



CHAPTER 5

ANTI-HIV ACTIVITY OF THE ISOLATED COMPOUNDS

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CHAPTER 5

ANTI-HIV ACTIVITY OF THE ISOLATED COMPOUNDS

5.1 Introduction

Globally, millions of people are infected and are still being infected with the human immunodeficiency virus (HIV) (Fig 5.1), the pathogen that causes Acquired Immunodeficiency Syndrome (AIDS) (Gurib-Fakim, 2006). AIDS is a collection of symptoms and infections in humans resulting from the specific damage to the immune system by the virus. The late stage of the condition leaves individuals prone to opportunistic infections and tumors (Marx, 1982). HIV uses cells of the immune system (macrophages and helper T cells) as sites for reproduction. Multiple copies of the viral genetic material (RNA) are made and packaged into new viral particles ready for dispersal into a new viral host. More and more cells of the immune system are killed or damaged with each round of infection, while millions of viral particles may be produced each day. Despite the production of antibodies and helper T cells that fight the disease, eventually the virus prevails and the infections and cancer associated with AIDS begins to appear (Gurib-Fakim, 2006). Although treatments for AIDS and HIV exist to slow the virus's progression, there is no known cure. HIV is transmitted through direct contact of a mucous membrane or the bloodstream with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, pre-seminal fluid and breast milk. This transmission can come in the form of anal, vaginal or oral sex, blood transfusions, contaminated needles, exchange between mother and baby during pregnancy, childbirth or breast feeding, or other exposure to bodily fluids (Mandell *et al.*, 2005).

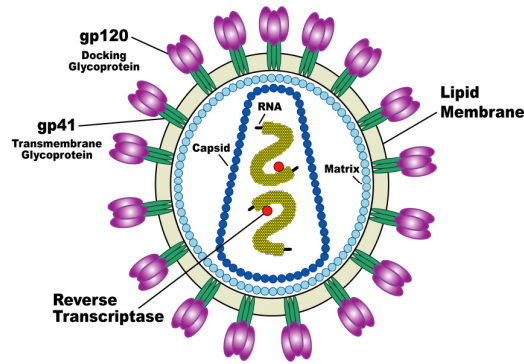


Figure 5.1: Human immunodeficiency virus (NIAID, 1998).

Most researchers believe that HIV originated in sub-Saharan Africa during the twentieth century (Gao *et al.*, 1999); it is now a pandemic with an estimated 38.6 million people living with the disease worldwide. As of January 2006, the joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organisation (WHO) estimated that AIDS has killed more than 25 million people since it was first recognised on June 5, 1981, making it one of the most destructive epidemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4-3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth and destroying human life (UNAIDS, 2006).

5.1.1 HIV in South Africa

A number of documents have described the seriousness of HIV/AIDS in the southern Africa region with particular emphasis on South Africa being the most affected (UNAIDS, 2000). The prevalent rate for South Africa is estimated to be 12.5%, which is one of the highest national prevalent rates in the world (James *et al.*, 2006). Women are the worst hit by the epidemic of HIV/AIDS. Of the 5.54 million people living with HIV in South Africa in 2005, 18.8% are adults aged 15-49 of which women account for approximately 55%. The infection is more pronounced in the age group 20-24 and 25-29 years where the HIV prevalence rates are 23.9% for women, 6% for men and 33.3% for women, 12.2% for men respectively (NSP, 2007). HIV was around 3% among children aged 2-14 year and nearly 4% for people in their sixties (Dinkelman *et al.*, 2005).

Children under the age of 18 years comprise 40% of the population of South Africa. In 2004, it was reported that 13% of them have lost either mother or father, half of which was due to AIDS. Children from deeply impoverished household were worst affected by the impact of AIDS (UNAIDS, 2004).

The severe effect of HIV/AIDS has led to a dramatic increase in the probability of death in South Africa's adult population. The latest forecast from the Actuarial Society of South Africa showed the likelihood of death before 60th birthday among men jumping from 36% in 1990 to 61% in 2008, whereas the likelihood of death among women increases from 21% in 1990 to 53% in 2008 (Collins and Leibbrandt, 2007).

5.1.2 Anti-HIV compounds

Currently, there is no cure for HIV/AIDS. Antiretroviral treatments reduce both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries. Antiretroviral therapy (ART) consists of four major treatment modalities, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion or entry inhibitors (Grinspoon, 2005). The HIV/AIDS stigma is more severe than that associated with other life-threatening conditions and extends beyond the disease itself to providers and even volunteers involved with the care of people living with HIV (UNAIDS, 2006).

New anti-HIV compounds from natural sources are reported almost daily, some essentially unproven and others with distinct promise based on *in vitro* research. Some of these compounds are at different stages in clinical trials while further studies on others have stopped because they are found not to be suitable for use as drugs (Gurib-Fakim, 2006). The search for molecules from natural resources with anti-HIV activity or the ability to treat AIDS related infections needs to be intensified and accelerated so that the possibility of developing a drug will soon be realised.

5.1.3 Reverse transcriptase (RT)

Reverse transcriptase is a DNA polymerase that will either use an RNA or DNA strand as a primer. It is responsible for the production of a double stranded DNA copy of the single stranded RNA genome that is contained in the HIV virus particle. RT from HIV-1 is of tremendous medical interest as it is the target enzyme for the best known anti-AIDS drug, AZT, which acts by causing chain termination of the polymerase reaction. Knowledge of its detailed three dimensional structures will greatly assist the development of new anti-AIDS drugs (Arnold *et al.*, 1995).

5.1.4 Replication of HIV

Infection typically begins when an HIV particle, which contains two copies of the HIV RNA, encounters a cell with a surface molecule called cluster designation 4 (CD4). Cells with this molecule are known as CD4 positive (CD4+) cells. One or more of the virus's glycoprotein (gp120) molecules binds tightly to the CD4 molecule(s) on the cell's surface (Fig. 5.2). The membrane of the virus and the cell fuse, a process that probably involves the envelope protein of the HIV (NIAID, 1998).

Although CD4+ T cells appear to be the HIV's main target, other immune system cells with CD4 molecules on their surfaces are infected as well. Among these are the long-lived cells called monocytes and macrophages, which apparently can harbour large quantities of the virus without being killed, thus acting as reservoirs of HIV. CD4+ T cells also serve as important reservoirs of HIV: a small proportion of these cells harbour HIV in a stable, inactive form. Normal immune processes may activate these cells, resulting in the production of new virions (NIAID, 1998).

In the cytoplasm of the cell, HIV reverse transcriptase converts viral RNA into DNA, the nucleic acid form in which the cell carries its genes. Seven of the 11 antiviral drugs approved in the United States for the treatment of people with HIV infection namely zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), stavudine (d4T), lamivudine (3TC), neviraine (NVP) and delavirdine (DLV), work by interfering with this stage of the viral life cycle.

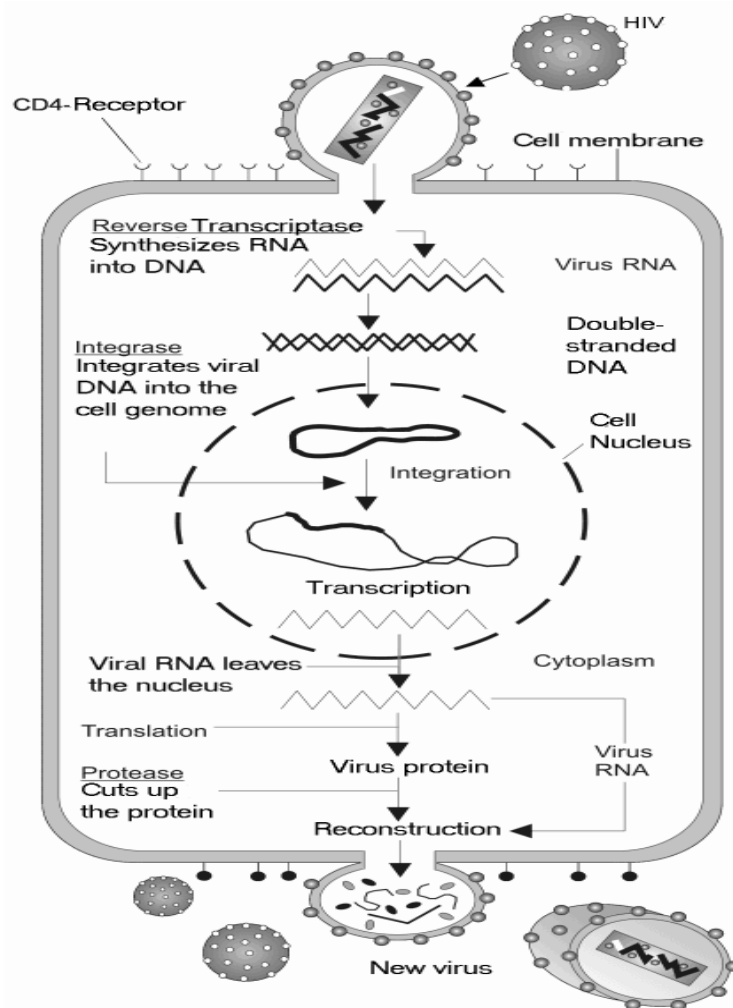


Figure 5.2: The HIV replication cycle (NIAID, 1998).

The newly made HIV DNA moves to the cell's nucleus, where it is spliced into the host's DNA with the help of HIV integrase. Once incorporated into the cell's genes, HIV DNA is called a "provirus." Integrase is an important target for the development of new drugs. For a "provirus" to produce new viruses, RNA copies must be made that can be read by the host cell's protein making machinery. These copies are called messenger RNA (mRNA). Production of mRNA is called transcription, a process that involves the host cell's own enzymes. Transcription requires the presence of transcription factors, the most important of which is NF kappa B (NF- κ B). Proteins, cytokines, that are involved in the normal regulation of the immune response also may regulate transcription. Molecules such as necrosis factor (TNF)-alpha and interleukin (IL)-6 that are secreted in elevated levels by the cells of HIV-infected people

may help to activate HIV “proviruses”. Early efforts at stopping HIV replication focused on these chemicals. Other infections, by organism such as *Mycobacterium tuberculosis*, also may enhance transcription (NIAID, 1998; Hopkins, 1999).

After HIV mRNA is processed in the nucleus, it is transported into the cytoplasm. HIV proteins are critical to this process: for example, a protein encoded by HIV’s *rev* gene allows HIV structural proteins encoding mRNA to be transferred from the nucleus to the cytoplasm. Without the *rev* protein, structural proteins are not made. In the cytoplasm, the virus co-opts the cell’s protein-making machinery including structures called ribosomes to make long chains of viral proteins and enzymes, using HIV mRNA as a template. This process is called translation result in immature viral particles which at this stage are not yet infectious. The long chains of proteins and enzymes that make up the immature viral core are now cleaved into smaller pieces by a viral enzyme called protease. This step results in infectious mature viral particles. Drugs called protease inhibitors (saquinavir, ritonavir, indinavir and nelfinavir) interfere with this step of the viral life cycle (NIAID, 1998).

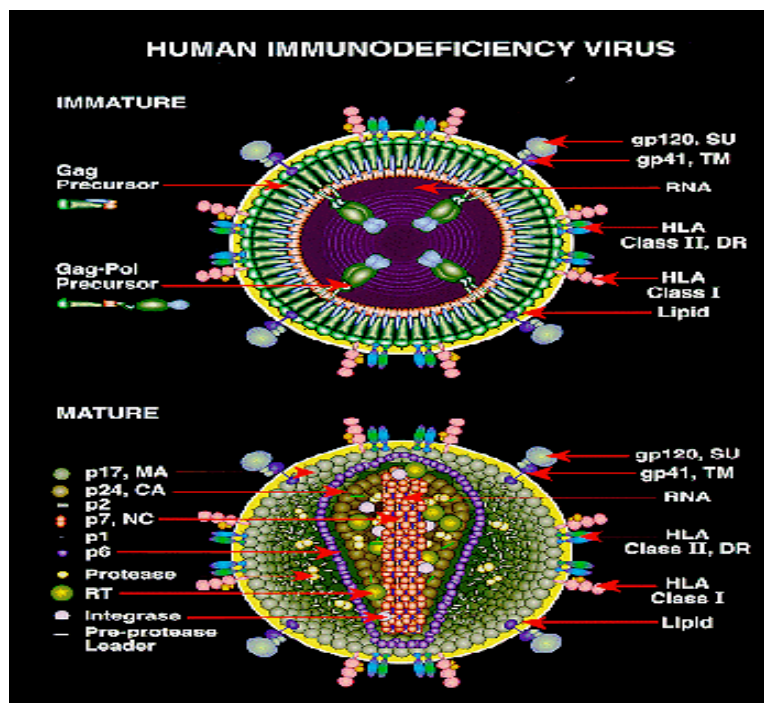


Figure 5.3: The immature and mature forms of the HIV (NIAID, 1998).

Several reports have linked human immunodeficiency virus (HIV) infection and prolonged usage of highly active antiretroviral therapy (HAART) with increased risk of cardiovascular disease (CVD), premature atherosclerosis and development of metabolic syndrome (dyslipidemia, insulin resistance, fat redistribution and hypertension) (Maggi *et al.*, 2007; Friis-Møller *et al.*, 2003). The relative risk rate of myocardial infarction is said to increase by 26% per year of HAART exposure (Grinspoon, 2005).

The fact that currently there is no cure in sight and that available treatment predisposes patients to risky side effects, is reason enough for desperate and radical search for new remedy with curative or preventive properties. Hence, the compounds isolated were assessed for anti-HIV activities.

5.2 Materials and Methods

5.2.1 Materials

The preparation of the crude extract and the isolation of compounds were as described in Chapter 2. The reverse transcriptase assay kit was purchased from Roche Applied Science, the dimethylsulfoxide (DMSO) from Sigma-Aldrich and the sterile 96-well microplates from Fishers Scientific.

5.2.2 Method

The anti-HIV activity of the compounds isolated was determined with the reverse transcriptase colorimetric assay. The protocol outlined in the kit was followed. The pure compounds were tested in triplicate at 50 µg/ml final concentration. Sterile 96-well microplates were used for the experiment. One positive controls and one negative control were included in each assay. The well for the positive control contained Doxorubicin at 100 µg/ml with 3.3% DMSO, 20 µl of the 83.33 ng/ml enzyme, 20 µl lysis buffer and 20 µl reaction mixture. The negative control was made of 40 µl lysis buffer with 20 µl reaction mixture without the enzyme. All other wells contained 20 µl of the 83.33 ng/ml enzyme, 50 µg/ml of the compounds with 3.3 % DMSO and 20 µl reaction mixture. The plate was then incubated at 37°C for an hour after which it was washed five times with 250 µl washing buffer per well per washing

cycle. Two hundred micro-litres antibody solution was added to each well and the plate was incubated at 37°C for an hour. The plate was washed again five times with 250 µl washing buffer per well per washing cycle. After 200 µl of the ABT substrate solution was added to each well, the plate was incubated for 10 minutes before being measured at 412 nm wavelength in an ELISA plate reader.

5.3 Results

Steenkrotin A, steenkrotin B, the indane and quercetin were each tested against reverse transcriptase *in vitro*. Indane showed 6% activity against the enzyme at 50 µg/ml. Eriodictyol and tamarixetin was not tested because the quantities isolated were very small. Quercetin, steenkrotin A and steenkrotin B were not active at the concentration (50 µg/ml) at which they were tested.

5.4 Discussion

Many natural products have been found to be inhibitors of HIV-1 RT. These compounds belong to diverse structural classes which include coumarins, flavonoids, tannins, alkaloids, lignans, terpenes and quinines (Mahidol *et al.*, 2002). For this reason the possible anti-HIV activity of the isolated compounds was explored. Mahidol *et al.* (2002) reported anti-HIV activity of quercetin-3-O- α -L-rhamnoside. The fact that quercetin had no anti-HIV activity can only be attributed to structural change (absence of glycosidic side chain attached to the molecule). The two diterpenes (steenkrotin A and steenkrotin B) also did not show anti-HIV activity at 50 µg/ml concentration. This can also be linked to differences in molecular structure of the compounds as a furanoid labdane diterpene was reported to exhibit antiviral activity (Kittakoop *et al.*, 2001). Of the compounds tested only the indane showed weak activity of inhibiting reverse transcriptase. Its observed cytotoxicity was very low (chapter 6). However, the mechanism of action leading to the observed activities is not clear at the moment. It could be due to the acidic property of the compound (presence of the carboxylic acid side chain in the molecule).

Furthermore, in order to fully explore the possible anti-HIV activity of the compounds isolated, they may have to be tested at higher concentrations and



different assays methods may have to be used. Possible derivatives of the compound could be made and tested for anti-HIV activity.

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