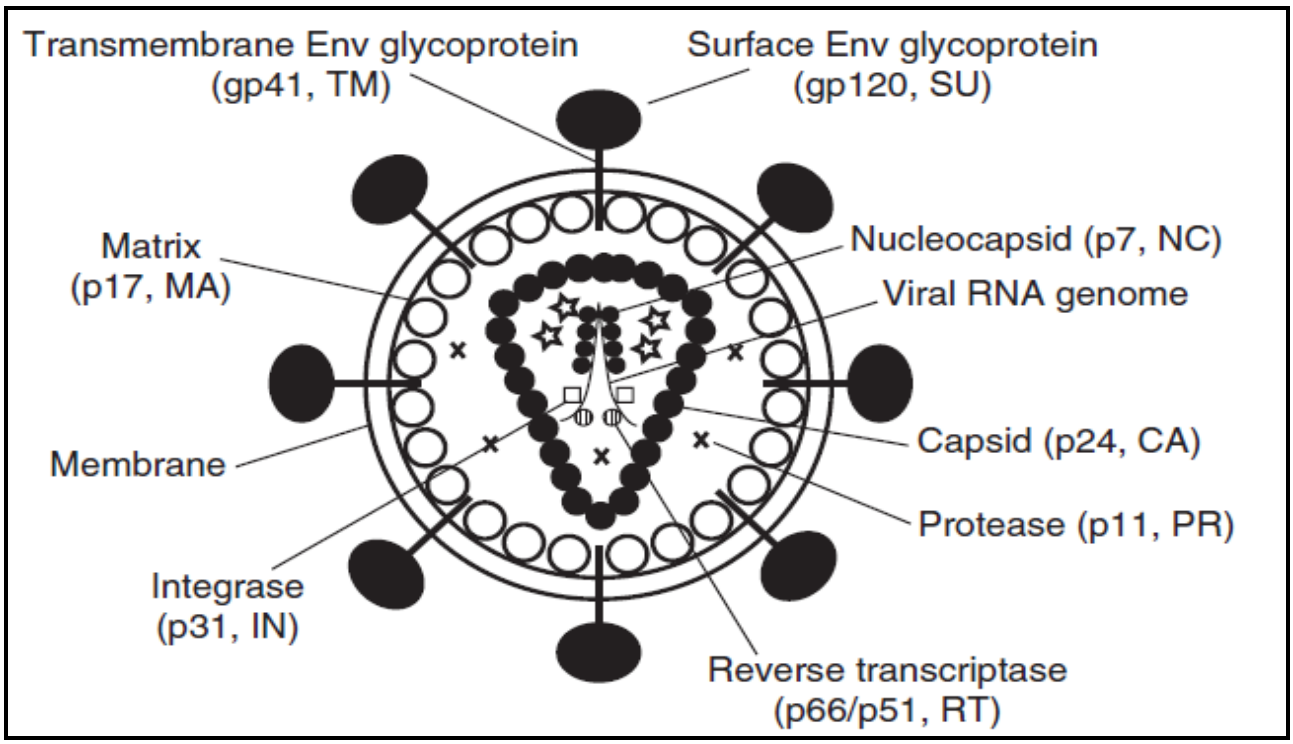

Figure 2.4: The structure of a mature HIV virion. The figure shows important viral proteins and their arrangement within the virion. This figure was taken from, Adamson and Freed (2007).

2.1.4 Life Cycle and Course of Infection

The life cycle of HIV involves three mains steps including (1) entry and integration, (2) transcription and translation and finally (3) budding (Lever, 2005). Figure 2.5 shows the key aspects of the life cycle as well as selected drug targets namely; (a) virus fusion, (b) reverse transcription, (c) proteolytic processing, (d) 3' processing and (e) strand transfer (ST) steps.

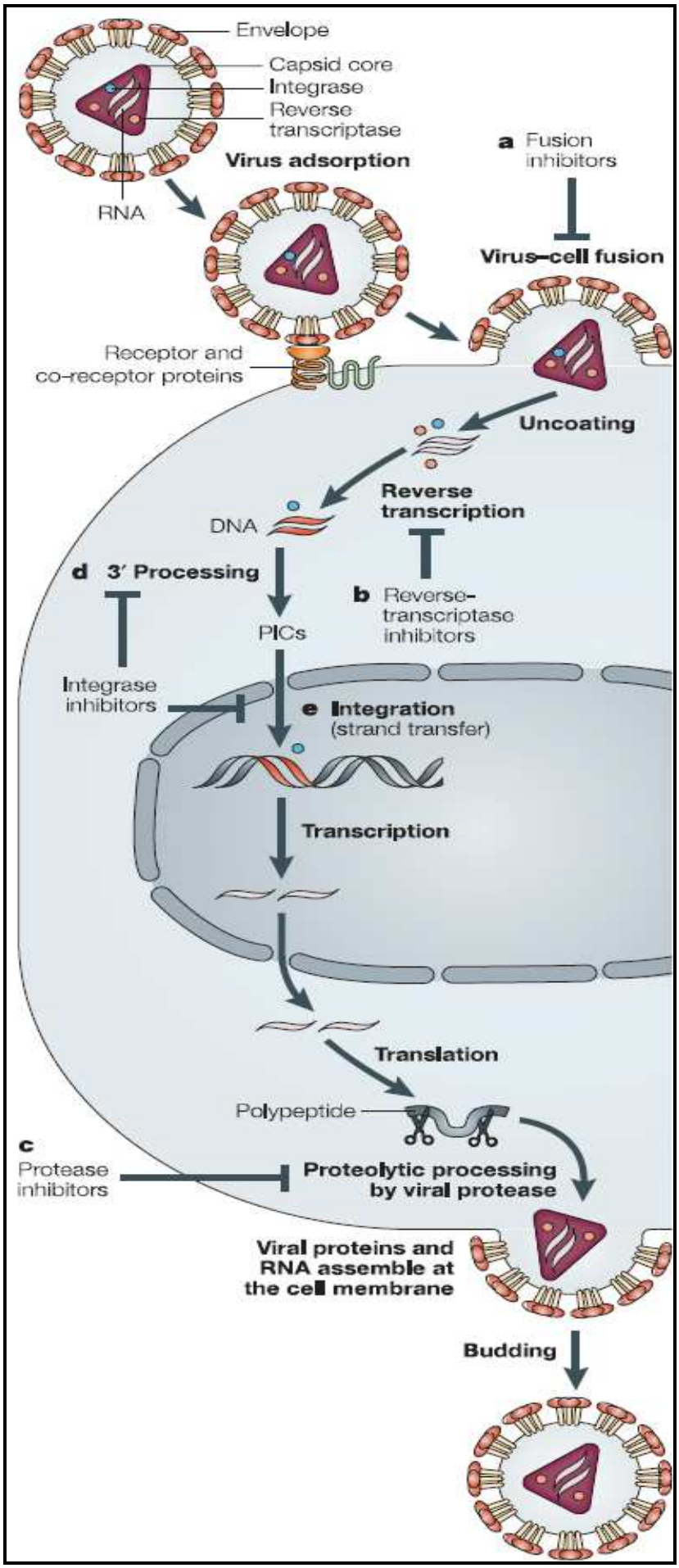

Figure 2.5: Key aspects of the life cycle of HIV. Important drug targets are also shown. The figure was taken from Pommier *et al*., (2005).

In the **entry and integration** steps of the life cycle, the virion bearing two copies of ribonucleic acid (RNA) binds to CD4+ receptors and chemokine co-receptors (CCR5 or CXCR4) on the cell surface. This is followed by fusion where the viral core is inserted into the host cell followed by uncoating and the release of viral contents within the cytoplasm of these cells. The viral RNA is reverse transcribed by RT to a complementary deoxyribonucleic acid (cDNA) strand, which is subsequently transported into the nucleus as the pre-integration complex (PIC). Here it is integrated into the host genomic deoxyribonucleic acid (DNA) by the viral enzyme, IN.

Transcription of the viral DNA leads to the production of viral genomic RNA and **translation** of viral proteins that are then processed and assembled in the cytoplasm by HIV PR. HIV PR further catalyses the maturation of the viral particles through proteolytic processing into infectious virions which then **bud** off from the cells

As the virus replicates and makes new copies, the course of infection in the infected individual is the gradual loss and destruction of naive and memory CD4+ T cells leading to AIDS which is the final stages of the infection course (shown in Figure 2.6, Forsman and Weiss, 2008). The primary acute infection stage (4-8 weeks) is characterised by high plasma viremia and low CD4+ cells and the absence or very little HIV specific antibodies. The viremia drops as cytotoxic T lymphocytes (CTLs) develop leading to an individual viral set point in the course of chronic infection (5-15 years).

In the final stages of infection, when opportunistic infections like tuberculosis and infections from *Pneumocystis, Cytomegalovirus* (CMV), cerebral *Toxoplasma* or *Candid*a occur (CD4+ count usually around 200 cells/µL of blood, World Bank, 1996), the viral load increases and CD4+ count continues to decrease ultimately leading to AIDS and death over a time course of 2-3 years. A striking new finding is the blow that the virus causes on the human body's largest lymphoid "organ" i.e. the gut and mucosal tissues, which is the significant depletion of mucosal CD4+ cells (Paiardini *et al*., 2008, Brenchley *et al*., 2006) not seen when circulating CD4+ cells in peripheral blood are sampled (Figure 2.6). This in turn could be the cause of the severe chronic immune activation noted throughout the course of infection such that recommendations for novel therapies aimed at targeting immune activation have been proposed (Forsman and Weiss, 2008). Other notable changes observed in lymphoid tissues are generalised lymphadenopathy, tonsillar enlargement and splenomegaly noted in early infection (Kilby, 2001). These features are associated with lymphocyte proliferation and the recruitment of inflammatory cells from the circulation. The enlargement gradually decreases in a majority of patients after seroconversion but may persist in others. In advance disease (and in the absence of treatment), the architecture of the lymphoid tissues changes resulting in an involution and lymphoadenopathy becomes less prominent (Pantaleo *et al.*, 1993).

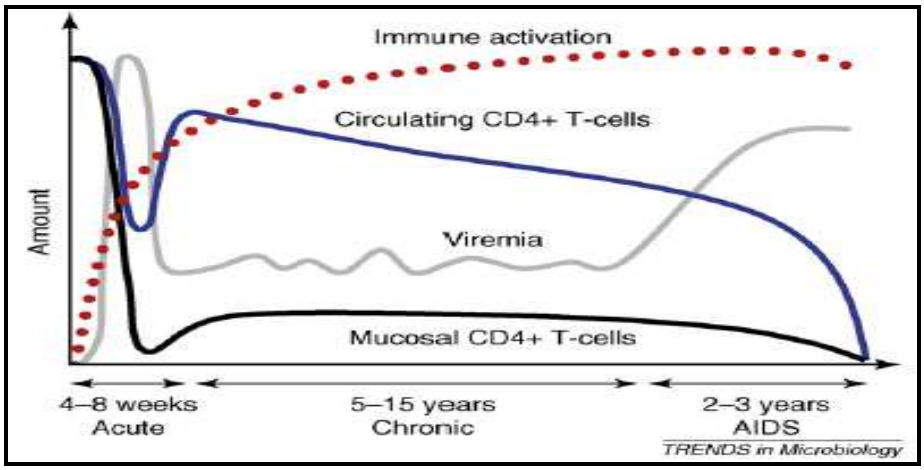

Figure 2.6: A schematic representation of the typical time course of HIV pathogenesis. The time course of adaptive immune responses in relation to viremia levels from acute infection to AIDS defining conditions is also depicted. The figure was taken from Forsman and Weiss (2008).

2.1.5 HIV and the Immune System

Infection with HIV triggers both B-cell (humoral) and T-cell (cell mediated) immune responses. These adaptive immune responses which unlike immediate and non specific innate immune responses, develop over days or weeks after exposure to the antigen as a result of clonal expansion and differentiation of B and T lymphocytes (McMichael and Dorell, 2005). These responses unfortunately fail to clear the infection (McMichael and Dorrell, 2009, Young, 2003). The CD4+ T cell subset which tends to be depleted in HIV infection is important in the adaptive immune response since these cells recognise antigen presented by major histocompatibility complex (MHC) class II and respond by turning on B lymphocytes to secrete antibodies against the antigen (humoral response). After recognising antigen, the CD4+ T cell gets activated and produces a heterogeneous group of proteins (cytokines) that are secreted to exert an effect on target cells (Goldsby *et al*., 2000) and which aid in the stimulation and recruitment of the CD8+ T cell subset or CTLs. Along with the decrease in CD4+ T cells in HIV infection, is the corresponding increase in CD8+ T cells (Musey *et al*., 1997, Koup *et al*., 1994). The latter targets and lyses virally infected cells through recognition of the foreign antigen bound by host proteins (Goepfert, 2003). In addition to killing the infected CD4+ cells, CTLs also release cytokines and chemokines which tend to block viral entry into other CD4+ cells (McMichael and Dorrell, 2009). The CTLs just like the antibodies play a critical role in the control of the infection.

Infection with HIV also kills CD4+ cells by direct cytopathic effect of the virus or through means which trigger apoptosis (a normal process for the elimination of unwanted cells), Young, (2003). This direct cytopathic effect of CD4+ T cells by the virus is one of the means by which the virus evades the immune system since by killing these cells, they also destroy immune effectors (Gougeon, 2005). The interaction of Fas ligand (which is a cell surface molecule belonging to the tumour necrosis factor family) on CTL surfaces with Fas molecules on the target cells e.g. CD4+ cells is also one of the ways by which apoptosis and lysis of the infected cells occurs (Garcia *et al*., 1997) and constitutes an indirect cytopathic means.

Figure 2.7 (taken from Gougeon, 2005) is an illustration of how HIV depletes the immune system of T helper cells as elaborated above. Through cognate interaction (cell-to-cell contact), CD4+ T cells recognise antigen from an antigen-presenting cell (APC, in this case a dendritic cell, DC) bearing MHC II complex. This interaction could either lead to apoptosis (direct or indirect viral cytopathic effect or CTL response), anergy (no response by the immune system) or to an activation state driven by cytokines such as IL-2 in the clonal expansion phase. Associated with this activation is the susceptibility to infection and destruction through activated T cell autonomous death (ACAD) and activation induced cell death (AICD) by virions. Proapoptotic virion particles such as gp120, Tat, Nef, Vpr or Vpu also cause HIV-protein mediated apoptosis. Following the massive cell death that occurs after activation (in the contraction phase, Figure 2.7) is the resulting loss of antigen specific CD4+ T cells. At this point cytokines such as IL-7 and IL-15 may rescue T cells from death, allowing for memory T cell generation. A fraction of the cells at this point still contain a reservoir of proviral DNA that is hidden from the immune system.

Given the central role played by the T helper cells on both the humoral and cellular arms of the immune response, it is easy to envision how their depletion as a result of the direct and indirect cytopathic effects and the CTL response can eventually lead to immune failure, opportunistic infections and death. It is therefore not surprising that targeting CD4+ T cell activation is now being recommended for novel HIV therapies (Forsman and Weiss, 2008) and has been shown to be effective in several clinical trials and studies (Lori *et al*., 2005, Lori *et al*., 1997, Frank, 1999, Rustchman *et al*., 1998). In addition, the role played by cytokines such as IL-7 in this sequence of events in rescuing T cells further elaborates its significance in boosting immune parameters as seen in the mice cleared of an HIV-like virus (Pellegrini *et al*., 2011).

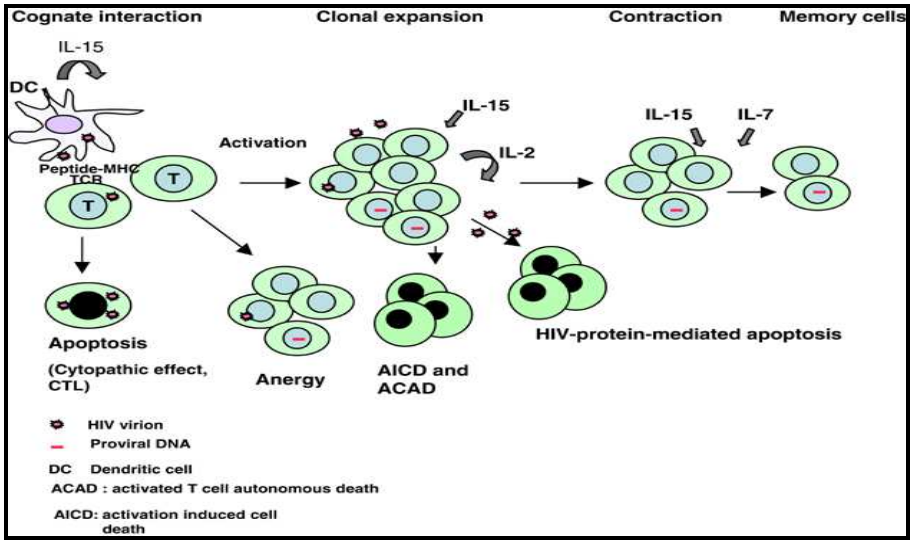

Figure 2.7: An illustration of the mechanisms of depletion of HIV specific CD4+ T cells during infection. Upon recognition of antigen, T cells are either killed by direct viral cytopathic effect or CTL response. Some cells undergo a state of anergy (no response) while others become activated and are destroyed through AICD and ACAD or by HIV-protein mediated apoptosis. Cytokines such as IL7 and IL-15 secreted in the course of the activation may rescue the cells allowing for memory T cell generation. This figure was taken from Gougeon (2005).

2.1.6 Vaccine Development

There is no doubt that an effective vaccine remains the most practical way of addressing and preventing new infections from HIV. Traditional vaccine strategies such as those that have been effective for pandemics like smallpox, polio, measles and yellow fever depended on the production of neutralising antibodies (Arrode-Bruses *et al*., 2010). The prevention and control of HIV infection on the other hand strongly depends on the development of high-frequency, broadly targeted, polyfunctional T-cell responses specific to the virus (Johnston and Fauci, 2008, Betts *et al*., 2006). Unfortunately efforts towards such a vaccine have not kept pace with basic scientific research except for some exciting advances that were made in 2009. These include the first partial protection in humans from an HIV vaccine in the RV144 trial in Thailand (Rerks-Ngarm *et al*., 2009). In this trial, four priming injections of a recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]) and two booster injections of a recombinant gp120 subunit vaccine (AIDSVAX B/E) were evaluated in a randomised trial involving 16,402 healthy men and women. Vaccine efficacy of up to 31.2% was observed. Other developments include evidence for significant vaccine induced control of SIV in non-human primates (Hansen *et al*., 2009) with others that involved the use of live-attenuated SIV/HIV (Mansfield *et al*., 2008, Johnson *et al*., 1997). The risk that pathogenic forms of such vaccines can redevelop makes them ineligible for human use (Arrode-Bruses *et al*., 2010). The identification of a new target for broadly neutralising antibodies on HIV's surface (Walker et *al*., 2009) was also a significant move towards vaccine development. Recent findings on vaccine development reported by BBC News on Health, May 11th 2011 (http://www.bbc.co.uk/news/health-13362927) by US researchers suggests protection of 13 of 24 rhesus macaques monkeys from infection with SIV. This exciting new finding involved the use of a genetically modified form of rhesus cytomegalovirus (CMV) engineered to produce antigens to attack SIV. Again safety concerns are an issue here in terms of translating these findings to humans considering that the CMV virus is disease causing.

While preventative vaccines studies are being pursued, the development of therapeutic vaccines needed to boost the immune system of people already living with the virus is also gaining grounds. Following reports that indicated the induction of protective anti-viral immunity in hu-PBL-SCID (mice model appropriate for HIV research) mice upon the adoptive transfer of autologous dendritic cells loaded *in vitro* with aldrithiol-2 (AT-2)-inactivated HIV-1 (Lapenta *et al.,* 2003, Yoshida *et al*., 2003), *in vivo* toxicity and efficacy studies were performed (Lu *et al*., 2004). This first *in vivo* study on the toxicity and efficacy of an HIV therapeutic vaccine resulted in viral suppression and HIV specific immunity after immunisation by 90% in 8 of 18 subjects, with the only clinical manifestation being the increase in size of peripheral lymph nodes (Lu *et al*., 2004). The efficacy of this vaccine still had to be proven in a randomized trial with appropriate controls (Lu *et al*., 2004). In a recent report by Garcia *et al.*, (2011), using the same AT-2-inactivated HIV-1 vaccine, with the inclusion of a control arm, weak HIV-1 specific

T cell responses were observed unlike the sustained responses observed by Lu *et al*., (2004). The differences in the responses in the two trials was not clear but might be related to the inactivation method of the virus used for the treatments (Garcia *et al*., 2011). Four therapeutic DNA vaccines with promising activity were presented at the XVIII International AIDS Conference in Vienna (Austria, 2010). The identification of a therapeutic vaccine for HIV will be advantageous over current medication because of the associated reduced toxicity (Fiorentini *et al*., 2010).

These recent advances in both preventative and therapeutic vaccines call for more investment in vaccine development. In the case of preventative vaccines, the immediate aim should be to increase the efficacy that has been demonstrated by the live attenuated SIV vaccine in non-human primates while focusing on ways to minimise the development of pathogenic strains; also a main concern in the CMV engineered vaccine. In addition, focusing on the development of vaccines that can illicit neutralising antibodies in the long term (Koff, 2010) is also desired so as to maintain extended protection.

2.1.7 Therapy

The US Food and Drug Administration (FDA) has approved a total of 25 ARV drugs for the treatment of HIV infection (de Béthune, 2010). These available drugs belong to six different classes and include; eight NRTIs, four NNRTIs, ten PR inhibitors and one IN inhibitor which all target viral enzymes, a fusion inhibitor which prevents the fusion of the viral envelope with the host cell membrane and a CCR5 inhibitor which blocks the interaction of the virus with one of its receptors on the host cell (De Clercq, 2009). Until recently, therapy largely involved the virally encoded targets RT, IN, PR, and gp41 (Adamson and Freed, 2010) and has only more lately been expanding to include viral-host protein interactions and cellular targets. The combination of these drugs (mostly RT and PR inhibitors) in what is known as HAART has led to substantial improvement in the clinical management of HIV infection in terms of delaying disease progression, prolonging survival and improving quality of life (Antiretroviral Therapy Cohort Collaboration, 2008). This simultaneous use of multiple drugs is required because of the ease with which HIV can develop drug resistance to any single inhibitor (Simon *et al*., 2006, Temesgen *et al*., 2006). In the following subsections, the various viral targets and structural examples of some of the drugs targeting each will be discussed followed by a brief discussion on novel targets that are being explored as future therapeutic intervention points.

2.1.7.1 HIV reverse transcriptase and inhibitors

The RT enzyme of HIV is a heterodimer consisting of 66- and 51-kDa subunits (Fields, 1996) and is involved in converting viral RNA to cDNA. This multifunctional enzyme is involved in RNA dependent polymerisation, DNA dependent polymerisation, strand displacement synthesis and strand transfer, and degrades the RNA strand in the RNA/DNA hybrid (Schultz

and Champoux, 2008). It performs these functions through its **polymerase function** (for which there are two classes of inhibitors, the NRTI and NNRTIs) and an **RNase H function** that is unique to the C terminus of the p66 subunit (Su *et al.,* 2010). The polymerase domain of the enzyme is found in the N-terminal two-thirds while the RNase H domain is in the C-terminal one-third (Telesnitsky and Goff, 1993) of the p66 subunit. The polymerase function requires either RNA or DNA as the template (Sarafianos *et al*., 2009). In addition, like most DNA polymerases, it needs a primer and makes use of a host transfer RNA (tRNA) as primer ($tRNA_{lys3}$). RNase H activity is required for processing the tRNA primer to begin minus-strand DNA synthesis and degradation of viral RNA during synthesis followed by preparation of the polypurine tract DNA-RNA hybrid which serves as the primer for positive strand DNA synthesis (Fields, 1996, Hansen *et al*. 1988). All these processes (reviewed by Sarafianos *et al*., 2009) result in the copying of a single stranded RNA to a double stranded DNA (Schultz and Champoux, 2008).

The crystal structure of RT in complex with some active site inhibitors are shown in Figure 2.8 while the structures of some **RT inhibitors** in clinical use (NRTIs and NNRTIs) are provided in Figure 2.9. The earliest inhibitor of HIV was the NRTI, azidothymidine (AZT) which initially had potential as an anti-cancer agent (Wlodawer and Vondrasek, 1998). Although RNase H inhibitors have been described, none has yet been approved for clinical use (Sarafianos *et al*., 2009). NRTIs function by terminating the elongation of the growing cDNA strand and thus function like deoxynucleotide triphosphate (dNTPs) or analogues of the natural substrates of DNA synthesis. These inhibitors lack the 3'-OH normally present in the natural substrates and act as chain terminators when incorporated into viral DNA by RT (Sarafianos *et al*., 2009). Examples are zidovudine and didanosine (shown in Figure 2.9). NNRTIs on the other hand are allosteric inhibitors that inhibit the polymerase function by binding to a pocket that is approximately 10 Å close to the NRTI site, (Sarafianos *et al*., 2009, Gotte, 2006). The structures of two NNRTIs (nevirapine and delavirdine) are shown in Figure 2.9.

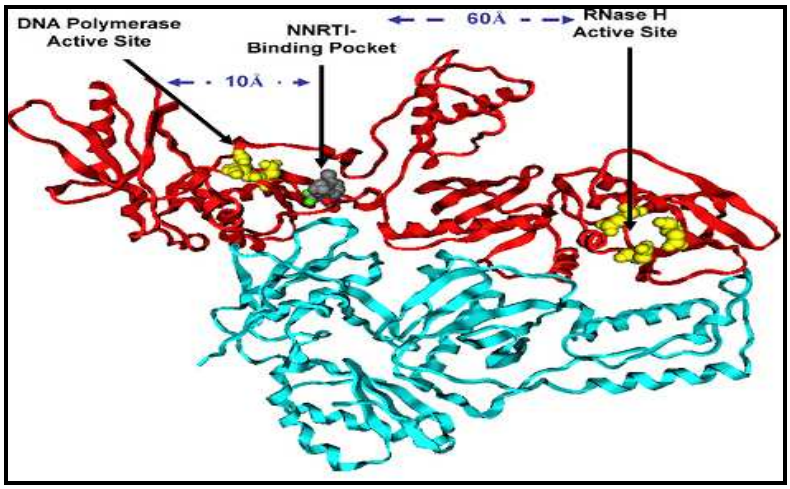

Figure 2.8: Ribbon representation of HIV-1 RT in complex with active site inhibitors. The DNA polymerase, the NNRTI binding pocket and the RNase H active site are shown, all within the p66 domain (red). The p51 subunit is shown in green. This figure was taken from Sluis-Cremer and Tachedjian, (2008).

The importance of these NNRTIs and the NRTIs in HAART is significant. The NRTIs form the basis of HAART with at least two of them usually included in combination with one NNRTI or a PR inhibitor (Pommier *et al*., 2005). Unfortunately, the greatest shortcoming with HAART therapy amongst others is not only the high genetic diversity of HIV within an individual patient resulting from high replication and frequent recombination events. The error prone nature of RT (Simon *et al*., 2006, Svarovskaia *et al*., 2003) also results in the development of viral strains resistant to RT inhibitors and other drug targets. In addition these drugs like most of the other classes of HAART drugs are toxic, with adverse effects ranging from lactic acidosis to hepatotoxicity (Montessori *et al*., 2004). More details on these limitations will be provided in the next section.

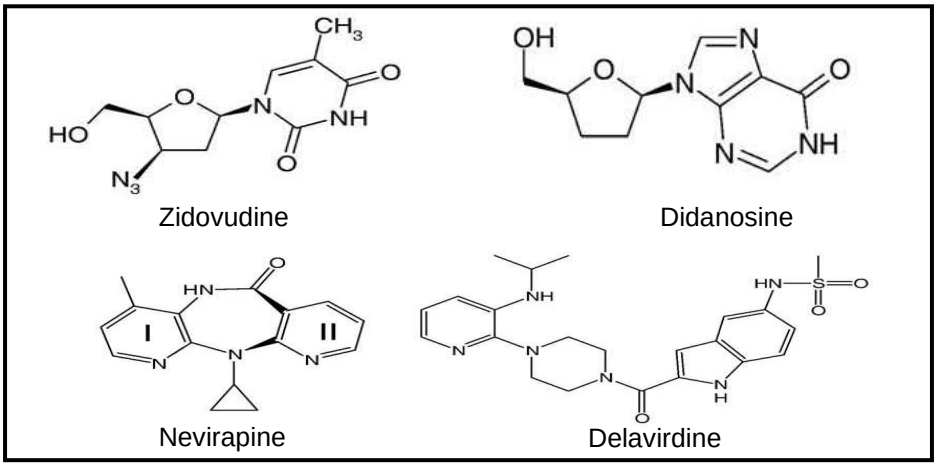

Figure 2.9: Structural representation of some RT inhibitors currently in clinical use. Zidovudine and didanosine are examples of NRTIs while delavirdine and nevirapine are NNRTIs. This figure was adapted from Sarafianos *et al*., (2009).

2.1.7.2 HIV protease and inhibitors

HIV protease is an aspartic PR that is involved in the processing of the viral gag and gag/pol polyproteins (Debouck, 1992), a step that is necessary for the production of infectious virions. It does so by hydrolysing the polyproteins to functional protein products that are necessary for viral assembly and subsequent activity. This maturation process occurs as the virion buds from the cell. A functional HIV PR enzyme exists as a dimer of identical 99 amino acids with a twofold axis of symmetry through the substrate binding site (Purohit *et al*., 2008). This enzyme is very important in HAART therapy since its inhibition prevents the formation of infectious virions (Wlodawer and Vondrasek, 1998). A crystal structure of HIV PR is shown in Figure 2.10 with two catalytic residues (Asp25 from each monomer) shown as ball and stick diagrams. The early knowledge of the crystal structure of HIV PR facilitated structure-based drug design for this target (Wlodawer and Vondrasek, 1998).

The structures of two of the ten clinically approved **PR inhibitors** are shown in Figure 2.11 namely ritonavir and indinavir. Both inhibitors have hydroxyl groups (boxed) that are important in the formation of hydrogen bonds with the active site aspartates (Wlodawer and Erickson, 1993). Resistance to PR inhibitors is also common and develops rapidly because of the site-specific mutations that occur in the enzyme at one or more locations (Rose *et al*.,

1996, Baldwin *et al*., 1995). This is also linked to the high error rate of RT since the nucleotide sequence of PR ends up changing over generations. Toxicity (e.g. hepatotoxicity), is also a common adverse effect associated with the use of PR inhibitors (Montessori *et al*., 2004).

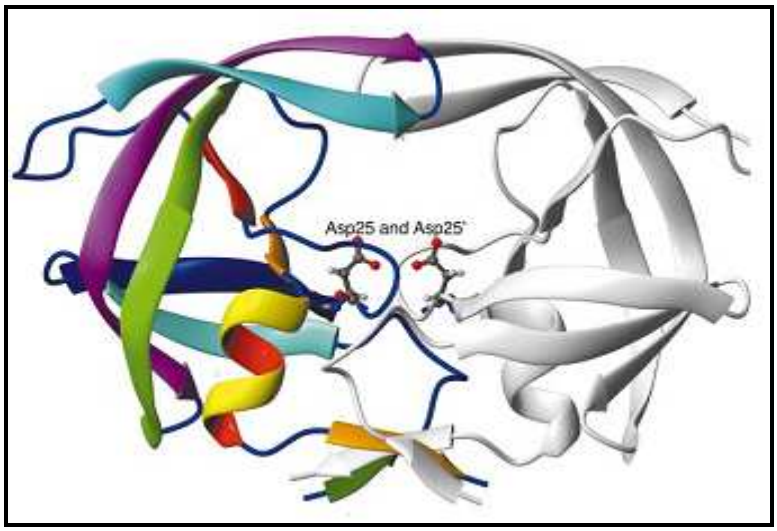

Figure 2.10: Structure of HIV PR. Catalytic residues Asp25 from each monomer are shown in ball and stick notation just below the binding site pocket (Adapted from Zoete *et al*., 2002).

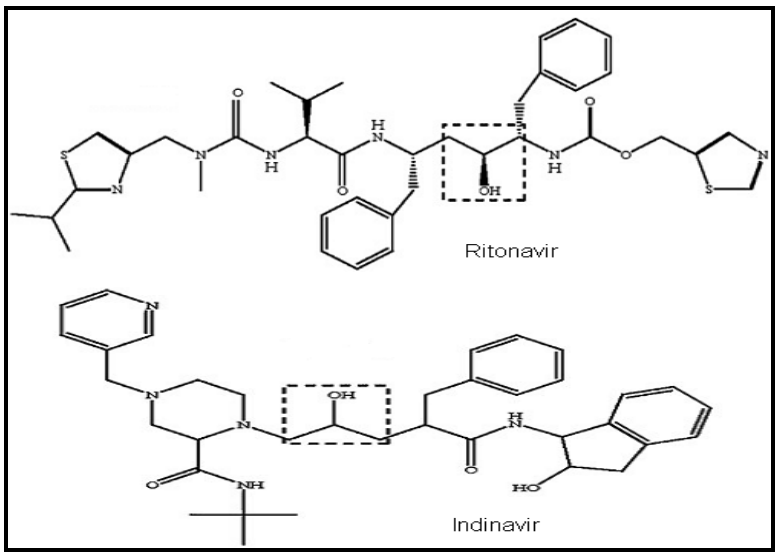

Figure 2.11: Structure of some HIV PR inhibitors in clinical use. The boxed hydroxyl group in both inhibitors is critical in forming hydrogen bonds with the active site aspartates. This figure was adapted from Wensing *et al*., (2010).

2.1.7.3 HIV integrase and inhibitors

HIV IN is an attractive drug target because there are no homologues in eukaryotic systems that could negatively affect host cell viability (Dolan *et al*., 2009). The enzyme is a DNA recombinase that catalyses two endonucleolytic reactions (Michel *et al*., 2009). These are the **3'processing (3'P) reaction** in which IN cleaves a dinucleotide from each of the 3' ends of viral cDNA thereby exposing a 3'-OH group at each end and the st**rand transfer reaction** where the enzyme generates a double-strand break in the host DNA and joins the newly formed ends to the viral 3' ends by transesterification (Engelman *et al*., 1991). Host DNA repair proteins then remove the two nucleotide overhangs and fill in the DNA gaps to complete the integration reaction (Yoder and Bushman, 2000). The enzyme consists of three

structural and functional domains (shown in Figure 2.12). The N-terminal zinc-binding domain (residues 1-49), which is required for 3' P and ST *in vitro*, binds viral DNA sequences and promotes IN multimerisation (Engelman *et al*., 1993), the central catalytic core domain (CCD; residues 50-212) binds specifically to viral DNA and the C-terminal domain (residue 213-288) that interacts with RT (Eijkelenboom *et al*., 1999).

IN inhibitors were only recently approved for anti-viral therapy with one of the greatest limitations having been the fact that the crystal structure of full length IN or in complex with DNA has not yet been resolved (Savarino, 2007). The first drug raltegravir has already been successfully used in the clinic (Chirch *et al*., 2009). Even though the enzyme represents an attractive HIV drug target, resistance (Wielens *et al*., 2010) and cross resistance problems between raltegravir and a second IN inhibitor in clinical trials (elvitegravir) have already been reported (Marinello *et al*., 2008). Figure 2.13 portrays the structures of raltegravir and elvitegravir and two other compounds, 5-CITEP (1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone) and a diketo acid B which have demonstrated *in vitro* inhibition of IN.

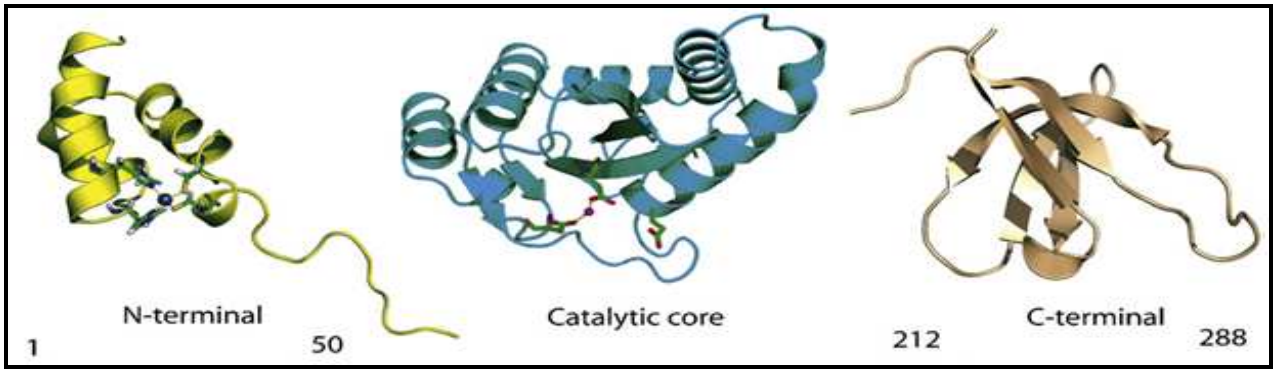

Figure 2.12: Structural and functional domains of IN. The N-terminal domain which is also the zinc binding domain consisting of residues 1-49, the CCD consisting of residues 50-212 which binds specifically to viral DNA and the C-terminal domain which interacts with RT and consist of residues 213-288. Residue numbers for each of the domains are shown (but not for RT and PR above) since the structure of full length IN has not been resolved. The figure was adapted from Mouscadet *et al*., (2010).

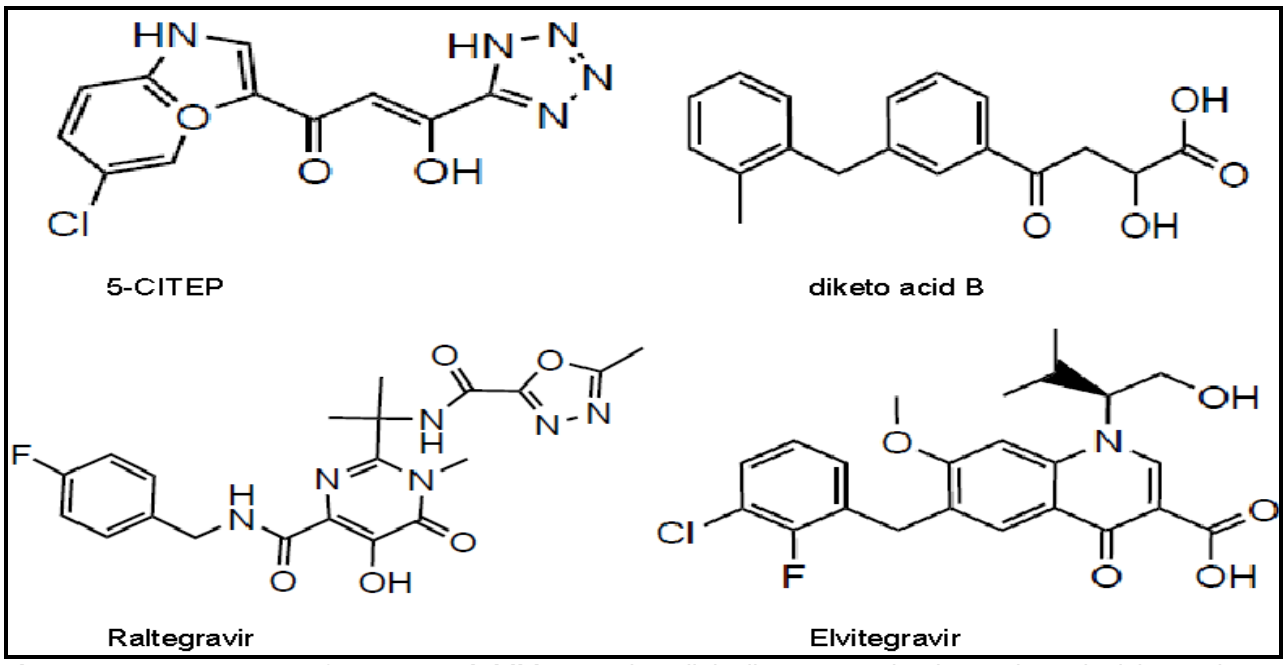

Figure 2.13: Structure of some IN inhibitors. The clinically approved raltegravir and elvitegravir are shown. 5-CITEP and the diketo acid B have inhibited IN *in vitro*. This figure was taken from Savarino, (2007).

2.1.7.4 Viral entry and inhibitors

The viral entry process is a complex multistep event that involves (a) attachment to host cells and CD4 binding, (b) co-receptor binding and finally (c) membrane fusion (see Figure 2.14A, reviewed by Tilton and Doms, 2010). Entry is initiated by the attachment of gp120 found on the viral surface with the CD4 receptor of the host cell. This is followed by conformational changes involving the V3 loop (on gp120) of the virus, allowing for binding with the co-receptor (Huang *et al*., 2005 Trkola *et al*., 1995). The gp41 fusion peptide is then inserted into the host membrane followed by the formation of a six-helix bundle that brings both viral and host membranes together. This leads to the formation of a fusion pore allowing for the entry of HIV capsid into the host cell.

Entry inhibitors consist of compounds that prevent one of the multistep processes involved in entry i.e. attachment and CD4 binding, co-receptor binding and fusion. Two entry inhibitors (maraviroc and enfuvirtide) have been approved for the treatment of HIV infection and a number of new drugs are in development (Tilton and Doms, 2010). The currently approved entry inhibitors block CCR5 binding e.g. maraviroc and fusion e.g. enfuvirtide (structures shown in Figure 2.14B). These drugs are ideal for patients harbouring strains resistant to RT and PR and can therefore serve as salvage therapy. Such drug types are also recommended for use in microbicides (Tilton and Doms, 2010) since they can prevent entry.

Resistance to entry inhibitors is also possible since the viral envelope which is targeted either directly or indirectly has high diversity and can vary between patients (Tilton and Doms, 2010). Mutations indicative of resistance have been seen in patients on enfuvirtide (Xu *et al*., 2005, Poveda *et al*., 2004).

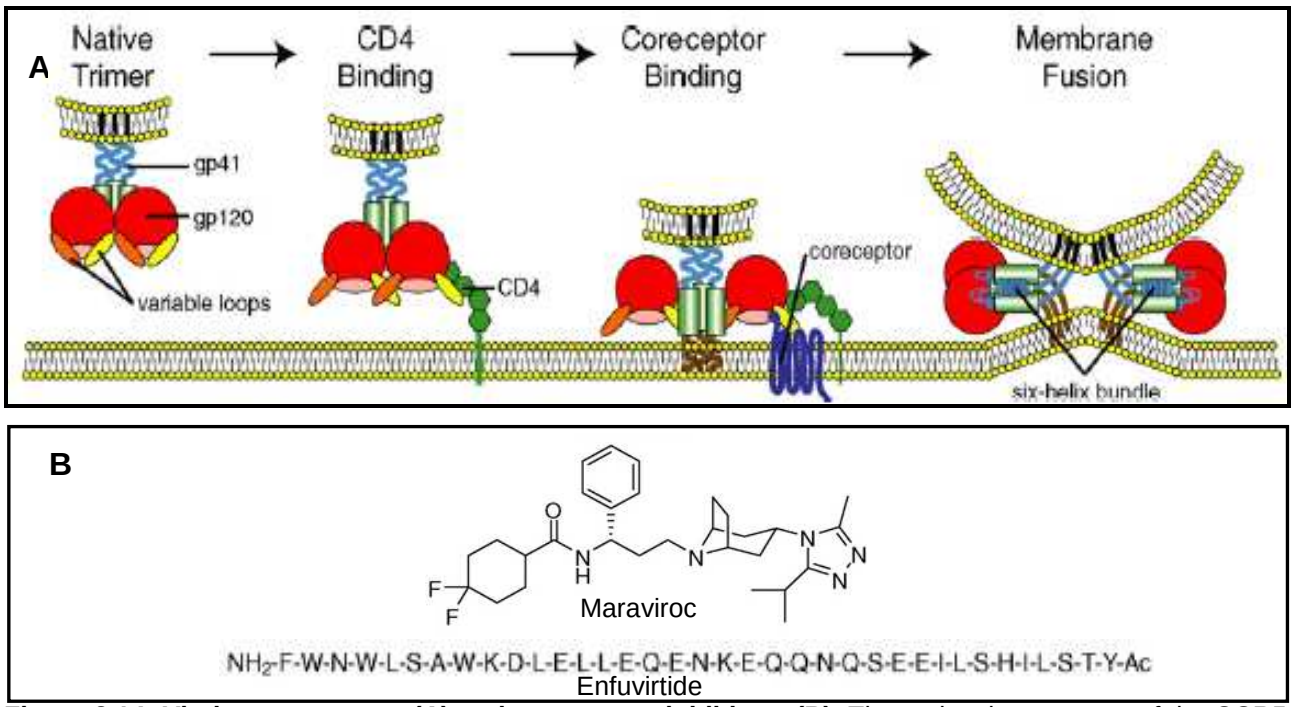

Figure 2.14: Viral entry process (A) and some entry inhibitors (B). The molecular structure of the CCR5 antagonist maraviroc and that of the fusion inhibitor, enfuvirtide are shown in B. This figure was taken from Tilton and Doms, (2010).

2.1.7.5 Cytostatic inhibitors and virostatic combinations

Drugs used in the clinic for HIV treatment have until recently, largely focused on those that target the virus directly. The problem with such medication is the rapid development of drug resistant strains of the virus (Simon *et al*., 2006, Svarovskaia *et al*., 2003, Holmes *et al*., 1992). In order to address drug resistance, recent research efforts have aimed at indirectly inhibiting the virus through cellular targets (Lori *et al*., 2005, Mayhew *et al*., 2005, Lori *et al*., 2007, Lori, 2008). Compounds that cause a cytostatic effect on cells such as hydroxyurea (HU), trimidox and didox have been shown *in vitro* and all the way to clinical trials (Lori, 2008, Mayhew *et al*., 2005, Lori *et al*., 2005, Lori, 1999, Lori *et al*., 1997) to be less prone to resistance when compared to the current HAART combinations. Cytostasis is the ability of an agent to prevent cell growth and multiplication. Hydroxyurea has a history in the hematology field for the treatment of myeloproliferative disorders and cancers (Lori, 1999) because of its cytostatic effects. The cytostatic effect of HU lowers dNTP pools within the cells resulting in a reduction in viral replication since the virus requires host dNTPs for synthesising viral cDNA. HU is known to inhibit RNR which normally converts ribonucleotides to dNTPs (Lori, 1999) and specifically reduces the synthesis of deoxyadenosine triphosphate (dATP, Slabaugh *et al*., 1991, Bianchi *et al*., 1986). For this reason, it is often combined with adenosine dideoxynucleoside analogues e.g. didanosine (ddI). In this combination, HU lowers the concentration of the natural substrate needed for DNA synthesis (i.e. dATP) thereby increasing the concentration of the analogue (ddI) leading to an overall decrease in viral replication. Other ways by which HU inhibits HIV is through its immune modulating effects in which case the compound decreases CD4 T cell numbers, reducing the number of activated cells that are primed for killing by HIV. There are concerns that such agents may be very toxic especially when administered to already immunocompromised individuals (Lori *et al*., 2005). However, even though HU alone may be toxic (to very sick people) compelling evidence now suggests that the combination of HU or HU-like agents with compounds that have a direct anti-viral effect such as ddI, results in the boosting of immune parameters such as CD4+ cell increases and decreases in viral load. This combination forms what is now considered a new and emerging class of anti-HIV agents known as virostatics and defined by Lori *et al*., (2007, 2005) as the combination of a drug directly inhibiting virus e.g. ddI (*viro*) and one indirectly inhibiting virus (*static*) e.g. HU.

The anti-viral and cytostatic mechanism of virostatics is illustrated in Figure 2.15A and B and the structure of HU is provided in Figure 2.16. Didanosine is clinically used as a NRTI and its structure was provided earlier in Figure 2.9. In the anti-viral mechanism (A), in the absence of treatment, more viral particles are produced and upon treatment with HU, there is a decrease in viral particles. In the absence of treatment (cytostatic mechanism, B), proliferation increases and so does viral particles but upon treatment with HU, there is a

decrease in viral particles and an optimal number of CD4+ cells. The combination of the anti-viral and cytostatic effects results in an overall shift to an optimal state.

Virostatic cocktails have shown promising results both *in vitro* and in clinical trials (Clouser *et al*., 2010, Lori *et al*., 2005, Mayhew *et al*., 2005, Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al*., 1998). The outstanding advantage is the observed improved resistance profile, which makes this combination unique over current HAART schedules.

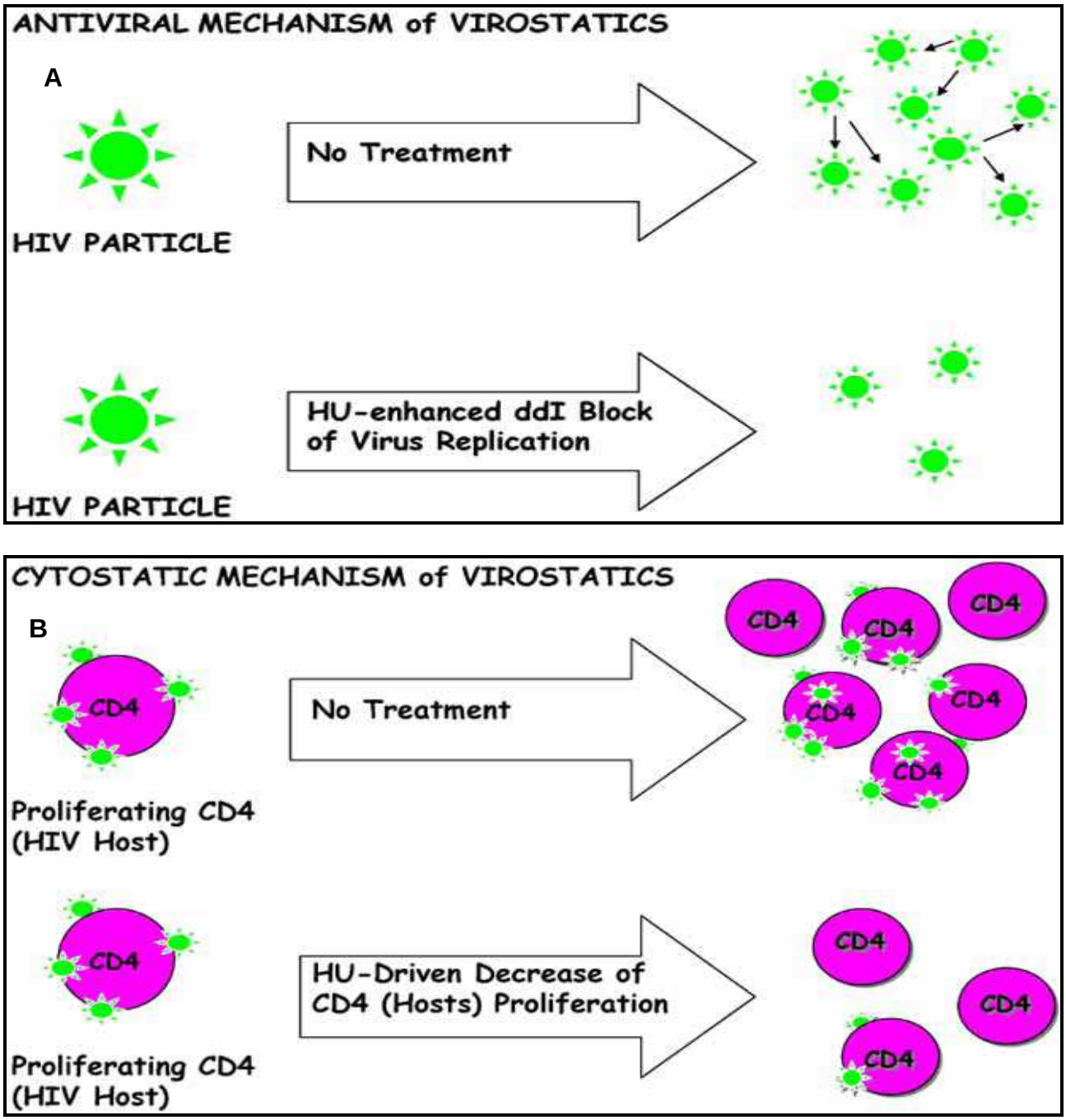

Figure 2.15: The anti-viral and cytostatic mechanism of virostatic agents. In (A), in the absence of treatment, the virus makes more copies of itself and upon treatment with HU-ddI combination, less viral particles are present. In B, in the absence of treatment, the virus divides more as the cells get activated and proliferation increases but upon treatment with HU, the number of viral particles reduces and the CD4 cell number shifts to an optimal but intermediate level. These figures were taken from Lori *et al*., (2007).

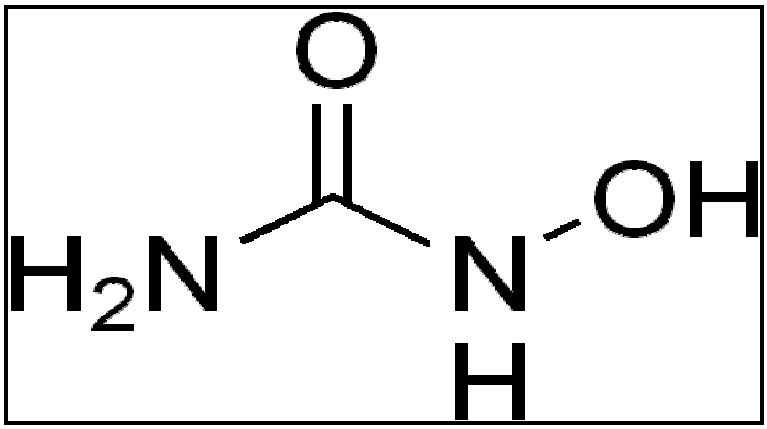

Figure 2.16: Structure of hydroxyurea, an important cytostatic agent. The figure was taken from http://upload.wikimedia.org/wikipedia/commons/c/c9/Hydroxyurea.png (accessed on the 5/05/2011).

2.1.7.6 Novel targets

Until a cure is developed for HIV, sustained continual development of anti-viral therapy is required since it is the only lifeline for infected individuals. The driving force for novel drug development is the resistance problems associated with HAART (Adamson and Freed, 2010). Identifying new therapeutic targets for inhibiting the virus is important and as such new ones are continually being sought. In addition to exploring new viral targets, viral host protein interactions and cellular targets are also being explored (Adamson and Freed, 2010). Novel targets relevant to this study include the RNase H site of RT and the IN cofactor or lens epithelium derived growth factor (LEDGF) or p75 binding site which are both post entry targets (Adamson and Freed, 2010). The RNase H site as mentioned earlier is involved in the reactions that result in the conversion of viral RNA to cDNA during the reverse transcription process. There are currently no known RNase H inhibitors in the clinic but a lot of research into their possible use is ongoing. RNase H is a viable target because point mutations within its domain have shown that its endonuclease activity is required for viral infectivity (Kirschberg *et al*., 2009).

LEDGF on the other hand is a cellular cofactor involved in the integration process by tethering IN to the chromosome of infected cells (Poeschla, 2008, Maertens *et al.,* 2003). Various studies have shown that by inhibiting the LEDGF-IN (protein-protein) interaction, the integration process catalysed by IN can be allosterically blocked (Christ *et al*., 2010). The cofactor interacts with the enzyme's catalytic core domain using its C terminal integrase binding domain (IBD). The structure of the LEDGF-IN complex is shown in Figure 2.17. Only the IBD of LEDGF is shown.

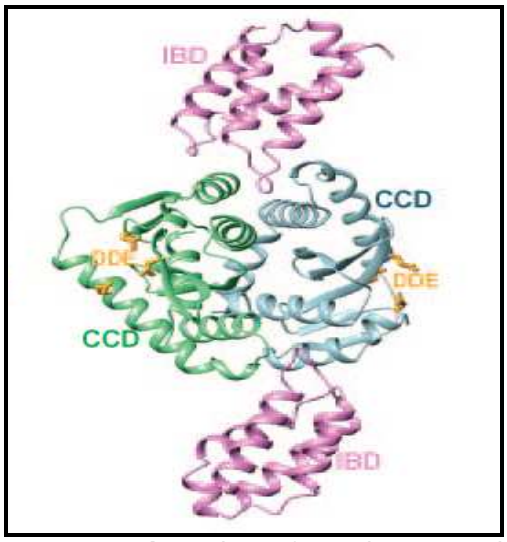

Figure 2.17: Structure of IN-LEDGF complex. The cofactor interacts with the CCD of IN using the IBD. The CCD of IN chains A and B are coloured green and blue. The integrase binding site of LEDGF is coloured purple. Yellow sticks represent the catalytic triad of IN active site. The LEDGF binding site is different from the catalytic site. This figure was taken from Cherepenov *et al.,* (2005).

2.1.8 Therapy Complications and the Need for Novel Drug Development

Treatment with antiretroviral therapy (ART) has greatly improved and prolonged the lives of infected individuals. This development has unfortunately been met with numerous challenges. The major ones which include toxicity to the host and resistance to drugs by the virus will be discussed together with some of the adverse effects that stem from the use of these drugs.

2.1.8.1 Viral resistance to available drugs

Drug resistance has been noted for all the known inhibitors of HIV i.e. those aimed at viral enzymes (RT, PR and IN) as well as those aimed at viral entry. This has been attributed to a number of factors. The extensive genetic variation that the virus has within an individual host particularly in the hypervariable regions of the *env* genes (Holmes *et al*., 1992) means that different variants of the virus easily develop. Furthermore, the error prone nature of RT during the viral genome copying process also facilitates the development of resistance (Simon *et al*., 2006, Svarovskaia *et al*., 2003). The enzyme is known to make ~ 0.2 errors per genome during each replication cycle (Preston *et al.*, 1988). These errors end up causing mutations in the structure of RT, IN, PR as well as the viral envelope over generations of replication with the result being resistance to all the inhibitors of these targets. This is further enhanced by the high replicative ability that the virus has with a viral generation time of ~ 2.5 days, producing ~ 10^{10} -10^{12} new virions everyday (Perelson *et al*., 1996). In addition, recombination and natural selection pressures further propagate evolution and genetic diversity and thus increases resistance (Rambaut *et al*., 2004). The fact that most HAART drugs were developed for subtype B viral strains means that specificity for non-B subtypes is reduced. The result of this is the development of resistant strains by non-B subtypes that are different from those seen in

the subtype B strain, in addition to those normally present in subtype B strains (Kantor and Katzenstein, 2004). Resistance problems are also further complicated by cross resistance to the same class of compounds e.g. NNRTI (Johnson *et al*., 2005) and to the recently approved raltegravir and elvitegravir (a second IN inhibitor in phase III trials, Marinello *et al*., 2008). The identification of drugs that target non viral targets (e.g. those that inhibit ribonucleotide reductase or which lower immune activation by preventing antigen presenting cells from activating T cells) may arguably be the best remedy in curbing the rampant resistance problems associated with all classes of drugs currently used in HAART combinations and in salvage therapy. More specifically the use of virostatic combinations (please see section 2.1.7.5 for details on this) which reduce the development of viral resistance, may be the way forward.

2.1.8.2 Drug toxicity to host

Toxicity to the host is a major limitation of ARV agents and is evident in many ways that result in adverse clinical manifestations. Some toxicity examples include hepatotoxicity from the RT inhibitors (NRTIs and NNRTIs) and PR inhibitors, PR inhibitor-associated retinoid toxicity (reviewed by Montessori *et al*., 2004) and mitochondrial toxicity caused by NRTIs which all lead to a whole host of clinical manifestations that can be deadly (Montaner *et al*., 2003). The fact that therapy is life-long means toxicity problems cannot be ruled out during HAART. Other clinical complications from HAART, provided by Yeni (2006) include complications from NRTIs that lead to subcutaneous lipoatrophy peripheral neuroparthy, lactic acidosis and pancreatitis with the former two being life threatening conditions. Complications from NNRTIs could be skin rashes and toxic hepatitis and these usually occur during the onset of treatment. With regards to PR inhibitors, the major adverse effects are the accumulation of visceral fat and hyperlipidermia. A link between the duration of HAART and the incidence of myocardiac infarction has also been observed.

2.1.8.3 Other limitations

Other limitations of HAART that end up affecting treatment and treatment schedules are: (1) intolerable side effects such as bloating, nausea, diarrhoea (which may be temporary or may be throughout therapy, Carr and Cooper, 2000), fatigue, headaches and nightmares (Montessori *et al*., 2004). These effects usually lead to poor adherence. Poor adherence means suboptimal doses of the drugs are taken such that virus escape mutants result leading to increased drug resistance. (2) The costs involved in acquiring the drugs limits availability in resource restricted settings such as Sub-Saharan Africa where the infection burden is the highest. (3) Unfavourable drug-drug interactions resulting from the combination therapy and (4) the presence of latent forms of the virus in patients on HAART (Finzi *et al*., 1997) prevents

complete eradication. This last complication is the reason why therapy has to be life-long since upon discontinuation, the latent forms emerge and start replicating.

2.1.8.4 Cure limitations

In 2009, the New England Journal of Medicine published a report of a man who was cured of HIV after receiving stem cells transplanted from a donor homozygous for the *CCR5* delta32 gene as treatment for acute myeloid leukaemia (Hutter *et al*., 2009). Infection with HIV requires the presence of the CD4+ receptor and the CCR5 co-receptor. People with a 32 bp deletion in their CCR5 allele are reportedly resistant to HIV infection. Although possible, treating HIV infected people through this means has significant costs implications and finding the right donor could be very challenging as well. An important outcome of the study was the awareness of the significance of the CCR5 co-receptor in HIV infectivity that has encouraged investigations into identifying CCR5 inhibitors. Findings by Pellegrinii *et al.*, (2011) in which the clearing of an HIV-like virus (by the boosting of immune functions) from mice through the suppression of the *Socs3* gene by IL-7 still require significant research to translate to useful clinical application. As mentioned before, the identification of latent forms of the virus during treatment (meaning the virus can not be completely eradicated with HAART, Finzi *et al*., 1997) was one of the earliest shortcomings. Upon termination of treatment, these latent forms emerge and start replicating.

2.1.8.5 Local needs

In the South African context, identifying novel therapy specific to the subtype C strain is very important because this strain (which is prevalent in this part of the world, Nkolola and Essex, 2006, Wouter *et al*., 1997) has not been as widely studied as the subtype B virus found in developed countries. Currently administered medications were synthesised using the subtype B viral strain and even though these drugs are active against non B strains e.g. C, effectiveness is less with a resultant increase in the incidence of mutations (Kantor and Katzenstein, 2004). In addition, the cost of current medication cannot be met by the poor (Ford *et al*., 2007) making the identification of local, more effective and potentially cheaper therapies a necessary endeavour. This was one of the reasons that led to the creation of the Project AuTEK Biomed Consortium which is affiliated with two mining companies in South Africa (Mintek and Harmony Gold) and South African universities. The idea here was that the natural availability of pure gold deposits in South Africa could be exploited for possible health benefits by using gold in synthesising potential drugs.

Taken together, all the shortcomings in managing HIV and AIDS, coupled with the fact that no available vaccine or cure has been discovered necessitates the continuous search and identification of novel treatment options that can be used to supplement or replace currently available drugs. In the next section, an introduction to the drug development process will be

provided followed by a discussion on the use of metals in medicine with specific emphasis on gold-based compounds.

2.2 DRUG DEVELOPMENT

Drug development and discovery can be a very tough and long process both scientifically and financially for the pharmaceutical industry and it can typically take up to a decade for a drug to go through the different phases of drug discovery (Fishman and Porter, 2005), which are shown in Figure 2.18. These phases include the lead or target discovery phase during which important molecular targets are identified. This phase can typically take a year to several years (Fishman and Porter 2005). This is followed by the preclinical phase where toxicity, efficacy and dose response is determined using both *in silico* and *in vitro* techniques and involves technologies that range from traditional high throughput screening (HTS) to affinity selection of large libraries, fragment-based techniques and computer-aided design (Keseru and Makara, 2006). In phase I/II, biomarkers and response to treatment are monitored together with adverse responses and efficacy in humans. Successful candidates which go through these preliminary phases are finally entered into phase III/IV, a phase which involves the prediction of adverse responses and efficacy monitoring at a larger scale and finally approval and clinical application of the successful drug candidate.

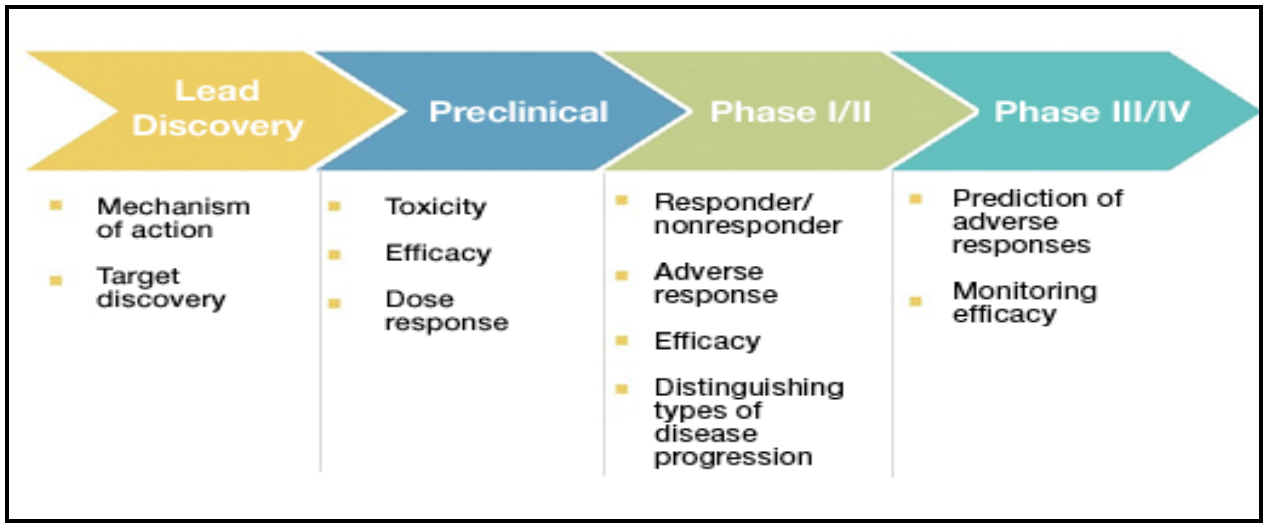

Figure 2.18: Drug discovery phases: a typical drug discovery phase diagram. This figure was taken from www3.bio-rad.com (accessed 26/01/2011)

In the course of the discovery process, drug-like properties which include; absorption, distribution, metabolism, excretion and toxicity (ADMET) are monitored. Compounds that are drug-like are defined as those compounds that have sufficiently acceptable ADMET properties to survive through the completion of human Phase I clinical trials (Lipinski, 2000). Identifying drug-like compounds has become increasingly important after it was observed in the late 1990s that the main causes of late-stage failures in drug development were as a result of poor pharmacokinetics and drug toxicity (Lombardo *et al*., 2003, van de Waterbeemd and Gifford,

2003). The introduction of ADMET screens in the early phases of drug discovery avoids loss in expenditure by pharmaceutical companies downstream the discovery process when it becomes apparent that the compounds are not drug-like.

In the past, focus on determining binding to the active site was a strong priority in discovery for medicinal chemists where HTS and traditional medicinal chemistry techniques were employed (Kerns and Di, 2008). The focus in modern day drug discovery is on structure activity relationships (SAR, Di and Kerns 2003). The latter has been enhanced by the development of virtual (*in silico*) screening techniques, which have been emerging in the past decade and are now perceived as complementary approaches to experimental HTS (Desai *et al*., 2006). Coupling experimental HTS and virtual screening with structural biology, promises to enhance the probability of success in the lead identification stage of drug discovery. The combinations of these techniques have not only led to increased output but through SAR or rational drug design studies, medicinal chemists can easily correlate pharmacological and biological properties (Kerns and Di, 2008). The earliest impact of this was the decrease in late failures from 39% in 1998 to 10% in 2000 (Kola and Landis, 2004).

While it is important that drugs should go through the various phases of drug design to ensure safety and efficacy, identifying a perfect drug has never been achieved in the pharmaceutical industry (Joshi, 2007). As such, finding drugs that are tolerable such that management and patients could eventually benefit has been the trend. In this regard, physician intervention at the point of administration is an important point to consider during therapy.

2.3 METALLODRUGS

2.3.1 Brief Background

With close to three decades that have passed since the discovery of HIV as the causative agent of AIDS, many investigators have dedicated enormous efforts to finding promising drug leads, both synthetic and natural (De Clercq, 1995) to supplement existing treatments. Although many potential medicinal products (crude extracts and single molecules) have shown efficacy against HIV *in vitro* and *in vivo* (Gambari and Lampronti, 2006), mostly organic synthetic agents have been clinically approved. Advances in inorganic chemistry suggest a significant role of metals especially those of the transition metal series as being important in synthetic medicinal chemistry (Rafique *et al*., 2010). These advances are facilitated by the inorganic chemists' knowledge on coordination chemistry and redox properties of metal ions (Kostova, 2006). In addition, the wide scope that metals have in interactions with biological systems means that they could easily be accommodated in drugs. This ease of interactions results from the fact that metals easily lose electrons and get converted to an ionic state, which is soluble and electron deficient (Orvig and Abrams, 1999). In this state, metals tend to interact with proteins and DNA which are electron rich (Orvig and

Abrams, 1999). An example is iron, contained in the protein haemoglobin which binds to oxygen. Others are manganese, copper, zinc and iron that are incorporated into enzyme structures producing metalloenzymes which facilitate important chemical reactions in the body (Orvig and Abrams, 1999).

Metal-based drugs or metallodrugs have a history dating back to the earliest times (Higby, 1982) have advantages over traditional organic medicine. The drugs make use of metal-drug synergism where there is enhancement of the activity of the parental drug after complexation which is chemical reaction involving a metal and an organic moiety (ligand) with the metal (Navarro, 2009, Beraldo and Gambino, 2004). This activity enhancement is thought to be as a result of structural stabilisation from the coordination/complexation of the metal to the organic moiety (Navarro, 2009) or the ligand to form what is known as a complex. The metal complex or coordination complex as defined by Rafique *et al*., (2010) is a structure consisting of a central atom, bonded to a surrounding array of molecules or anions.

In some cases, complexation with metals has been reported to lead to decreases in toxicity of the metal ions since the organic portion of the drug makes it less available for unwanted interactions that could lead to toxicity (Sánchez-Delgado and Anzellotti, 2004). Coordination may also lead to significant reduction in drug resistance because of improved specificity (West *et al*., 1991, Kostova, 2006). The metals in metallodrugs form covalent bonds and ionic forces, unlike organic molecules, which form van der Waal forces and hydrogen bonding. Since these covalent and ionic forces are stronger, the drugs tend to stay at the active site longer thereby increasing efficacy and resulting in a synergistic effect from the organic and metal moieties (Navarro, 2009).

Some metals with medicinal properties are iron, ruthenium and silver, among others (reviewed by Rafique *et al*., 2010). A typical example of a medicinally significant metal-based compound is cisplatin (a platinum-based drug), an anti-cancer agent. The discovery of this compound renewed interest in medicinal inorganic chemistry (Fricker, 2007, Zhang and Lippard, 2003). Gold-based metallodrugs also exist and are the focus of this study.

2.3.2 Gold Compounds as Metallodrugs

The use of gold in medicine (known as chrysotherapy) dates back to 2500 BC in ancient China (Fricker, 2007) probably mostly from anecdotal evidence. Its modern day application goes as far back as 1890 when Robert Koch discovered [$KAu(CN)_2$] as a bacteriostatic agent effective against the tubercle bacillus (Navarro, 2009). This led to the subsequent use of gold compounds for the treatment of tuberculosis (Berners-Price and Sadler, 1996) without success and later for the treatment of rheumatoid arthritis (RA) for which remission has been largely successful. In the following subsections, the activity of gold compounds both *in vitro* and *in vivo* will be discussed with emphasis on the anti-rheumatoid arthritic, anti-cancer, anti-malarial and anti-HIV effects.

2.3.2.1 Gold compounds as anti-rheumatoid arthritic agents

Rheumatoid arthritis is an inflammatory disease characterized by progressive erosion of the joint resulting in deformities, immobility and a great deal of pain (Fricker, 1996). It is an autoimmune disease that causes progressive destruction of the connective tissue in joints (Sutton, 1986). As early as 1935, Jacques Forrestier reported on the beneficial effects that gold salts had in slowing down RA (Sutton, 1986). Some gold compounds that have been used for the treatment of RA are the thiolate compounds in which the gold is coordinated to sulphur-containing ligands. The earliest gold compounds used for the treatment of RA were the injectable thiolates; aurothioglucose (also called solganol) and aurothiomalate (also called myochrisin). Auranofin (also called radiura) is orally administered and was identified as a potential anti-rheumatoid arthritic agent in 1972 (Sutton *et al*., 1972) and was latter approved for clinical use. This compound has better pharmacokinetic properties and reduced toxicity than the injectable drugs. The structures of the thiolate compounds (aurothioglucose, aurothiomalate and auranofin) are shown in Figure 2.19. A fourth compound which is of medical importance (also represented in Figure 2.19) is the bis(diphos)gold(I) chloride compound which demonstrated promising anti-cancer activity and will be discussed in the next subsection.

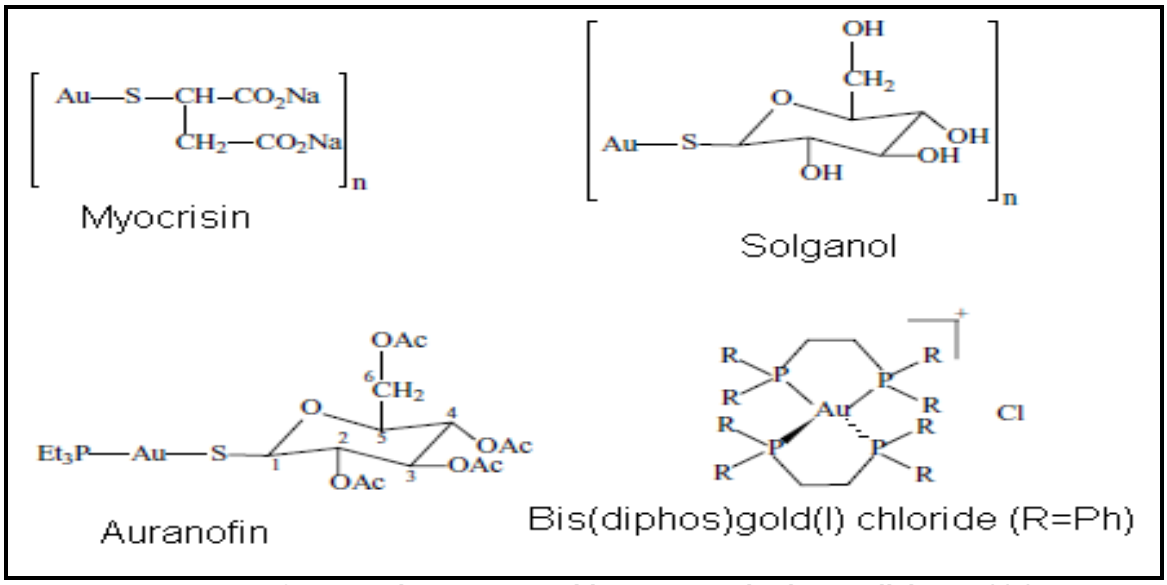

Figure 2.19: Structure of some important gold compounds in medicine. Thiolate compounds coordinated to Au through a S atom include myocrisin, solganol and auranofin. Myocrisin and solganol are injectable drugs while auranofin is orally available. The bis(diphos)gold(I) chloride is also represented as Au(DPPE) chloride (Adapted from Ahmad, 2004).

2.3.2.2 Gold compounds as anti-cancer agents

The discovery of cisplatin and its derivatives as anti-cancer drugs prompted the search for other metal containing anti-cancer agents (Arnesano and Natile, 2009). Early studies showed that the anti-rheumatoid arthritic agent, auranofin was toxic to some tumour cells in culture and *in vivo* against P388 leukaemia (Lorber *et al*., 1979) but because of inactivity *in vivo* on most cancer cells, further testing was not pursued. A bis(diphos) gold(I) chloride

containing compound, $Au(DPPE)_2Cl$ (Figure 2.19) known in full as [1,2-bis(diphenylphosphino)ethane]gold(I) chloride was reported in the 80s as having promising anti-tumour activity (Fricker, 1996, Berners-Price *et al*., 1986, Mirabelli *et al*., 1986) but latter dropped because of pre-clinical toxicity (Hoke *et al*., 1989). This compound and auranofin are both gold(I) complexes.

Since gold(III) complexes are similar to cisplatin isostructurally and isoelectrically (Bruni *et al*., 1999), these complexes were favoured for anti-cancer testing. Despite the similarity, little literature information existed on the use of gold(III) complexes as anti-cancer agents (Tiekink, 2002) up to the late 1990s. The reason for this is because of the high redox potential and poor stability that these compounds have in the biological milieu (Fricker, 1996). It has only been in the last decade that gold(III) complexes with promising anti-cancer activity *in vitro* have been identified leading to a rekindled interest. This is attributed to the identification and coordination to more stable ligands that are not readily reduced. Ligands such as polyamines, terpyridines, and phenathrolines are favoured (Milacic and Dou 2009). Some examples of stable gold(III) complexes that employed stabilising ligands are $[Au(cyclam)](ClO_4)Cl_2$, $[Au(terpy)Cl]Cl_2$ and $[Au(phen)Cl_2]Cl$ (where terpy = terpyridine and phen = phenathroline) which were active against the A2780 ovarian cancer cell line and on a cisplatin resistant variant (Marcon *et al*., 2002, Messori *et al*, 2000). The gold(III) porphyrins are other examples which are stable in the presence of glutathione and exerted higher potency than cisplatin to human cervix epitheloid cancer cells (Che *et al*., 2003). These new gold(III) compounds which have demonstrated greater efficacy than cisplatin require further pharmacological testing (Nobili *et al*, 2010) to establish their possible role in anti-cancer therapy.

2.3.2.3 Gold compounds as anti-malarial agents

Since the landmark report by Navarro *et al.,* (1997) on the anti-malarial activity of a Au-CQ complex (CQ=chloroquine) and their 2004 report (Navarro et al., 2004) further supporting these findings, other authors (Khanye *et al*., 2010, Gabbiani *et al*., 2009, Sannella *et al*., 2008) have also shown that gold-based compounds demonstrate such activity. Khanye *et al*., (2010) investigated the anti-malarial activity of gold(I) thiosemicarbazone-based complexes against the malarial cysteine protease, falcipain 2. The authors showed that there was an enhanced efficacy of the gold(I) thiosemicarbazone-based complexes against CQ sensitive (D10) and CQ- resistant (W2) strains compared to the parent ligand through the inhibition of falcipain 2. Gabbiani *et al*., (2009) also reported on the anti-plasmodial activity of a panel of metal complexes consisting of one mononuclear gold(III) complex (Aubipy where bipy represents bipyridenes) and three dinuclear gold(III) complexes. In another report, Sanella *et al*., (2008) showed that auranofin which is a potent inhibitor of mammalian thioredoxin reductases (which causes severe oxidative stress) was capable of inhibiting the growth of the malaria parasite which is known to be sensitive to oxidative stress. This interesting revelation which displays

the potent antiplasmodial effect of auranofin (a drug already in clinical use for the treatment of RA) has the advantage of lowering costs in drug discovery in the emerging field known as drug repositioning.

2.3.2.4 Gold compounds as anti-HIV agents

Various reports on the *in vitro* activity of gold compounds as anti-HIV agents have been recounted by various authors (Sun *et al*., 2004, Traber *et al*., 1999, Tepperman *et al*., 1994, Okada *et al*., 1993, Blough *et al*., 1989). *In vivo* activity has also been reported (Lewis *et al.,* 2011, Yamaguchi *et al*., 2001, Shapiro and Masci, 1996). Shapiro and Masci noted an increase in the CD4+ count of an HIV positive patient who was being treated for psoriatic arthritis with auranofin. Since the natural progression of HIV is characterised by a decrease in CD4+ count and considering the patient was not on anti-HIV medication, the assumption was that auranofin must have caused the improvement in the patient's status. The anti-HIV activity of gold-based compounds was reviewed by Fonteh *et al*., (2010) as part of this PhD project and the full article is provided at the end of this thesis. The activity of these compounds is linked to their inhibition of HIV RT (Blough *et al*., 1989, Okada *et al*., 1993, Sun *et al*., 2004), immunomodulatory effects (Yamaguchi *et al*., 2001, Traber *et al*., 1999) and also to infectivity inhibition (Okada *et al*., 1993). In their 1996 report, Shapiro and Masci postulated that the remission that was observed for the HIV patient might have been as a result of inhibition of RT by auranofin or to the fact that proliferating cells were able to escape viral cytopathic effects. More recently, Lewis *et al*., (2011) demonstrated remission of a primate lentiviral infection through the restriction of viral reservoirs in a monkey AIDS model when auranofin was administered.

2.3.3 Some Anti-HIV Mechanisms of Gold Compounds

Gold-based metallodrugs have been used for the treatment of RA and have shown activity against cancers and a wide range of microorganisms including HIV as discussed above. This implies that these compounds have various mechanisms by which they function. In the next subsections, the mechanism of action of gold-based compounds will be discussed with particular focus on anti-HIV modes of action.

2.3.3.1 Ligand exchange reactions

Ligand exchange reaction is one of the mechanisms by which gold-based compounds interact with biological materials for example in the interaction with the sulfhydryl group of cysteine residues in the active site of proteins (Shaw III, 1999, Sadler and Guo, 1998). This is because gold readily binds to atoms of relatively low electronegativity such as sulphur, phosphorus or carbon (Parish and Cottrill, 1987). Another notable observation that suggested that gold in gold complexes undergoes ligand exchange reactions was the identification of

$Au(CN)_2$ (a metabolite of gold compounds) in the urine of most patients after the administration of gold drugs and very minute amounts of the administered complex (such as auranofin and solganol, Elder *et al*., 1993). This observation meant that the drugs are prodrugs and that the active form was not the one administered but was produced as a result of the original compound being converted to the active form through ligand exchange reactions (Shaw III, 1999).

Ligand exchange reactions were implicated in the inhibition of HIV infectivity by aurothioglucose (AuTG) and aurothiomalate which did so through the formation of the reactive species bis(thiolato) gold(I) with acidic thiol groups exposed on the surface proteins of the virus (Okada *et al*., 1993). The bis(thiogluocse)gold(I) - bisAuTG reactive intermediate is formed upon the addition of AuTG to thiol ligands (such as thioglucose) that are capable of interacting with thiol groups of cysteinyl residues on the surface of proteins (Shaw III, 1999). In their work, Okada *et al.,* (1993) demonstrated that the bisAuTG intermediate could undergo ligand exchange with thiol groups exposed on the surface of viral proteins. BisAuTG, was able to protect MT-4 cells from infection and lysis by HIV-1_{NL4-3} (Okada *et al*., 1993). Inhibition of viral entry or infectivity was reportedly through its reaction with Cys^{532} on gp160, a viral coat protein. BisAuTG was much more active than AuTG but unfortunately, lacked activity against more virulent strains of HIV.

The inhibition of RT as seen for $Au(CN)_2$ (Tepperman *et al*., 1994) was also attributed to ligand exchange reactions where gold binds to sulfhydryl groups in the active site of RT (Allaudeen *et al*., 1985).

2.3.3.2 Stripping of peptides from class II MHC

De Wall *et al*., (2006) suggested that metal-based compounds such as gold compounds prevent the progress of autoimmune diseases like RA by stripping peptides from class II MHC proteins. Class II MHC proteins are essential for normal immune system function but also drive many autoimmune responses. This is done through the binding of peptide antigens in endosomes and presenting them on the cell surface for recognition by CD4+ T cells (Watts, 1997). The findings by De Wall *et al*., (2006) that metals can strip peptides from class II MHC supports the hypothesis of Best and Sadler (1996) that gold has the ability to alter MHC class II peptides. Ultimately, a small molecule inhibitor such as a gold-based compound could therefore potentially block an autoimmune response by disrupting MHC-peptide interactions. De Wall and colleagues (2006) proposed this mechanism based on the identification of noble metal complexes as allosteric inhibitors of class II MHC proteins. The authors also showed that the noble metal inhibitors were able to block the ability of antigen presenting cells from activating T cells. This proposed mechanism might also be related to how gold compounds inhibit HIV. Considering that immune activation results in increased viral replication and decrease in CD4+ count (Forsman and Weiss, 2008), compounds that block

this activation might reduce viral replication and hence slow disease progression. The metal ions shown to possess this property shared similar characteristics such as being able to form square planar, four coordinate complexes which are isoelectric (i.e. having d^8 electronic configuration). A typical example of a metal complex with these characteristics is the platinum-based complex, cisplatin. Gold(III) compounds form similar complexes to cisplatin and are favoured over gold(I) compexes for such a mechanism.

2.3.3.3 Modulation of cytokine production

Chrysotherapy has been shown to reduce the production of IL-6 and 1L-8 in serum (Madhok *et al*., 1993) and cells such as monocytes (Crilly *et al*., 1994), macrophages (Yanni *et al*., 1994) and synovial cells (Loetscher *et al*., 1994). IL-6 and 1L-8 are all cytokines under nuclear factor kappa beta (NF-κB) regulation. This nuclear factor is also known to be a potent activator of HIV gene expression through the triggering of the transcription of viral genes. Gold compounds possibly act by down regulating NF-κB leading to a reduction in the production of these cytokines. The result is prevention of activation of the transactivator *Tat* gene which in turn prevents explosive increase in HIV replication (Traber *et al*., 1999).

In another report, weekly treatment of LP-BM5 murine leukemia virus-infected mice with aurothiomalate resulted in prolonged survival (Yamaguchi *et al*., 2001). LP-BM5 murine leukemia virus causes a disease in mice that presents as immunosuppression and lymphoproliferation with features similar to AIDS. The mice had less cervical lymph node swelling and generally had fewer abnormalities in the expression of cell surface markers such as CD4.

2.3.4 Side Effects of Gold-Based Therapy

Like many medications, clinically available gold compounds demonstrate toxicity and various side effects. The side effects are strongly linked to the ligand used in synthesising the particular gold complex (Ott, 2009). Systemic toxicity e.g. nephrotoxicity is one of the noted toxicological effects of gold compounds (Nobili *et al*., 2010). Side effects noted in the course of gold therapy develop after the drugs have accumulated in the body and these effects affect the skin, blood, and kidney and occasionally cause liver toxicity (Parish and Cottrill, 1987). Side effects on the skin include rashes, dermatitis and stomatis (Ott, 2009). Major side effects such as proteinurie and thrombocytopenia have been reported (Taukumova *et al*., 1999, von dem Borne *et al*., 1986, Tosi *et al*., 1985). The majority of the noted side effects have been linked to the polymeric (injectable) gold compounds. The reason for this is because these compounds take up to two months to reach a steady state in blood and generally have a very long half life (Parish and Cottrill., 1987). Only 70% of gold drugs are excreted after 10 days of administration (Jones and Brooks, 1996). Gold is rapidly cleared from the blood and distributed to various tissues like the kidneys where it causes the already mentioned

nephrotoxicity. The orally available monomeric auranofin is much more tolerable but then has lower efficacy than the injectable drugs (Jones and Brooks, 1996) while $Au(CN)_2$ is known to accumulate in cells with relatively low cytotoxicity (Zhang *et al*., 1995).

The ability of gold-based compounds to alter MHC II peptide complex is thought to result in both the advantageous and disadvantageous qualities these drugs have (Best and Sadler, 1996). Depending on the type of interaction (i.e. binding to MHC protein directly or to the peptide directly) that the gold drugs make with the MHC II peptide complexes, therapeutic (inhibition of specific T cells) or side effects (stimulation of new set of T cells) could result respectively. Gold-induced dermatitis is known to result from significant lymphocyte proliferation in response to gold therapy (Verwilghen *et al*., 1992). Gold-specific T cell clones that proliferate when exposed to either gold(I) or gold(III) *in vitro* have been isolated from patients who developed hypersensitive reactions to gold therapy (Romagnoli *et al*., 1992).

While these limitations are a concern, it should be noted that some patients tolerate the drugs more than others and because of the popularity that these drugs have in providing long lasting remission from rheumatoid arthritis (De Wall *et al.,* 2006, Merchant, 1998) their therapeutic effect cannot be ruled out.

2.4 HYPOTHESIS AND MAIN RESEARCH QUESTIONS

The important role that gold plays in medicinal inorganic chemistry (section 2.3) coupled with the need for identifying novel compounds that could serve as anti-HIV agents prompted research into identifying gold-based anti-HIV agent(s). The research **hypothesis** was that: gold-containing compounds can inhibit HIV replication directly through action on viral enzymes and indirectly through action on host cells (e.g. immune modulation) and can serve as drug leads for further analysis and development. To investigate this hypothesis, the following main research questions were asked.

2.4.1 Were the Gold Compounds Drug-Like?

The mentioned side effects of gold-based drugs (section 2.3.4) suggest that for consideration as treatment, there was a need to determine how drug-like the compounds in the current study were. This need was further supported by the fact that one of the major reasons for late failures in drug development stems from the lack of drug-like or ADMET properties (Lombardo *et al*., 2003, van de Waterbeemd and Gifford, 2003). A very important drug-like property that was also investigated was the stability of the compounds in the dimethylsulfoxide (DMSO) solvent used for dissolution over time.

2.4.2 What were the Effects of the Gold Compounds on Host Cells and Whole Virus?

There is no point in developing a drug for human use if the source material is toxic to human cells. Primary cells and continuous cell lines represent an easy way of determining

drug interactions with host cells in an inexpensive manner prior to animal studies which are more costly and involve complex ethical issues (Allen *et al*., 2005). As noted earlier, gold-based compounds such as AuTG and the reactive intermediate bisAuTG were capable of preventing viral infectivity of host cells (Okada *et al*., 1993). Aurothiomalate was shown to enhance CD4+ cell frequency in a mouse AIDS model and prolonged the life of the mice after weekly treatments (Yamaguchi *et al*., 2001). Based on this background, the effects of the compounds on immune system cells and on cell lines susceptible to HIV infection were also investigated. The cell-based analysis included; determining cytotoxicity (i.e. if the compounds had adverse effects that could lead to interference with structures and processes essential for cell survival) and monitoring of compound effect on cell proliferation (increasing cell number) patterns. Additionally the effect of the compounds on viral infectivity and immune system cells (frequency of CD4+ and CD8+ cells from both HIV positive and negative donors and the effect on inflammation by assessing IFN-γ and TNF-α levels within the CD4+ and the CD8+ cells) was also investigated. The effect of the compounds on T cell frequency and on the inflammation caused by HIV will be referred to as immunomodulatory effects which are defined as immunological changes in which one or more immune system molecules (such as IFN-γ and TNF-α) are altered through suppression or stimulation.

2.4.3 Could the Gold Compounds Inhibit Viral Enzymes, and How?

Current anti-HIV medications inhibit three important viral enzymes i.e. HIV RT, PR and IN. Viral resistance has become a major problem for compounds that target these enzymes. The identification of new inhibitors for existing drug resistant viruses or inhibitors that can inhibit important viral functions catalysed by these enzymes that are not blocked by existing drugs (Himmel *et al*., 2009) is important. In this project, tests to identify inhibitors of these enzymes were performed in direct enzyme assays (where compound effect on purified enzyme was studied in the presence of substrate). These direct enzyme assays provide information on whether a compound inhibits a viral enzyme or not, but does not provide information on the type of binding site interactions that occur or information on whether the compound is an active site or allosteric inhibitor. In order to probe the binding interactions of the compounds with viral enzymes, *in silico* computer aided screening also known as docking was performed. Docking refers to procedures aimed at identifying orientations of small molecules called ligands in the binding pocket of a protein or a receptor and to predict the binding affinity between the two (Krovat *et al.*, 2005). Through this method, compounds with the potential to inhibit specific enzyme functions such as the polymerase or RNase H function of RT could be identified. In addition, medicinal chemistry information can be obtained that should aid in rational drug design such as information on functional group preference for enzyme active sites.

2.5 SCREENING STRATEGY AND METHODOLOGY

In a proof of concept study, sixteen compounds were tested for toxicity to human cells, cellular uptake, HIV RT and PR inhibition, and for effects on the production of the viral core protein, p24 (Fonteh *et al*., 2009, Fonteh and Meyer 2009, Fonteh and Meyer, MSc. Dissertation, 2008). These compounds consisted of four ligands and eleven gold complexes. The compounds demonstrated stability in DMSO solution after one week when ^{31}P and ^{1}H NMR spectra were obtained. Uptake into both primary cells and continuous cell lines were demonstrated by inductively coupled plasma atomic emission spectrometry (ICP-AES, Traoré and Meyer, 2001) as positive. Eight of the complexes significantly inhibited HIV-1 RT at concentrations of 25 and 250 µM and three of the eight did so at 6.25 µM. In a fluorogenic substrate assay against HIV-1 PR, four of the gold complexes demonstrated inhibitory activity at 100 µM. The gold compounds were selectively toxic to cell lines but not to primary cells. One of the complexes (EK231) significantly ($p=0.0042$) reduced p24 production at a non-toxic concentration of 25 µM.

Present Study: Based on the inhibitory effects of the gold compounds on RT and PR, the present study was initiated. Important questions such as the drug-likeness of the gold compounds, effects on host cells (immunomodulatory and whole virus) and binding interactions with viral targets such as RT and PR were desired. The effect of the compounds on the activity of a third viral enzyme, IN, was also sought.

Eleven new compounds were included in the new study leading to a total of twenty seven compounds (eight ligands or gold compound precursors and nineteen complexes) consisting of five classes (I – V) which were based on the ligand types used for synthesis. The classes included; (I) gold(I) phosphine chloride-containing complexes, (II) the bis(phosphino) hydrazine gold(I) chloride-containing complexes, (III) the gold(I) phosphine thiolate-based complexes, (IV) the gold(III) Tscs-based complexes and (V) a gold(III) pyrazolyl-based complex. In addition to determining drug-likeness, the compounds' effect on immune system cells was also determined. Direct enzyme assays were performed to determine the compounds' effects on viral enzymes (RT, PR and IN) followed by *in silico* binding predictions to determine active site binding modes. To establish whether the different ligand types (and oxidation states i.e. +1 or +3) conferred unique class properties that could be exploited for further therapeutic use, activity and drug-likeness was compared.

Strategy: A schematic overview of the screening strategy that was used is shown in Figure 2.20. In the figure, twenty seven compounds from five classes (I-V) were tested for drug-likeness, effects on host cells and viral enzymes followed by statistical analysis for differences between controls and treatments.

In this report, the gold compound precursors (the ligands) and complexes are collectively referred to as the compounds. In the molecular modelling section in Chapter 5, the terminology used for the compounds will be ligands, to comply with molecular modelling

terminology for the complementary partner molecule that binds or interacts with a receptor. All the compounds tested here were received through Project AuTEK Biomed for bioscreening. Detailed synthesis (chemistry information) is not important for this work and is reported elsewhere but the basic chemical characteristics of the compounds are provided in chapter 3.

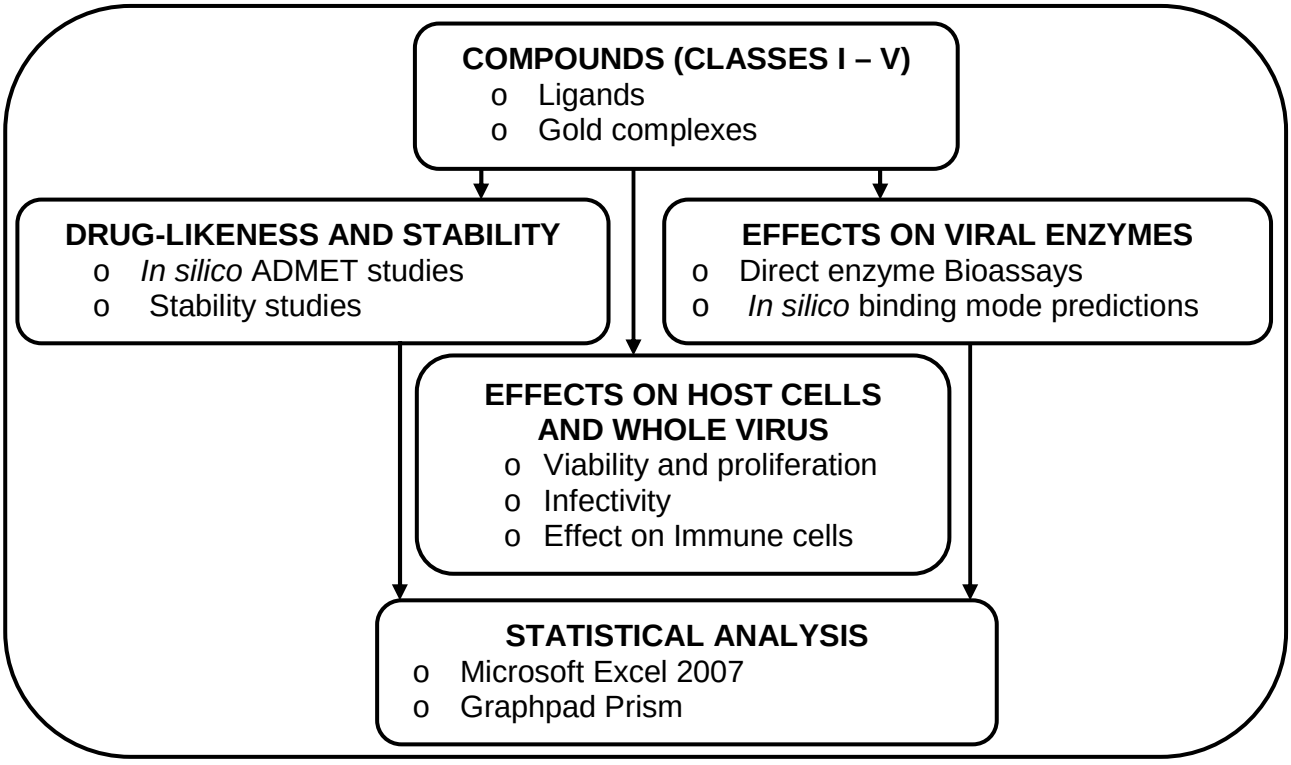

Figure 2.20: Schematic representation of the screening strategy. Twenty seven compounds from five classes (I – V) were tested for drug-likeness, and for effects on host cells and viral targets.

In silico techniques were used in this study, for optimisation and as complementary approaches to *in vitro* experimental assays. This is because the compounds tested had already been synthesised using traditional medicinal chemistry knowledge such as analysis of available biological data and chemical structure (Ohlstein *et al.,* 2000), lipophilicity and anti-viral activity of the relevant ligands and the history of gold-based compounds as anti-HIV agents. Other considerations were the fact that complexation of the ligands with gold and other metals usually led to an enhanced synergistic medicinal effect. Therefore, the screening approach was not rational drug design-based, where *in silico* predictions precede synthesis and experimental analysis. However, as soon as ADMET predictions were determined, the more favourable drug-like compounds were prioritised for further screening. Compounds with less favourable ADMET predictions were only tested further with the hope that efficacious compounds based on beneficial experimental data can be recommended for structural modification to improve activity.

Preliminary assays included determining the ADMET properties and enzyme inhibitory effects of the compounds. This was done using *in silico* drug-likeness predictions and *in vitro* cytotoxicity studies as well as direct enzyme assays respectively with emphasis on high throughput screening (96 well plate formats for analysis). Subsequent assays included determining the immunomodulatory effects of the compounds, effect of compound on ability to

prevent whole virus from infecting host cells (infectivity) and *in silico* enzyme mechanistic studies. In most cases, especially for the non HTS such as immunomodulatory studies, only compounds with favourable ADMET properties or which had shown activity in the direct enzyme assays were tested.

Methodologies: Methodologies employed were standard biochemical techniques and included: (1) Nuclear magnetic resonance spectroscopy which was used in determining the stability of the compounds by monitoring structural changes from spectral chemical shifts, (2) spectrophotometric studies for determining absorbance (related to activity) after colour reaction in viability dyes and for the colorimetric RT and IN assays, (3) flow cytometry for determining the properties of single cells in suspension such as scatter, viability, proliferation and immune state, (4) fluorescent-based methods for monitoring the fluorescence of a fluorogenic HIV PR substrate, (5) luminescent methods for determining the luminescence of the luciferase gene product used in measuring infectivity levels and (6) *in silico* techniques for predicting drug-likeness and the bindings modes of the compounds to enzyme active sites using protocols in Discovery Studio® (DS) (Accelrys®, California, USA). In the next subsections, more details on what each of the main research questions entailed is provided.

2.5.1 Drug-likeness Studies

The drug-likeness of the compounds was determined using *in vitro* cytotoxicity techniques and *in silico* ADMET predictions. *In vitro* methods included the determination of the compound's effect on the viability of human cells. This was assessed by monitoring the optical density of viability dyes by spectrophotometry and fluorescence properties of stained cells by flow cytometry as well as by monitoring the effect of the compounds on the proliferation of these cells. The *in silico* predictions involved the use of the ADMET protocol in the DS® software program (Accelrys®, California, USA) to predict human intestinal absorption (HIA), aqueous solubility, blood brain barrier (BBB) penetration, cytochrome P450 (CYP) inhibition, plasma protein binding (PPB) and hepatotoxicity. In addition, predictions for lipohilicity and polar surface area (hydrogen bonding ability) were also deduced. Stability studies were performed by storing the compounds at different temperatures and monitoring structural changes using NMR spectroscopy. This is important because structural stability means the original chemical entity obtained at the point of synthesis still has the same characteristics. Additionally, stability information can lead to deductions on shelf life.

2.5.2 The Effect of the Compounds on Host Cells and on Whole virus.

To investigate the effects of the compounds on host cells and whole virus, cell-based assays were performed. The assays included viability studies and proliferation studies to determine cytotoxicity, viral infectivity inhibition studies and the effect of the compounds on

immune cell frequency and cytokine production. Inhibition of infectivity was measured as a reduction in luciferase reporter gene expression in the TZM-bl reporter cell line.

The immunomodulatory assays were done using a multi-parametric flow cytometry assay to determine the production of the important bio-molecules such as cytokines that are altered during infection. For this project the intracellular production of two representative cytokines within T cells (CD4+ and CD8+) was monitored. Only representative cytokines were chosen because of the complexity of the immune system. These were; (1) the anti-inflammatory cytokine, interferon gamma (IFN-γ). This cytokine although also labelled as a pro-inflammatory because of its bimodal role in HIV (causes both enhancement and suppression of HIV replication, Alfano and Poli, 2005), was evaluated in this study as an anti-inflammatory cytokine. IFN-γ is known to prevent systemic inflammation and has been associated with a decrease in HIV disease progression and pathogenesis (Ghanekar *et al.*, 2001, Francis *et al.*, 1992). The second cyotokine (2) was tumour necrosis factor alpha (TNF-α) which is a pro-inflammatory cytokine that is known to promote systemic inflammation and which has been associated with HIV disease progression *in vivo* (Caso *et al*., 2001). In addition, the choice of TNF-α as a representative pro-inflammatory cytokine was based on the fact that it is a prototype of pro-inflammatory cytokines and activates the production of other pro-inflammatory cytokines such as IL-1 (Barrera *et al*., 1996).

Proliferation studies simultaneously provide information about cell viability and mechanistic information such as mode of cell death (apoptosis, necrosis or cytostasis). In addition, proliferation patterns provided clues on the possible stimulatory or inhibitory effects the compounds could have on T cell proliferation (i.e. if the compounds could have therapeutic benefits or if they could have adverse side effects, Best and Sadler, 1996). These were performed using the flow cytometric carboxylflourescein succinimidyl ester dye dilution technology and the xCelligence impedence-based technology on an RT-CES device.

2.5.3 The Effects of the Compounds on Viral Enzymes

The inhibition of three viral enzymes was performed using direct enzyme bioassays. These assays involve combining of recombinantly purified enzymes and their substrates in the presence of the compounds followed by activity monitoring either spectroscopically by measurement of absorbance from a colour reaction (for RT and IN) or by fluorescence measurement (PR). A complementary *in silico* assay using molecular modelling (docking) was performed for compounds which showed promise in the direct enzyme assays so as to predict the type of inhibitory mechanism involved as well as to confirm direct enzyme assay findings. With regards to inhibitory mechanism, it was necessary to know if compounds which inhibited these enzymes in the bioassays did so by binding to the active site or if they were allosteric inhibitors. In the case of RT, since the enzyme has both a polymerase and an RNase H

function, it was important that the exact function inhibited by the compound be determined. For IN, knowledge of whether the compounds interacted with the DNA binding or LEDGF binding site was necessary.

2.5.4 Statistical Analysis

Statistical analyses were performed using Microsoft® Office Excel® 2007(Microsoft Corporation, Washington, USA) and Graphpad Prism® (San Diego, California, USA). Some of the calculations that were performed included: standard error of means (SEM), p values, correlation coefficients, means, standard deviations, medians, CC_{50}s and IC_{50}s. Detailed explanations of what the statistical terminologies mean are provided in the appendix, chapter 8 (Table A2.1 where A= appendix)

For this project, assays were performed at least 4 times and up to 6 times for experiments that needed optimisation unless stated otherwise.

2.6 OTHER RESEARCH OUTCOMES

Other research outcomes include publications, awards (travel and fellowships) and the presentation of results at conferences. More details on these are provided in the preface section of this document, after acknowledgments (on page VIII).