The HIV-1 glycoprotein gp120 elevates NF-κB levels in human cardiomyocytes which may be reversed with the treatment of a sesquiterpene lactone isolated from *Vernonia staehelinoides*

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Master of Science.

Johannesburg, January 2009

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Dayaneethie Coopusamy

this day of 2009

ABSTRACT

Twenty five years of studying HIV-1 structure and replication have improved diagnosis and treatment of individuals infected with the virus. In particular, the introduction of highly active antiretroviral therapy (HAART) has significantly altered the course of HIV-1 infection by increasing life expectancy and reducing opportunistic infections. However, chronic cardiovascular complications such as HIV associated cardiomyopathy (HIVCM), which manifest later during the course of HIV-1 infection, have become increasingly evident. Despite its growing incidence, with high cardiovascular morbidity and mortality in young and middle-aged adults, the molecular mechanisms of HIVCM remain poorly understood. A number of pathways have been implicated in HIVCM, including damage initiated by HIV-1 surface glycoprotein, gp120, and dysregulation of NF-κB. NF-κB is a universal transcription factor and it regulates a number of genes, many of which are involved in inflammation, injury and stress response. The ability of HIV-1 to manipulate host signalling pathways, including elevated NF-κB levels, has resulted in efficient viral replication and gene expression. The elevation of NF-κB has also been shown to be involved in animal models of HIVCM but very little work has been conducted on human cells. For this reason, the primary objectives of this thesis were to establish the level of NF-κB in a cellular model of HIVCM by challenging human cardiomyocytes with HIV-1 or gp120 and to mitigate the effect on NF-κB using natural compounds derived from South African indigenous plants. The effect of gp120 and HIV-1 on NF-κB levels in human cardiomyocytes was tested by an ELISA-based assay and immunocytochemistry. This was to determine whether the damage induced by HIV-1 and gp120 is mediated by NF-κB. The results shows that gp120 significantly increased NF-κB levels in human cardiomyocytes compared to control unstimulated cardiomyocytes ($p<0.001$). One plant compound, the sesquiterpene lactone 106A, significantly reduced the NF-κB response by human cardiomyocytes to gp120 stimulation ($p<0.05$). Taken together, this study suggests that the activation of NF-κB by gp120 has a role to play in a cellular model of HIVCM and that the sesquiterpene lactone 106A could prove valuable in further studies on the modulation of cellular responses due to gp120 and HIV-1 induced stress in human cardiomyocytes.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
ARV	antiretrovirals
BCA	bicinchoninic acid
cTnI	cardiac troponin I
CD4/40	cluster of differentiation 4/40
CCR5	chemokine (C-C motif) receptor 5
DMSO	dimethyl sulphoxide
DMEM	Dulbecco's Modified Eagle's medium
EDTA	ethylenediaminetetra-acetic acid
EDC	N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide
FCS	foetal calf serum
HIV-1	human immunodeficiency virus type-1
HIVCM	HIV associated cardiomyopathy
HCl	hydrochloric acid
iNOS	inducible nitric oxide synthase
IKK	inhibitory kappa kinase
IL	interleukin
LTR	long terminal repeat
MWCO	molecular weight cutoff
MDM	monocyte-derived macrophages
NF-κB	nuclear factor kappa B
NHS	N-hydroxysuccinimide
NLS	nuclear localisation sequence
PMA	phorbol 12-myristate 13-acetate
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
RHD	Rel homology domain
RU	response units
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TMB	3.3', 5.5'-tetramethylbenzidine
TNFR	Tumour necrosis factor receptor

1 INTRODUCTION

1.1 The HIV/AIDS pandemic

In 1981 a handful of young homosexual men in the United States were found to have a novel type of immunodeficiency (CDC, 1981). It was soon found that this type of immunodeficiency was associated with a depletion of $CD4^+$ T helper cells (Gottlieb *et al.*, 1981; Masur *et al.*, 1981). The discovery of the disease in intravenous drug users and recipients of blood transfusions as well as haemophiliacs receiving pooled clotting factors led scientists to believe that the causative agent was viral (CDC, 1982; Ragni *et al.*, 1983; Weiss, 2008). Due to the infectious nature of the disease and depletion of the immune system cells, it was called Acquired Immunodeficiency Syndrome (AIDS). In 1983 a group of scientists at the Pasteur Institute in Paris isolated a previously unknown retrovirus from a patient diagnosed with AIDS (Barre-Sinoussi *et al.*, 1983). This virus was eventually named Human Immunodeficiency Virus type-1 (HIV-1). In 1984, a year after the discovery of HIV-1 and three years after the identification of AIDS, there was sufficient data to persuade scientists that HIV-1 was the causative agent of AIDS (Weiss, 2008).

Twenty five years of study of this agent's structure and replication has enabled doctors to better diagnose, monitor and treat HIV-1 infection (Weiss, 2008). The advances in HIV-1 detection have made it possible to detect the virus early during infection, which has helped make blood donation safe. The understanding of the action of viral proteins has lead to antiretrovirals that inhibit viral replication, which has greatly prolonged the lifespan of HIV-1 positive individuals (Kinloch-De Loes *et al.*, 1995; Ruprecht *et al.*, 1990; Weiss, 2008). Knowledge that the virus infects and kills $CD4^+$ cells during its replication has allowed the monitoring of disease progression by $CD4^+$ cell counts and viral load.

Even with the advances in detection and treatment, HIV-1 prevention programmes have not been as effective. The World Health Organisation reports that the worldwide estimate for people living with HIV-1 in 2007 was 33.2 million (WHO, 2008). Sub-Saharan Africa continues to be the epicentre of the epidemic with a total of 22.5

million HIV-1-infected individuals living in this region in 2007, constituting approximately 67% of the worldwide HIV-1-infected population (WHO, 2008).

1.2 HIV-1 structure and infectious cycle

A mature HIV-1 virion contains two copies of viral genomic RNA in its core in addition to the protease, reverse transcriptase and integrase viral proteins, which are required for early replication events. The outer surface of the virus consists of spikes formed by the association of the surface glycoproteins gp120 and gp41, which are arranged as trimers. The viral genome is less than 10 kilobases and contains nine genes (Figure 1) (Haseltine, 1991). It has, however, managed to hijack host cellular pathways while hiding from the immune system.

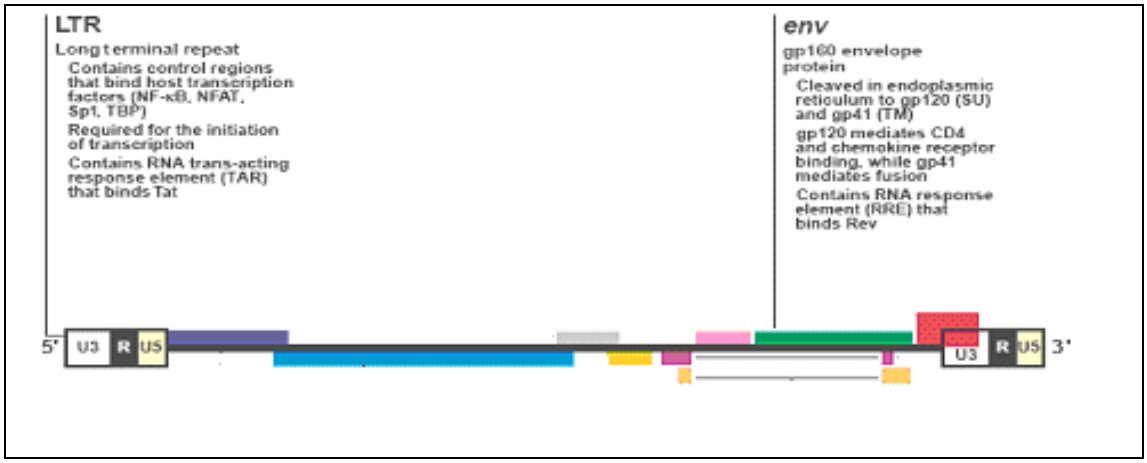


Figure 1: An overview of the HIV-1 proviral genome, genes and proteins together with a summary thereof. The figure was adapted from Greene and Peterlin (Greene and Peterlin, 2002).

The binding of gp120 to the CD4 receptor on macrophages and T cells initiates viral entry. This induces conformational changes in gp120 that allow a second interaction between the viral protein and either the CCR5 or CXCR4 chemokine co-receptor, although other co-receptors have been noted (Eckert and Kim, 2001; Greene and Peterlin, 2002). The chemokine receptor binding allows for fusion with the cell and the release of the viral core into the cytoplasm of the host cell (Eckert and Kim, 2001). The viral reverse transcriptase enzyme converts the viral RNA genome to DNA once the viral core has disassembled (Haseltine, 1991). The reverse transcriptase enzyme has no proofreading ability and is error prone, producing a

heterogenic population. Host and viral factors move the proviral DNA to the nucleus of the cell where it is inserted into the chromosomal DNA by the viral protein integrase. The inserted proviral DNA may be transcriptionally active or latent depending on the location that it is integrated into (Greene and Peterlin, 2002). HIV-1 uses the host cell's machinery to replicate and express its genome. Viral proteins move to the cell membrane where they assemble into immature virus particles, acquire a lipid envelope and bud off from the cell (Greene and Peterlin, 2002). The viral protease cleaves the Gag-Pol HIV-1 protein to produce mature virions. Without this final step, virions remain non-infectious and cannot replicate in other cells (Simon *et al.*, 2006).

1.3 HIV pathogenesis

Active replication can be seen early in infection via the mucosal or parenteral route and results in a high initial viral load. This may be accompanied by fever, diarrhoea and lymphadenopathy (Barre-Sinoussi *et al.*, 1983; Weiss, 2008), as this replication takes place in the regional lymph nodes. The initial state of high viral load then resolves to a lower level. This new viral load is predictive of the rate of progression to AIDS, with a higher viral load together with a low $CD4^+$ cell count leading to a worse prognosis (Mellors *et al.*, 1997). At any given point viral load is predictive of transmission risk, with transmission risk being exceptionally high during acute infection and co-infection with sexually transmitted diseases (Simon *et al.*, 2006). As the infection progresses towards AIDS, the final disease stage, a number of clinical syndromes manifest including HIV-associated dementia, nephropathy and cardiomyopathy (Moroni and Antinori, 2003).

1.4 HIV-associated Cardiomyopathy

Preceding the introduction of highly active antiretroviral therapy (HAART), cardiac disease was not clinically significant in HIV positive individuals. This was because most cases were silent or overshadowed by clinical symptoms in other organs, mainly the brain and lungs (Sudano *et al.*, 2006). The arrival of HAART has significantly altered the course of HIV disease, resulting in increased life expectancy and the reduction of opportunistic infections (Barbaro, 2001; Barbaro *et al.*, 2001). However, this has brought with it some side effects in the form of certain chronic conditions such as HIV-associated cardiomyopathy (HIVCM). Even though HAART has

reduced the incidence of opportunistic infections, the number of cases of coronary syndromes is increasing because of the long term effects of HIV infection, HAART and opportunistic infections (Barbaro *et al.*, 2001). The Heart of Soweto study conducted in 2006 showed 844 new cases of heart failure, 29 of which were attributed to HIV-related cardiomyopathy (Stewart *et al.*, 2008). The Data Collection on Adverse Events of Anti-HIV Drugs study found that of the 33 347 patients in this study, 517 HIV positive patients developed myocardial infarction. Of this group, 509 individuals were exposed to ARV (Sabin *et al.*, 2008), which if taken together with the increasing number of people receiving ARV shows increasing rates of cardiac events linked to HIV. Regimens of HAART that include reverse transcriptase and protease inhibitors have been shown to reduce the rate of mortality for congestive heart failure but increase acute coronary syndromes (Murphy and Barbaro, 2003). Unfortunately studies conducted prior to the introduction of HAART are still applicable as the vast majority of HIV-1-infected individuals do not have access to these drugs (Barbaro *et al.*, 2001; Murphy and Barbaro, 2003).

Many causes of HIVCM have been proposed including both direct and indirect effects of HIV-1. Opportunistic infections such as cytomegalovirus, *Toxoplasma gondii*, *Staphylococcus aureus* and Epstein-Barr virus have also been shown to be contributing factors in the advance of cardiac complications (Hajjar *et al.*, 2005; Moroni and Antinori, 2003). Certain nutritional deficiencies have been directly or indirectly linked to HIVCM that shows left ventricular dysfunction, with selenium supplementation in nutritionally depleted patients showing a reversal of cardiomyopathy (Barbaro, 2001; Barbaro *et al.*, 2001; Lewis, 2000). In addition to these, autoimmune response, in the form of cardiac-specific autoantibodies, as well as drug toxicity have been suggested, although it is most likely that a number of these causes act in concert (Barbaro *et al.*, 2001).

The molecular mechanisms of the disease are still poorly understood. It has been found that this process is inflammatory and mediated by HIV-infected and cyclooxygenase 2-expressing $CD68^+$ activated macrophages together with other inflammatory cells (Liu *et al.*, 2001). Most of these inflammatory cells were found to have CD3 and CD8 receptors. Productive infection, together with expression of the viral proteins gp120 and Nef, was found only in macrophages and T cells (Liu *et al.*,

2001). This discounts direct infection of cardiomyocytes by HIV as previously suggested (Grody *et al.*, 1990), but instead alludes to an indirect mechanism of cardiomyocyte damage initiated by inflammatory cells and/or circulating cytokines (Fisher *et al.*, 2003). Several different cellular products have been put forth to explain this including nitric oxide (Barbaro *et al.*, 1999; Kan *et al.*, 2000) and cytokines, particularly tumour necrosis factor-α (TNF-α) and interleukin (IL)-1 (Barbaro *et al.*, 2001; Lewis, 2000). Although not produced in significant amounts by HIV-1-infected cells, TNF-α has been found in high levels in the plasma from 46% of HIV-1 positive patients with acute myocarditis (Barbaro *et al.*, 1999; Fisher *et al.*, 2003). It was also found to be expressed in 100% of endomyocardial biopsies from HIV-infected individuals showing myocarditis, together with IL-1b, IL-6 and IL-8 (Fisher *et al.*, 2003). This group also showed that the intensity of staining for TNF-α and iNOS in these biopsy samples inversely corresponded with CD4 count, with ARV not affecting these results (Barbaro *et al.*, 1999). Cardiomyocyte contractility is affected by a number of factors in HIVCM including TNF-α (Fisher *et al.*, 2003) and gp120 (Chen *et al.*, 2002), suggesting that these factors have a direct effect on cardiomyocyte dysfunction. The damage to cardiomyocytes due to HIV-1 infection is possibly due to an interaction between viral proteins such as gp120 (Chen *et al.*, 2002; Kan *et al.*, 2000) and cellular factors leading to the apoptosis of cardiomyocytes, a critical mechanism in HIVCM (Twu *et al.*, 2002). A study that found that gp120 stimulates the production of nitric oxide in cardiomyocytes through the activation of nuclear factor-κB adds weight to this theory (Kan *et al.*, 2000).

1.5 Nuclear factor-κB

Nuclear factor-κB (NF-κB) was first characterised as a nuclear factor necessary for the transcription of immunoglobulin κ light chains in B-cells (Sen and Baltimore, 1986b). Originally thought to be present in these cells only, NF-κB was found in the cytoplasm of most cells from insect to human, sequestered by inhibitory proteins (Ghosh *et al.*, 1998). More than twenty years since the discovery of these transcription factors, research into the function and regulation of NF-κB continues at a brisk pace (Hayden and Ghosh, 2008).

The functional transcription factor consists of homo- or heterodimers made up of subunits from the Rel/NF-κB family of proteins (Tak and Firestein, 2001). This

family consists of five members; p50/p105, p52/p100, RelA (p65), c-Rel and RelB encoded by genes that share an N-terminal Rel homology domain (RHD) (Baldwin, 1996; Tak and Firestein, 2001). The most studied of the NF-κB proteins is the p50:p65 heterodimer. The first two are synthesised as inactive precursor molecules that undergo processing to produce transcriptionally active proteins (May and Ghosh, 1998). The RHD is responsible for the dimerisation of subunits and DNA binding, in addition to containing the nuclear localisation sequence (NLS) (Barkett and Gilmore, 1999; Ghosh *et al.*, 1998; Hayden and Ghosh, 2008). The inhibitory κB (IκB) proteins are a family of structurally and functionally related molecules. They contain sequences known as ankyrin repeats, which associate with the RHD of NF-κB dimers (May and Ghosh, 1998). NF-κB dimers are inactive in resting cells, either due to this association with the IκB proteins or the inactivating precursors (p100 and p105). These inactivating agents mask the NLS, maintaining NF-κB predominantly in the cytoplasm. The most studied member of IκB, IκBα, only partially masks the NLS of the NF-κB dimer but contains a nuclear export sequence, leading to shuttling of inactive molecules between the nucleus and cytoplasm (Hayden and Ghosh, 2008; Perkins, 2007). Once activated, transcription factors move from the cytoplasm and bind to the κB promoter sites on DNA where they enhance the transcription of target genes (Ghosh *et al.*, 1998). The availability of these transcription factors allows for rapid response when signalled as it does not require protein synthesis for the signal to be transmitted (Ghosh *et al.*, 1998; May and Ghosh, 1998).

There are two major pathways that lead to active NF-κB transcription factors binding to their target genes: the canonical (classical) and noncanonical (alternative pathways). The canonical pathway involves the degradation of IκBα. The activated inhibitory κ kinase (IKK) complex, containing IKKα, IKKβ and other proteins, causes the phosphorylation of IκBα at Ser32 and Ser36. This leads to the degradation of IκBα by the 26S proteasome, which is ubiquitin induced. The degradation of IκBα exposes the nuclear localisation sequence of the p50:p65 NF-κB heterodimer. This allows the heterodimer to be translocated to the nucleus (Ghosh *et al.*, 1998; May and Ghosh, 1998; Perkins, 2007). The phosphorylation of the p65 by several kinases is necessary for transcriptional activity as this phosphorylation allows the binding of transcriptional co-activators to p65 and enhances transcription. In the absence of phosphorylation, this dimer binds to DNA but represses transcription (Hayden and

Ghosh, 2008). The noncanonical pathway depends on the phosphorylation of p100 by active IKKα. This induces ubiquitin-dependent 26S proteasomal processing of the p100 precursor molecule to active p52, allowing nuclear translocation of p52-containing dimers (Ghosh *et al.*, 1998; May and Ghosh, 1998; Perkins, 2007). The transcription of IκB proteins is NF-κB dependent creating a negative feedback loop and, together with measures that target DNA-bound dimers, this terminates the NF-κB response (Hayden and Ghosh, 2008).

Different pathways regulate distinct subsets of NF-κB, allowing the separate pathways to target different genes. A broad range of receptors and stimulants activate the canonical pathway (TNF-α, IL-1, LPS) while only those belonging to a subset of the TNFR superfamily (CD40, latent membrane protein-1 of Epstein-Barr virus, lymphotoxin β receptor) appear to activate the alternative pathway (Hayden and Ghosh, 2008; Pereira and Oakley, 2008). In addition to bacteria and viruses, NF-κB is also induced by physiological and physical stress such as haemorrhagic shock and irradiation respectively, as well as drugs and environmental stresses. The sequence variability of the κB promoter site together with the varied binding preferences of the different NF-κB dimers results in a staggering number of genes that are very precisely regulated by NF-κB. Most of these genes are involved in inflammation, injury and stress response (Ghosh *et al.*, 1998; May and Ghosh, 1998; Pahl, 1999). These genes include receptors essential for immune recognition, proteins required for antigen presentation and at least 27 different cytokines (Pahl, 1999). NF-κB therefore relays the message of a stress while simultaneously eliciting a response by activating the transcription of products to reduce the specific stress. This ensures that the reaction to a given stimulus is site and event specific and allows cells to respond to the external environment appropriately (Tak and Firestein, 2001).

The dysregulation of NF-κB has huge implications for inflammatory diseases. Rheumatoid arthritis shows an overexpression of NF-κB in the inflamed lining of the joint. This may increase the production of IL-1, IL-6, IL-8 and TNF-α, which are pro-inflammatory cytokines and recruit inflammatory cells to the area. High levels of p50 and p65 have been found in the synovium and mononuclear cells in the surrounding tissue (Han *et al.*, 1998). Gastritis-associated with *Helicobacter pylori* shows a marked increase in NF-κB levels, with the number of NF-κB induced cells

determining the severity of the gastritis (Pande and Ramos, 2005; Tak and Firestein, 2001; van Den Brink *et al.*, 2000). Inflammatory bowel disease shows macrophages in the lamina propria of the gastrointestinal tract with activated p50, c-Rel and exceptionally high p65. The level of pro-inflammatory cytokine production was modulated in these cells by their treatment with antisense p65 oligonucleotides (Tak and Firestein, 2001). Bronchial biopsies from asthma patients show NF-κB activation and nuclearisation. High levels of pro-inflammatory cytokines and chemokines were also found (Hart *et al.*, 1998). NF-κB has also been linked to the inflammation of the arteries seen in artherosclerosis. Activated NF-κB has been found in the cells of artherosclerotic plaques but not in their healthy counterparts (Pande and Ramos, 2005). Abnormal levels of NF-κB directly contribute to the pathogenesis of inflammation.

NF-κB has been shown to be involved in the cell cycle and cell differentiation. NF-κB is also involved in apoptosis but may be pro- or anti-apoptotic, depending on the cell type and stimulus (Barkett and Gilmore, 1999; Pahl, 1999). It follows that NF-κB has been implicated in oncogenesis, as cancer is a disruption of the healthy cell cycle and apoptosis (Barkett and Gilmore, 1999; Pande and Ramos, 2005). The transcription factor has been shown to specifically protect cancerous cells by suppressing the apoptotic pathways as well as increasing proliferation of these cells (Barkett and Gilmore, 1999; Pande and Ramos, 2005). A mutated IκBα gene found in Hodgkin's lymphoma was incapable of regulating NF-κB (Cabannes *et al.*, 1999). 3T3 cells in mice were found to have an enhanced tumorigenic potential when there was increased expression of p52 (Ciana *et al.*, 1997). The deletion of the ankyrin repeats in mice p100 showed increased gastric hyperplasia in these animals (Ishikawa *et al.*, 1997). Further evidence of NF-κB participation in oncogenesis has been shown with the blockage of tumour formation by NF-κB inhibition through the regulation of several proteins upstream of NF-κB (Pande and Ramos, 2005). NF-κB activation has been shown in almost all types of cancers in humans (Barkett and Gilmore, 1999; Pande and Ramos, 2005).

1.6 The role of NF-κB in HIV infection

The ability of HIV-1 to manipulate host signalling pathways has resulted in efficient viral replication and gene expression. Primary monocytes and myeloid cell lines

chronically infected with HIV-1 show elevated levels of NF-κB (Pande and Ramos, 2005). Ordinarily, this would trigger the expression of pro-inflammatory proteins and the immune system recognising and attacking the pathogen. HIV-1 has evolved to exploit this activation. Like many other viruses that induce NF-κB activity, HIV-1 contains NF-κB binding sites in the viral genome (Greene and Peterlin, 2002; Pahl, 1999). The activation of NF-κB in an infected cell due to viral infection or the host immune response results in enhanced HIV-1 replication. HIV-1 mediated NF-κB induction is mostly due to IKK activation but the exact mechanism remains unclear (Hiscott *et al.*, 2001). Although not essential for viral replication or gene expression, the absence of NF-κB slows growth rates of HIV-1 (Hiscott *et al.*, 2001).

The different subtypes of HIV-1 contain varying numbers of NF-κB binding sites in the promoter proximal region of the Long Terminal Repeat (LTR) of the viral genome. HIV-1 subtype B which is predominant in North America contains two NF-κB binding sites whereas the LTR of HIV-1 subtype C, the predominant subtype in Africa, contains three (Barkett and Gilmore, 1999; Hiscott *et al.*, 2001; Jeeninga *et al.*, 2000). Subtype C has higher LTR promoter activity than some HIV-1 subtypes (Jeeninga *et al.*, 2000), making it tempting to assume that there is a direct correlation between the number of NF-κB binding sites and rates of viral replication. However the control of viral replication by NF-κB is much more complex when transcription factors such as Sp-1 that act synergistically with NF-κB or compete for the same binding site (NFAT) are added to the mix (Hiscott *et al.*, 2001; Jeeninga *et al.*, 2000; Kinoshita *et al.*, 1997).

Several HIV-1 proteins play a part in the activation of NF-κB. HIV-1 Tat protein stimulates cytotoxicity and apoptosis by activating caspases and promoting the accumulation of reactive oxygen intermediates through NF-κB induction (Manna and Aggarwal, 2000). The HIV-1 accessory protein Vpr activates IL-8 expression in macrophages and T cells through an NF-κB dependent mechanism (Hiscott *et al.*, 2001; Roux *et al.*, 2000). The Nef protein may stimulate T cells through CD3 or CD28 to increase the NF-κB dependent secretion of IL-2, a known inducer of NF-κB. HIV-1 Nef protein would thereby enhance viral transcription and replication (Wang *et al.*, 2000). The HIV-1 envelope glycoprotein gp120 with its interaction with the CD4 receptor activates NF-κB via two different but closely related pathways, one through

the tyrosine kinase $p56^{lck}$ while the other works through phosphatidylinositol-3-kinase and IKK (Hiscott *et al.*, 2001).

1.7 The role of plant extracts in NF-κB inhibition

NF-κB has become an attractive target in the treatment of inflammation due to its influence on pro-inflammatory genes (D'Acquisto *et al.*, 2002). Inhibitors of NF-κB function in a number of different ways, some scavenge reactive oxygen intermediates, others hinder IκB or p100 degradation by affecting the 26S proteasome, while others still affect the transcriptional activity of NF-κB bound to DNA (Hehner *et al.*, 1998).

A number of groups have shown inhibition of NF-κB by certain plant extracts, especially those rich in sesquiterpene lactones. The first study on extracts from medicinal plants as a source of NF-κB inhibition focused on plants native to Mexico (Bork *et al.*, 1997). The study found that the extracts negatively interfered with NF-κB activation. The sesquiterpene lactones in the extract were subsequently inhibited by the addition of cystein and a loss of inhibitory activity on NF-κB was found, suggesting that the inhibition of NF-κB was due to the sesquiterpene lactones found in the extracts (Bork *et al.*, 1997). A further study found that one particular compound, parthenolide, had a strong inhibitory effect on NF-κB in Jurkat (human T cell leukaemia), L929 (mouse fibroblast) and HeLa cell lines (Hehner *et al.*, 1998). This sesquiterpene lactone was also shown to have a proapoptotic effect on cancer cells (Wen *et al.*, 2002) and reduce inflammation in a mouse model of artherosclerosis (Lopez-Franco *et al.*, 2006). Parthenolide has become the model compound for studying NF-κB inhibition by sesquiterpene lactones and an attractive possible pharmaceutical (Bremner and Heinrich, 2005). The mechanism of action of the sesquiterpene lactones is a murky one with one group stating that sesquiterpene lactones interfered with the induced degradation of IκB-α and IκB-β (Hehner *et al.*, 1998), while another finding that a sesquiterpene lactone selectively modifies the p65 subunit, thereby inhibiting the NF-κB signalling cascade (Lyss *et al.*, 1998).

Flavonoids have also shown an inhibitory effect on NF-κB. Green tea flavonoids as well as resveratrol found in red wine inhibit the action of IKK, thereby reducing the induction of NF-κB (D'Acquisto *et al.*, 2002; Holmes-McNary and Baldwin, 2000; Nomura *et al.*, 2000). Tea flavonoids that have been shown to have an effect on NF-

κB include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) (Wiseman *et al.*, 2001). Epigallocatechin-3-gallate lowered NF-κB levels in both normal human epidermal keratinocytes and A431 cancer cells (Ahmad *et al.*, 2000; Wiseman *et al.*, 2001). Decreased binding of NF-κB to DNA was seen in RAW264.7 macrophages treated with EGCG, in addition to the inhibition LPS-induced lethality in mice by reducing the activation of NF-κB (Wiseman *et al.*, 2001; Yang *et al.*, 1998).

2 OBJECTIVES

The aims of this thesis were:

- to measure NF-κB levels in a cellular model of HIV-associated cardiomyopathy, established using human stem cell-derived cardiomyocytes challenged with purified gp120, cell-free HIV-1 or HIV-1 infected macrophages, and
- to determine whether certain natural compounds derived from South African indigenous plants could downregulate or restore NF-κB activation in this model of HIVCM.

3 MATERIALS AND METHODS

3.1 Propagation and Maintenance of cell lines

The human epithelial cell line 293T (American Type Culture Collection, USA) and HeLa cells (Sigma, Germany) were propagated and maintained in DMEM (Lonza, USA), supplemented with 10% heat inactivated FCS (Sigma, Germany) and 50 mg/L gentamicin sulphate (Highveld Biological, RSA). The human cord-blood stem cell-derived cardiomyocytes (Celprogen, USA) were cultured in Human Cardiomyocyte Expansion Media (Celprogen, USA), a proprietary media containing serum, growth factors and antibiotics. All three cell lines grew optimally as monolayers at 37°C in a humidified incubator with 5% CO_2.

3.1.1 Cell count

Cells were counted using the Trypan blue exclusion method. Cells were washed with PBS pH 7.2 (Lonza, USA), treated with 0.25% trypsin, 0.02% Versene (EDTA)

(Lonza, USA) and the clumps removed by gentle pipetting. A 1:10 dilution of the cell suspension was made in 0.4% Trypan blue (Sigma, Germany) and loaded into a haemocytometer. Dead cells take up the dye and appear blue while live cells do not and therefore remain clear. The number of live cells per millilitre of media was obtained by multiplying the average number of live cells per haemocytometer square by 10^5.

3.1.2 Subculture of cell lines

All cell lines were subcultured as per normal tissue culture practises (Phelan, 2003). To detach cells from culture flasks, the monolayers were washed with room temperature PBS pH 7.2 and treated with 0.25% trypsin, 0.02% Versene (EDTA) for 2-5 minutes at 37 °C. Trypsinisation was stopped by adding Human Cardiomyocyte expansion media or 10% FCS supplemented DMEM pre-warmed to 37 °C and cell clumps dispersed by careful pipetting. The cells were pelleted at 56 × *g* for 5 minutes and re-seeded in the supplemented culture medium at the required cell density. Cells were not cultured beyond passage 20 as cells lost their phenotype with increasing passages. Cells were counted regularly and subcultured to maintain logarithmic expansion of cultures.

3.1.3 Freezing of cell lines

Cells no later than passage 6 were frozen to build a stock of cells. Cultures in the logarithmic phase of growth were trypsinised and centrifuged as in 3.1.2 and resuspended in 46.25% fresh medium, 46.25% conditioned propagation media, 7.5% DMSO (Sigma, Germany) at a density of 1 × 10^6-10^7 cells/ml. These cells were quickly aliquoted into 2 ml cryogenic vials (Corning, USA), frozen gradually (-1 °C per minute) and stored at -80 °C. To thaw the cells, the vial was quickly heated by rubbing in hands. The cells were transferred dropwise to 10 ml Human Cardiomyocyte expansion media or FCS supplemented DMEM pre-warmed to 37 °C in a sterile 50 ml Falcon tube (Corning, USA) and pelleted at 56 × *g* for 5 minutes. The cell pellet was resuspended in the propagation medium and seeded as required in a tissue culture flask.

3.2 Expression and purification of gp120

The mammalian 293T cell line was used to express gp120. These cells were transfected with plasmids containing the HIV-1 gp120 inserts from the CAP 45 or Du151 HIV-1 strains (National Institute of Communicable Diseases, RSA). The transfection was carried out using the ProFection® Mammalian Transfection System-Calcium Phosphate Kit (Promega, USA) once the monolayer of cells in a 150cm^2 cell culture flask (Corning, USA) reached 30-60% confluency. This kit was used to maximise the tranfection of cells with the plasmid, and thereby maximise protein expression. Three hours prior to transfection the medium in the flasks was replaced with fresh 2% FCS supplemented DMEM pre-warmed to 37 °C and the transfection reagents brought to room temperature. Sterile, deionised water was added to 10-20 µg plasmid DNA and 62 µl 2 M calcium chloride to a final volume of 500 µl, which was added dropwise to 500 µl 2 × HBS while gently vortexing. After incubating at room temperature for 30 minutes, the solution was added to the 293T cells and incubated at 37 °C in a humidified incubator with 5% CO_2.

The supernatant was harvested two days post-infection and purified using a *Galanthus nivalus* lectin-agarose column (Sigma, Germany) as previously described (Nkosi *et al.*, 2005). This column has affinity for glycosylated proteins and isolates gp120 form the supernatant. The supernatant was passed through the column then sequentially washed with 5 column volumes of 0.65 M sodium chloride (Sigma, Germany) in PBS pH 7.4 (Sigma, Germany), 1 column volume of PBS pH 7.4, 1 column volume of 1 M sodium chloride in PBS, and 1 column volume of PBS. Samples were eluted with 3 ml 1 M methyl-α-D-mannopyranoside (Sigma, Germany), dialyzed against PBS at 4 °C for 16 hours and an additional 2 hours using the 3.5 kDa MWCO Slide-A-Lyzer® dialysis cassette (Thermo Scientific, USA), and concentrated using a 30 kDa MWCO Amicon® Ultra-15 centrifugal filter devices (Millipore, USA).

3.3 Detection and quantification of 293T-expressed gp120

3.3.1 SDS-PAGE detection of gp120

The column flow through and purified gp120 were separated using denaturing 8% acrylamide gels using standard formulations for the gel and all buffers. Samples were diluted in sample buffer and heated for 5 minutes at 95 °C. After loading, samples were electrophoresed for 45 minutes at 200 V using a Mini-PROTEAN® 3 Cell

(BioRad, USA). The gels were either prepared for Western Blotting or stained with Coomassie Brilliant Blue solution [0.25% Coomassie Brilliant blue R250 (Merck, Germany), 50% methanol (Sigma, Germany), 10% acetic acid (Sigma, Germany)] for 30 minutes and destained (20% methanol, 10% acetic acid) until all background was removed.

3.3.2 Western Blot detection of gp120

Resolved bands on the SDS-PAGE gels were transferred electrophoretically to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, USA) in transfer buffer (25mM Tris base (Merck, Germany), 192 mM glycine (Merck, Germany), 20% methanol) at 90 mA, 4 °C for 16 hours using the Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad, USA). The membrane was washed in PBS with Tween® 20 pH 7.4 (Merck, Germany), blocked for an hour with 5% (w/v) fat free milk powder in PBS-Tween 20 and probed with a 1:15000 dilution of human serum from HIV-1 positive patients in 2.5% (w/v) fat free milk powder in PBS-Tween. The serum from HIV-1 positive patients contained high levels of antibodies against HIV-1 gp120. The membrane was washed thrice with PBS-Tween 20 for 5 minutes, and reblocked in 10% (w/v) milk powder in PBS-Tween 20. The anti-human HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA) was diluted 1:20000 in 2.5% (w/v) fat free milk powder in PBS-Tween 20. The membrane was probed with this diluted antibody for an hour at room temperature. The membrane was washed three times as above and detected using ECL Advance™ Western Blotting Detection kit (GE Healthcare, USA). First, equal parts of solution A and solution B were mixed. This was poured over the membrane and allowed to incubate at room temperature for a minute. Excess detection reagent was removed and the membrane wrapped in cling film. This was exposed to Amersham Hyperfilm ECL X-ray film (GE Healthcare, USA).

3.3.3 Quantification of purified gp120 by the bicinchoninic acid protein assay

Purified gp120 was quantified using the bicinchoninic acid protein assay [Pierce® BCA Protein Assay kit (Thermo Scientific, USA)]. This assay allows for the colorimetric detection and quantification of total protein content (Smith ***et al.***, 1985) even in samples that contain detergents, which are included in most protein extraction buffers. A standard dilution series (0.025 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5

mg/ml, 0.75 mg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml) was set up using the albumin standard. The BCA Working Reagent, which contains cupric sulphate and BCA, was prepared (50:1 Reagent A: Reagent B) and added to the standard dilutions and samples of unknown concentration in a 1:20 ratio. This was heated to 37 °C for 30 minutes, cooled to room temperature and the absorbance measured at 562 nm using the NanoDrop® ND-100 spectrophotometer (Thermo Scientific, USA). In the alkaline medium provided by the BCA Working Reagent, peptide bonds and the amino acids cysteine, tryptophan and tyrosine reduce the Cu^{2+} of cupric sulphate to Cu^{1+} (Wiechelman *et al.*, 1988). This ion chelates two BCA molecules, producing the purple colour measured at 562 nm. A standard curve was prepared from the albumin standards and the concentrations for the unknown samples determined from the graph.

3.4 Validation of HIV-1_{Du151} gp120 biological activity

The HIV-1_{Du151} gp120 expressed in 293T cells needed to be assayed for biological activity before use in further experiments. The binding of the HIV-1 gp120 monoclonal antibody IgG1 b12 to the expressed protein was employed to determine whether the protein was biologically active, as this antibody has been mapped to the CD4 binding site. The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas. The binding of expressed protein to IgG1 b12 was monitored using surface plasmon resonance measured by the BIAcore® 3000 (GE Healthcare, USA).

3.4.1 Immobilisation of HIV-1_{Du151} gp120 to sensor surface

The expressed HIV-1_{Du151} gp120 was bound to a CM5 sensor chip by amine coupling, using the BIAcore® amine coupling kit (GE Healthcare, USA) (Brigham-Burke *et al.*, 1992; Karlsson *et al.*, 1991). Equal amounts of 1 M N-hydroxysuccinimide (NHS) (GE Healthcare, USA) and 1 M N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC) (GE Healthcare, USA) were mixed and injected over a flow cell of the carboxymethylated dextran surface of the sensor chip (GE Healthcare, USA) to activate it. The HIV-1_{Du151} gp120 (20 µg/ml in 10 mM sodium acetate (Sigma, Germany) pH 4.7) was injected over the activated flow cell. This was followed by injection of 1 M ethanolamine-hydrochloride pH 8.5 (GE Healthcare, USA) to block remaining amino-ester groups. Finally, 10 mM glycine-HCl (Merck, Germany) pH

2.5 was injected over the flow cell to remove noncovalently bound protein. The flow rate for all BIAcore® experiments was set at 5 µl/min.

3.4.2 Interaction between immobilised gp120 and IgG1 b12 antibody

The IgG1 b12 antibody (50 µl, 14 µg/ml in 10 mM sodium acetate pH 4.7) was injected over the gp120-coupled flow cell. The flow cell was regenerated by passing 10 mM glycine-HCl pH 2.5 to remove the antibody from the coupled protein. The ligand buffer (10 mM sodium acetate pH 4.7) was injected over the protein-coupled flow cell to serve as a negative control. The baseline was set immediately before injection of antibody or buffer to determine the number of response units bound after injection relative to the baseline.

3.5 Isolation of human peripheral blood mononuclear cells from buffy coats and cultivation of human macrophages by monocyte differentiation

Human buffy coats were obtained from the Gauteng Blood Transfusion Services. Buffy coats are the residual material from donated blood after plasma, platelets, and red cell separation for patient use. Peripheral blood mononuclear cells (PBMC) were isolated form buffy coats as previously described (Khati *et al.*, 2003). Buffy coats were layered onto Ficoll-Paque™ PLUS (GE Healthcare, USA) and centrifuged at 900 × *g*, 20 ºC for 30 minutes. The serum layer was removed and heat inactivated at 56 ºC for 30 minutes to provide autologous serum. The PBMC layer was harvested, washed once with PBS pH 7.2 and recovered by centrifugation at 56 × *g*, 4 ºC for 5 minutes. The cell pellet was resuspended in 30 ml cold hypotonic ammonium chloride solution and incubated at room temperature for 20 minutes to allow for the lysis of residual red blood cells. The PBMC were pelleted by centrifugation and incubated in gelatine-coated tissue culture flasks containing RPMI medium (Sigma, Germany) with 5% autologous serum at 37 ºC, 5% CO_2 for 2 hours. Non-adherent cells were removed by washing with RPMI pre-warmed to 37 ºC and adherent monocytes incubated for an additional 16 hours. Monocytes were harvested by incubating the culture at 4 ºC for 30-45 minutes to allow for spontaneous detachment. The monocytes were re-seeded in X-VIVO 10 medium (Lonza, USA) containing 5% autologous serum. X-VIVO 10 is a conditioned media that allows for the differentiation of monocytes into macrophages in 7 to 10 days (Khati *et al.*, 2003).

3.6 Infection of monocyte-derived macrophages with HIV-1

Day 7 to 10 monocyte-derived macrophages (MDM) were infected with Du151 or CM9 strains of HIV-1 (National Institute of Communicable Diseases, RSA) or mock infected with PBS as previously described (Strober, 2004). The cells were exposed to 1×10^5 genome equivalents of the HIV-1 or PBS for 3 hours before washing with PBS and X-VIVO 10 supplemented with 5% autologous serum. The cells were maintained in X-VIVO 10 containing 5% autologous serum for 14 days, with half of the media being replaced on Day 7.

3.6.1 Determination of viral replication in MDM

Day 14 supernatant from the HIV-1 infection was tested for the presence of the HIV-1 protein p24 using the Vironostika® HIV-1 antigen Microelisa test (bioMérieux SA, France) according to the manufacturer's recommendations. The use of this kit to determine HIV-1 infection and growth rate is a standard protocol of the AIDS unit of the National Institute of Communicable Diseases where HIV-1 experiments were conducted. The kit contains a plate coated with an anti-HIV-1 p24 monoclonal antibody which captures the HIV-1 p24 protein. A standard dilution series and diluted culture supernatant were added to the wells and incubated at 37 ºC for 1 hour. The wells were washed three times with proprietary 1 × Wash Buffer and horseradish peroxidase-conjugated human anti-HIV-1 p24 antibody added. This was further incubated at 37 ºC for an hour and washed as above. Equal parts of the TMB and urea peroxidase solutions were premixed before addition to the wells. The absorbance was immediately measured at 650 nm as a kinetic reading using a Versa Max Pro microplate reader (MDS Analytical Technologies, USA).

3.7 Immunocytochemistry

HeLa cells or human cord-blood stem cell-derived cardiomyocytes were removed from their culture flasks by trypsinisation and incubated in 2-well chamber slides (Nunc, USA) overnight to allow cells to adhere and regain their morphology. Immunocytochemistry was performed as previously described (Baldwin, 1996; Watkins, 1989). After media was removed and cells washed in cold PBS pH 7.2, cells were simultaneously fixed and permeabilised by incubating in 2% paraformaldehyde (Merck, Germany), 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes on ice. Fixative was removed and cells washed in cold PBS pH 7.2. The fixed cells were

incubated first with primary and then fluorochrome-conjugated secondary antibody (Invitrogen, USA) for 1 hour at 4 °C, both of which were diluted in 1% serum to prevent nonspecific binding of antibody. Antibody incubations were followed by four PBS pH 7.2 washes of 5 minutes each. The upper structure of the chamber slide was removed and cells mounted using UltraCruz™ Mounting Medium (Santa Cruz Biotechnology, USA) containing DAPI for DNA counterstaining. The slides were subsequently visualized using the Olympus® BX41 System Fluorescent Microscope, together with the analySIS LifeScience® software (Wirsam Scientific and Precision equipment, RSA). This technique was used with two very different aims in mind. The first was to ensure that the cardiomyocytes retained their phenotype by staining for the cardiac specific protein troponin I (cTnI) by a monoclonal antibody (R&D Systems, USA). The second was to visualise the activation of NF-κB in HeLa cells and cardiomyocytes using either a mouse or rabbit monoclonal antibody raised against human p65 (Santa Cruz Biotechnology, USA), a subunit of the NF-κB p50/p65 heterodimer.

3.8 Cytotoxicity assays

Seven natural compounds derived from South African indigenous plants (Bioprospecting Group, CSIR Biosciences, RSA) were screened for cytotoxicity against HeLa cells, MDM and cardiomyocytes. These compounds were from three different classes of compounds; flavonoids, sesquiterpene lactones and a sesquiterpene (Table 1). Cells were seeded at a density of 2×10^4 cells per well in 96 microwell white optical bottom plates (Nunc, USA). HeLa cells and cardiomyocytes were allowed to adhere overnight, while monocytes were seeded and allowed to differentiate into macrophages in X-VIVO 10 containing 5% autologous serum over seven to ten days. The cells were incubated with 10-fold serial diluted (100 μM, 10 μM, 1 μM or 100 nM) plant compounds for 3 hours at 37 °C, 5% CO_2 with humidity. The experiments were conducted in triplicate, including cell controls, which were not treated. After the 3 hour incubation with the compounds, the cells were lysed and ATP levels measured as a measure of cell viability using the CellTiter-Glo® Luminescent cell viability assay (Promega, USA). This assay detects the number of viable cells in the culture as a function of ATP levels, as ATP levels correlate to cell viability. The CellTiter-Glo® Reagent lyses cells but prevents the degradation of ATP by ATPases. It then uses the bioluminescent luciferin-luciferase reaction to detect the

amount of ATP in the cell culture. The reagents were brought to room temperature before mixing the CellTiter-Glo® buffer with the CellTiter-Glo® substrate by gentle inversion to prevent the formation of bubbles. A volume of CellTiter-Glo® reagent equal to the media in the wells was added to the wells. This was incubated at room temperature to allow all cells to be lysed and the luminescent signal to stabilise. The luminescence measurement was acquired using the Biotek® FLx800™ Fluorescence microplate reader and the Gen 5™ Data Analysis software (Analytical and Diagnostic Products, RSA).

3.9 Quantitative measurement of NF-κB

3.9.1. Immunofluorescence

HeLa cells were seeded in sterile 96 microwell black optical bottom plates (Nunc, USA) at 1×10^4 cells per well and allowed to adhere overnight. Some cells were left unstimulated while others were simulated to release NF-κB by incubating with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 20 and 60 minutes at 37 °C, 5% CO_2 with humidity (Bork *et al.*, 1997). After medium was removed and cells washed in cold PBS, cells were simultaneously fixed and permeabilised by incubating in 2% paraformaldehyde (Merck, Germany), 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes on ice. Fixative was removed and cells washed in cold PBS pH 7.2. The fixed cells were incubated first with the p65 antibody (Santa Cruz Biotechnology, USA) and then fluorescein isothiocyanate-conjugated secondary antibody (Invitrogen, USA) for 1 hour at 4 °C, both of which were diluted in 1% serum. Each incubation of antibody was followed by four PBS washes of 5 minutes each. Secondary antibody staining only was used as a blank for background fluorescence. All conditions were carried out in triplicate. The plate was read at 520 nm on the Biotek® FLx800™ Fluorescence microplate reader and the Gen 5™ Data Analysis software (Analytical and Diagnostic Products, RSA).

3.9.2.1 Protein extraction

The whole cell lysate was obtained using the 5 × Reporter Lysis Buffer (Promega, USA). Lysis buffers which were freshly prepared were attempted but these yielded protein levels that were too low to be used in further experiments. Cells in 24-well plates (Corning, USA) were washed with room temperature PBS pH 7.2. The

Reporter Lysis Buffer was diluted five-fold and 125 µl 1 × Reporter Lysis Buffer was added to each well. The plate was incubated at room temperature with gentle rocking for 20 minutes. The plate was then frozen at -20 °C overnight. This was thawed to room temperature with gentle rocking for 30 minutes. The cells were scraped and centrifuged at 12 000 × *g* for 2 minutes at 4 °C. The supernatant, or whole cell lysate, was used undiluted in the ELISA-based NF-κB assay.

3.9.2.2 ELISA-based NF-κB assay

Previously electromobility shift assays have been used to show increases in NFκB. However, this used radioactivity and resulted in streaky gels that required densitometry to quantify these increases. In this thesis the TransAM™ NFκB Family Transcription Factor Assay kit (Active Motif, USA) was utilized to detect and quantify NF-κB. This assay is a modification on traditional enzyme-linked immunosorbent assays because it uses the NF-κB consensus sequence to immobilize NF-κB instead of the conventional capture antibody. This assay could have been prepared from scratch but would have been more expensive to purchase the individual components. Complete Binding Buffer (30 µl), which contained dithiothreitol and herring sperm DNA, was added to each DNA-coated well and 20 µl of the whole cell lysate added to this. The plate was sealed and incubated at room temperature with mild shaking for 1 hour. Each well was washed three times with 1 × Wash Buffer. The p65 antibody was diluted 1:1000 in 1 × Antibody Binding Buffer, 100 µl added to each well and the plate incubated at room temperature for 1 hour. After washing each well thrice with 1 × Wash Buffer, horseradish peroxidase-conjugated secondary antibody was diluted and incubated as above. The wells were washed thrice and 100 µl of the developing solution was added to each well at room temperature. This was incubated for 2-10 minutes protected from light until the solution turned a medium to dark blue. The reaction was stopped by adding 100 µl of the stop solution to each well. The absorbance was read within 5 minutes at 450 nm using the Biotek® ELx800™ Absorbance microplate reader and KC™ junior software (Analytical and Diagnostic Products, RSA).

3.10 Statistical Analysis

Statistical analyses (Normality and *t*-tests) were performed using the GraphPad Prism® software Version 3.02.

4 RESULTS

4.1 HIV-1 glycoprotein gp120 expression and validation

4.1.1 Expression, purification detection and quantification of gp120

Large scale expression (≥ 2 litres) of gp120 yielded approximately 0.4 mg/L and 0.2 mg/L of HIV-1_{Du151} and HIV-1_{CAP45} gp120 respectively as determined by the BCA assay. This yield was much lower than expected. After five months of poor yield and trouble shooting, a luminescence-based detection for mycoplasma enzymes found that the cells were contaminated with mycoplasma. This contamination caused a marked decrease in cell metabolism, thereby reducing transfection and expression efficiencies. New mycoplasma-free 293T cells were obtained, propagated and tranfected as described in section 3.2. The protein expressed using a new batch of 293T cells resulted in improved yield of about 2 mg per 200ml (equivalent to 10 mg/L) of HIV-1_{Du151} gp120 and 0.5 mg per 60 ml (approximately 8 mg/L) HIV-1_{CAP45} gp120 as measured by the BCA assay and confirmed by western blot (Figure 2). Lane 2 of the western blot contains the positive control HIV-1_{Ba-L} gp120 which runs at approximately 97 kDa on the blot and is therefore smaller than the 120 kDa protein expressed. This is because the positive control was expressed in insect cells and is only partially glycosylated, resulting in this protein being smaller than the fully glycosylated gp120 expressed in mammalian cells (Figure 2). Due to the amount of protein expressed, only HIV-1_{Du151} gp120 at a final concentration of 2 mg/ml was validated and used in subsequent experiments.

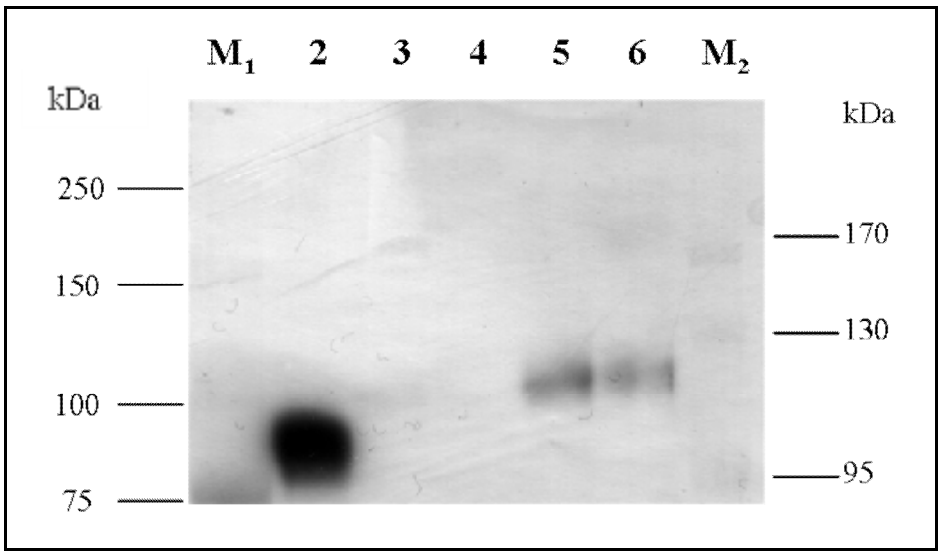


Figure 2: Western blot analysis of the lentil-lectin purified gp120. M_1 and M_2 represent two different molecular weight markers. Lane 2 contains 7 µg HIV-$1_{Ba\text{-}L}$ gp120, Lane 3 is flow-through from the lentil-lectin column of the HIV-1_{CAP45} gp120 transfection, Lane 4 contains flow-through from the HIV-1_{Du151} gp120 transfection, Lane 5 and 6 contain purified 4 µg HIV-1_{CAP45} gp120 and 4 µg HIV-1_{Du151} gp120 proteins respectively. The membrane was probed with serum from HIV-1 positive patients and anti-human HRP-conjugated secondary antibody, before detection of gp120-bound antibodies by chemiluminescence.

4.1.2 Validation of HIV-1_{Du151} gp120 biological activity

Following expression and purification, the functionality of HIV-1_{Du151} gp120 was validated by binding to IgG1 b12 using surface plasmon resonance technology. A total of 17500 RU of HIV-1_{Du151} gp120 was coupled to one flow cell of a CM5 sensor chip. The ligand buffer (10 mM sodium acetate pH 4.7) was used to dilute the antibody before passing it over the gp120-coupled flow cell. This buffer served as an excellent negative control as it showed how the buffer reacted with the protein. A small change of 120 RU was seen after the injection of the ligand buffer compared to the 3000 RU of IgG1 b12 bound (Figure 3). The sensogram curve of IgG1 b12 was typical of protein binding.

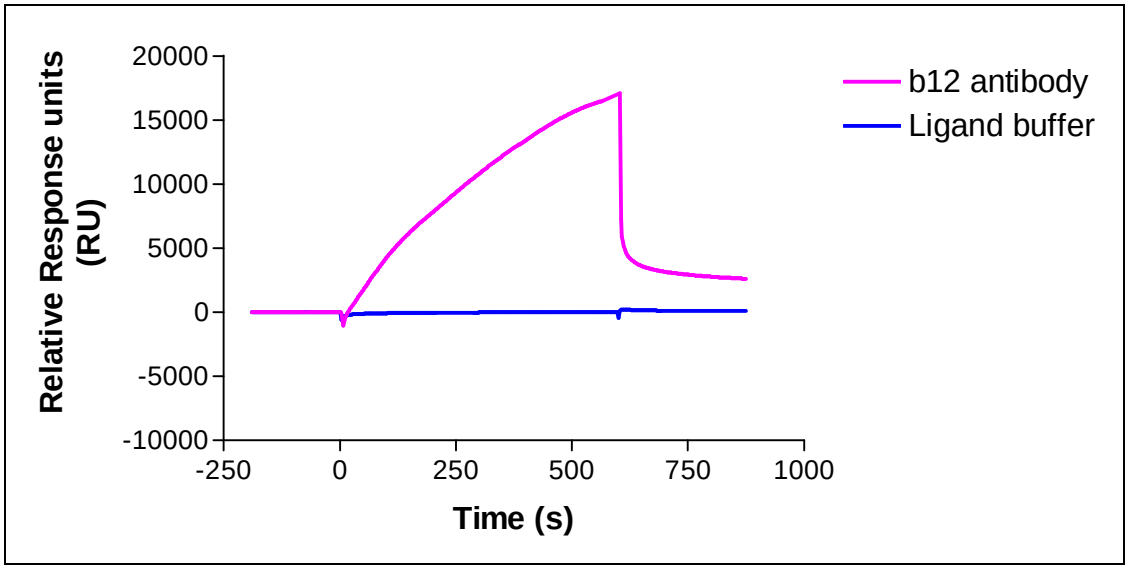


Figure 3: Sensogram showing the interaction between the anti-gp120 monoclonal antibody b12 and HIV-1_{Du151} gp120. The pink graph represents the binding of the b12 antibody to HIV-1_{Du151} gp120, the blue shows the ligand buffer passed over the gp120-coupled flow cell and corresponds to non-specific binding.

4.2 Culture and infection of monocyte-derived macrophages

4.2.1 Isolation of human peripheral blood mononuclear cells (PBMC) from buffy coats and cultivation of human macrophages by monocyte differentiation

Day 16 MDM were cultured and thereafter seeded in a chamber slide and bright field images obtained using Olympus® BX41 System Fluorescent Microscope, together with the analySIS LifeScience® software. The macrophages exhibited a heterogeneous population (Figure 4) and had strong adherence properties.

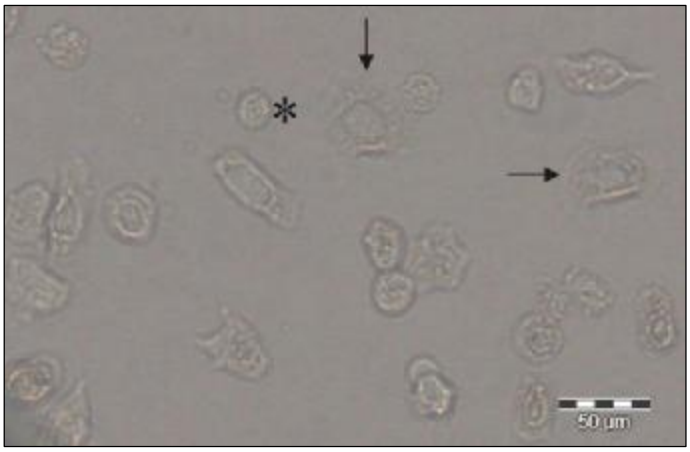


Figure 4: Light microscopy image of Day 16 monocyte-derived macrophages (Magnification, 100×). Different morphologies can be seen because MDM exhibit a diverse and heterogeneous population. The arrows indicate the fried egg-like shape typical of circulating macrophages and the asterisk shows a spherical macrophage.

4.2.2 Infection of MDM by Du151 and CM9 HIV-1 strains

Infection of MDM by HIV-1 was measured by the Vironostika® HIV-1 antigen kit. An increase of the viral protein p24 was used as a measure of viral replication. A standard curve was plotted after measuring the absorbance of the dilution series of the kit standard. Unfortunately the measurements for all infections on Day 14 were approximately 0 mg/ml, indicating that the macrophages were not infected with the two strains of HIV-1 used. It was also found that the viral stocks of HIV-1 $_{DU151}$ were no longer viable and needed to be re-established. Due to the amount of time this takes, a different viral strain HIV-1 $_{CM9}$ was used in subsequent experiments.

4.3 Culture and maintenance of cells lines

The HeLa cells or human cord-blood stem cell-cardiomyocytes were incubated in a chamber slide overnight and bright field images obtained using Olympus® BX41 System Fluorescent Microscope, together with the analySIS LifeScience® software. The HeLa cells rapidly proliferated with a high density (Figure 5A). The cardiomyocytes were spindle-shaped, as is typical of muscle cells (Figure 5B).

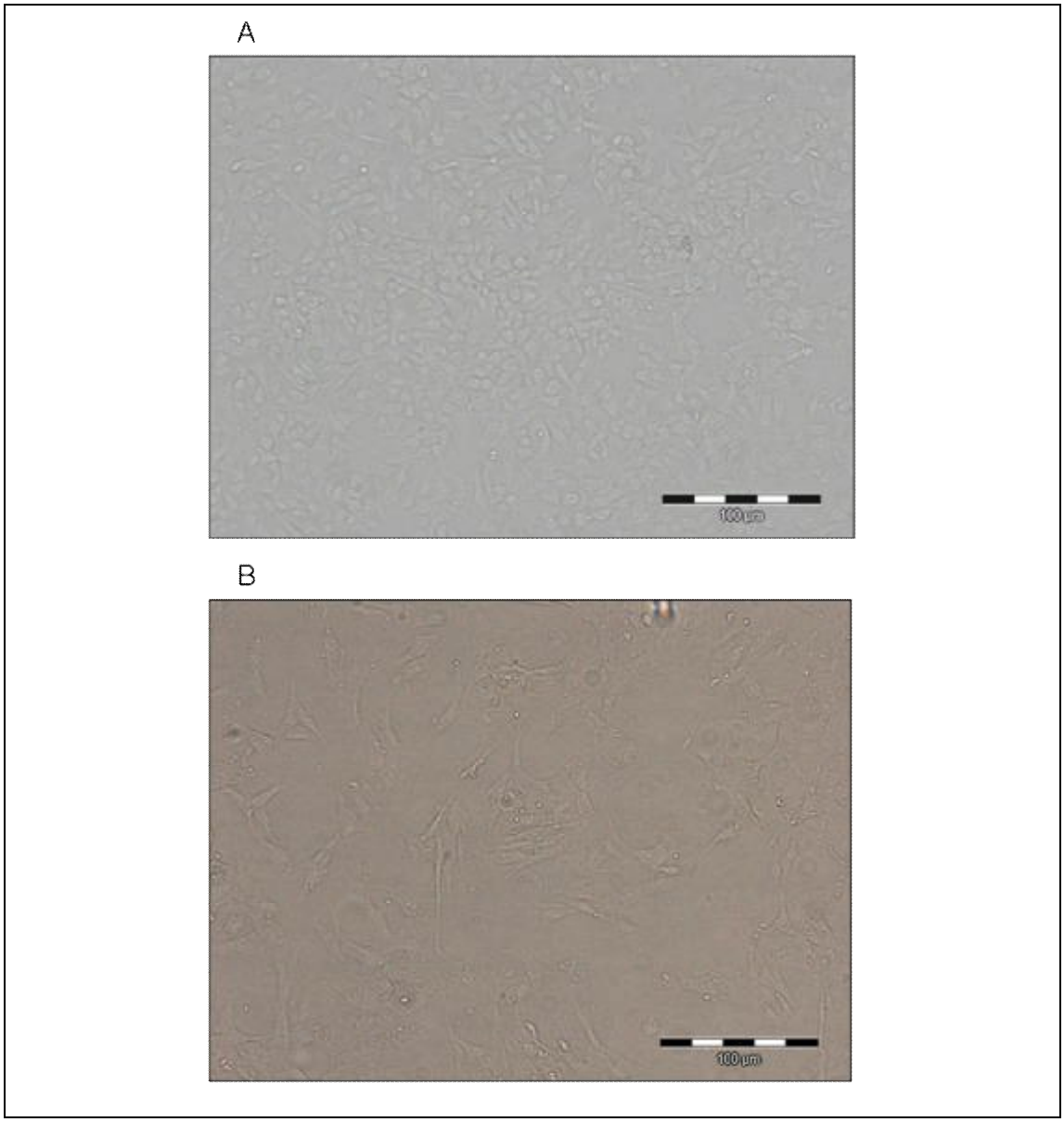


Figure 5: Light microscopy image of cultured cell lines; HeLa cells (*A*) or human cord-blood stem cell-derived cardiomyocytes (*B*). (Magnification, 100×)

The human cord-blood stem cell-derived cardiomyocytes were routinely tested to determine whether they maintained their cardiomyocyte phenotype. This was accomplished by immunocytochemistry, with staining for cTnI. Two negative controls were conducted, the first using HeLa cells to show specificity of the antibody to cardiomyocytes and the second using human cord-blood stem cell-derived cardiomyocytes stained with only rhodamine-conjugated secondary antibody (Invitrogen, USA) to determine whether the fluorochrome or secondary antibody showed any nonspecific binding (Figure 6 A and B respectively). The human cord-

blood stem cell-derived cardiomyocytes stained positive for cTnI (Figure 6C), confirming that these cells were cardiomyocytes.

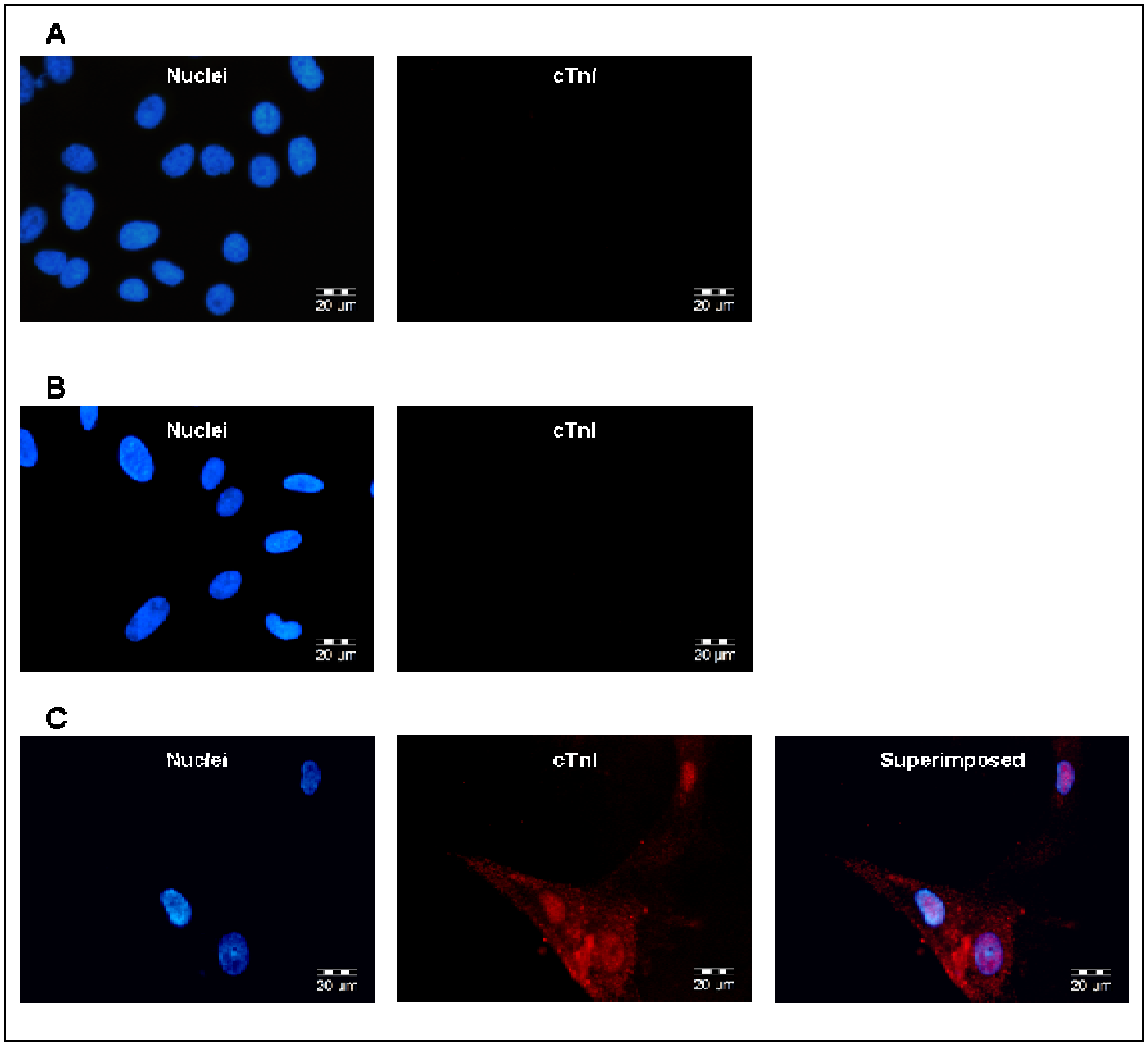


Figure 6: Phenotyping of cardiomyocytes using anti-cTnI. Fluorescence microscopy images of HeLa cells stained with anti-cTnI (*A*) as a negative control, human cord-blood stem cell-derived cardiomyocytes stained with secondary antibody only (*B*) as a second negative control, and with anti-cTnI (*C*). The red dye indicates the presence of cTnI and the blue the DAPI-stained nuclei. (Magnification, 500×)

4.4 Cytotoxicity of plant compounds

The plant compounds were diluted to a stock concentration of 2 mg/ml in DMSO and stored at -20 °C until use. The compounds were serially diluted in PBS pH 7.2 just before use. The serially diluted compounds were incubated with the respective cell lines to test their cytotoxicity. The compounds significantly reduced cell viability

(p<0.05) in very few cases; in HeLa cells with compounds 8B and 8C; and in MDM with compounds 8A and 10B. This significant reduction of cell viability was only seen with treatment at the highest concentration of 100 µM (Figure 7A and B respectively). However, a very significant decrease in cell viability was observed after incubation of the cardiomyocytes with compounds 106A and 38B at the highest concentration (p<0.001) (Figure 7C).

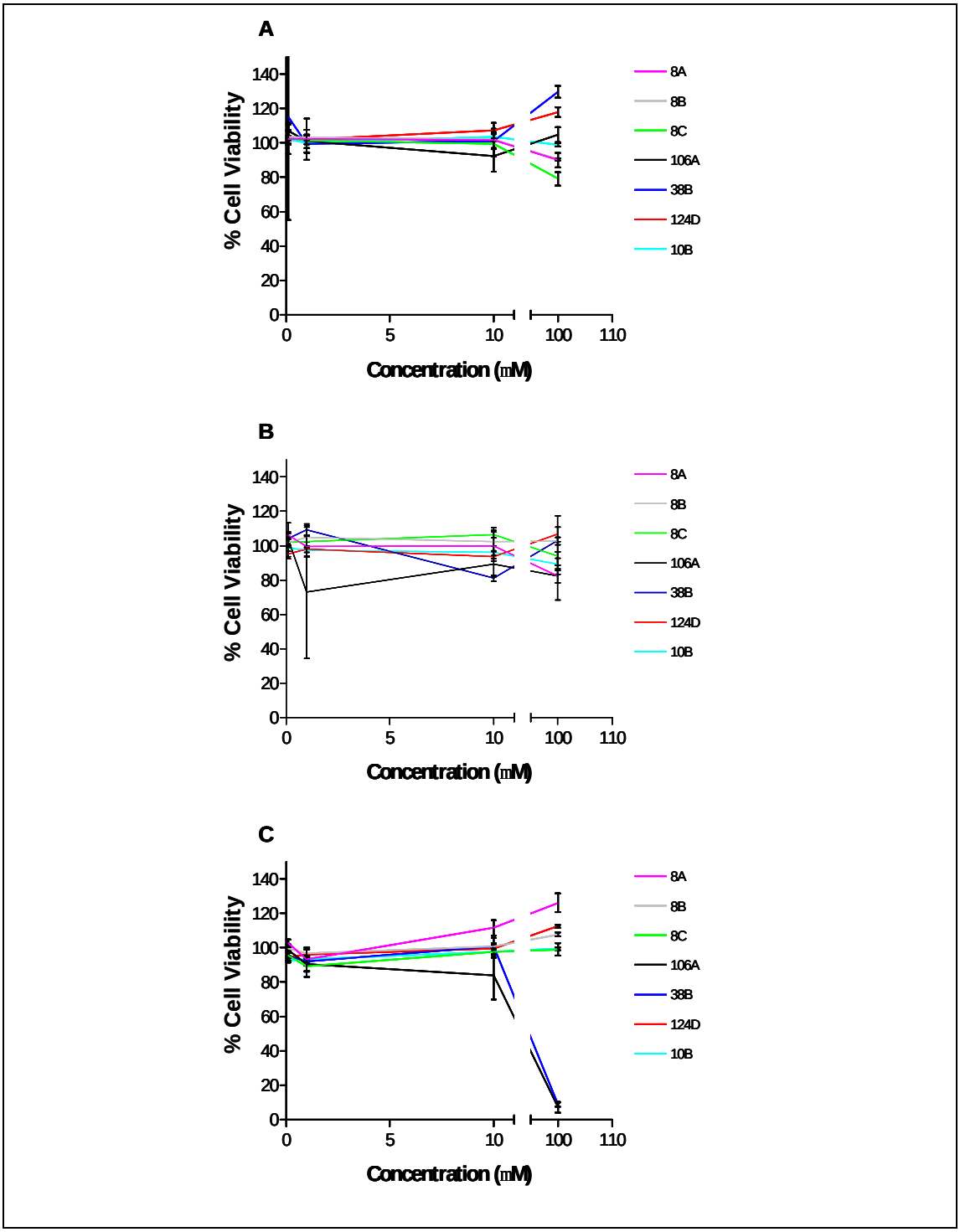


Figure 7: Cytotoxicity of plant compounds. Graphs showing the percentage cell viability after 3 hours of plant compound treatment of HeLa cells (*A*), MDM (*B*), and human cord-blood stem cell-derived cardiomyocytes (*C*).

4.5 Measurement of NF-κB

4.5.1 Immunocytochemistry

HeLa cells were used to illustrate the induction of NF-κB and the nuclear localisation thereof. This was to show the difference between resting cells and cells exhibiting constitutively activated NF-κB. Cells were stimulated with 50 ng/ml PMA for the stated time before staining with an anti-p65 mouse monoclonal antibody. This was visualised using a fluorescein isothiocyanate-conjugated secondary antibody (Invitrogen, USA) which emits a green fluorescence. Unstimulated HeLa cells exhibited low levels of NF-κB in their cytoplasm (Figure 8A). The incubation with PMA showed an increase in cytoplasmic NF-κB after 20 minutes (Figure 8B) and visible nuclear translocation after 60 minutes (Figure 8C).

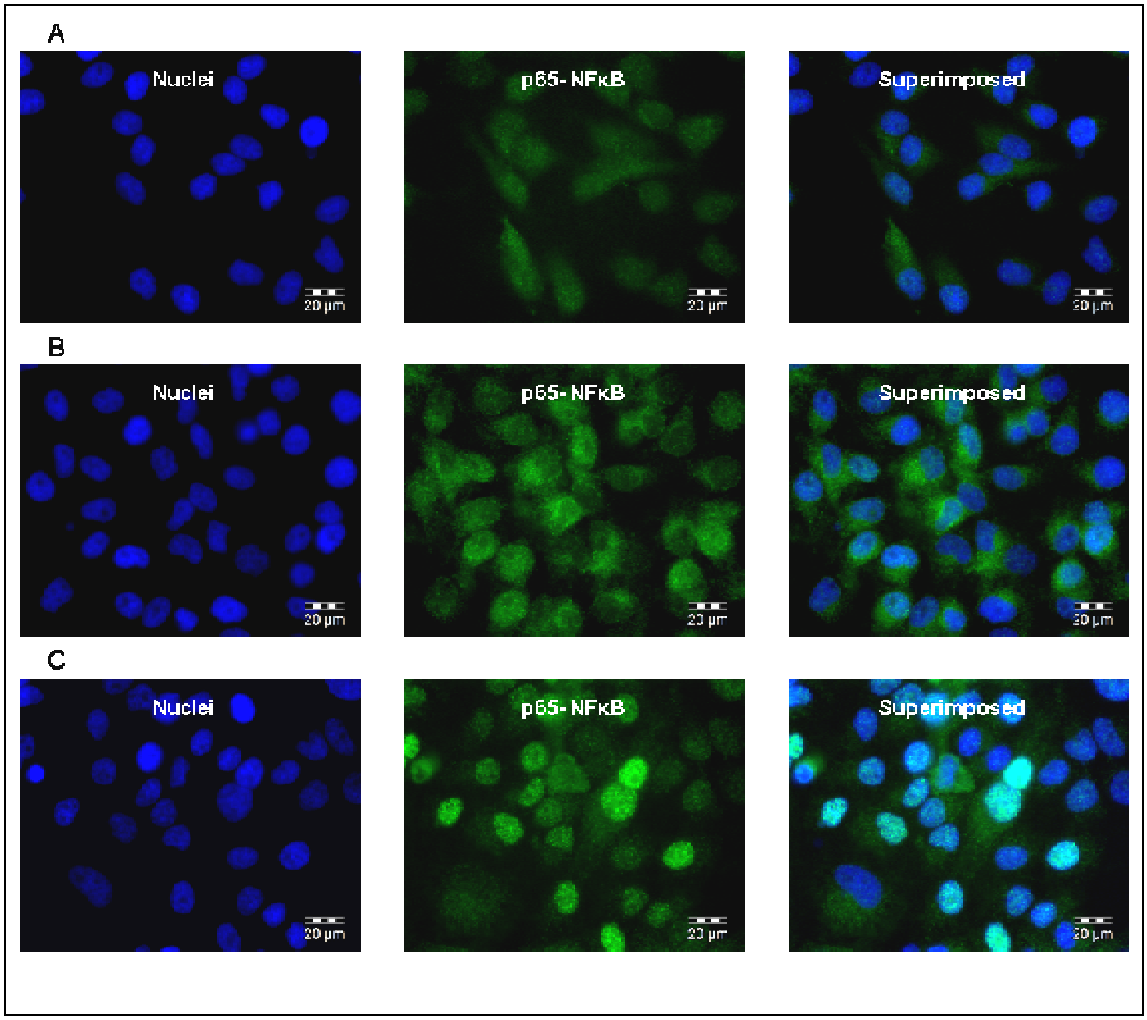


Figure 8: NF-κB induction and nuclear translocation in HeLa cells after PMA activation. Fluorescence microscopy images with green indicating NF-κB and blue the DAPI-stained nuclei. These images show untreated HeLa cells with low levels of NF-κB in the cytoplasm (*A*), cells stimulated with 50 ng/ml PMA for 20 minutes (*B*) clearly showing an increase in cytoplasmic NF-κB, and for 60 minutes (*C*), illustrating the movement of NF-κB to the nucleus. (Magnification, 500×)

4.5.2 Immunofluorescence

HeLa cells were cultured on a 96 well black optical bottom plate and stimulated to release NF-κB by incubating with 50 ng/ml PMA for 20 or 60 minutes or left untreated to show the difference between resting cells and those exhibiting activated NF-κB. Cells were stained for NF-κB with the p65 primary antibody and fluorescein isothiocyanate-conjugated secondary antibody before detection by the Fluorescence microplate reader (Relative fluorescence values can be found in the Appendix Figure 16). No significant difference between the resting and stimulated cells ($p>0.05$) could

be seen after measurement of fluorescence emitted (Figure 9). This may be due to the test not being sensitive enough to determine the difference between resting and stimulated levels of NF-κB or there being no significant difference between NF-κB levels in resting and stimulated cells. This could only be determined by using a different method to quantify NF-κB levels.

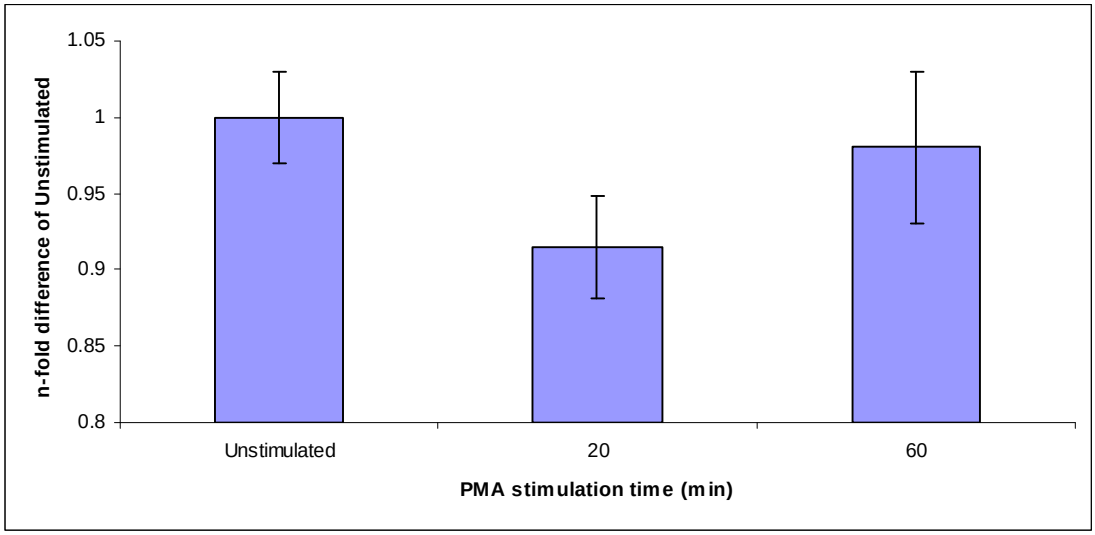


Figure 9: Quantification of NF-κB in resting and stimulated HeLa cells by immunofluorescence. NF-κB levels relative to unstimulated cells show no significant difference between unstimulated cells and those stimulated with PMA 20 and 60 minutes respectively ($p>0.05$).

4.5.3 ELISA-based NF-κB assay

HeLa cells were cultured on 24 well plates at the specified cell densities. Some of these cells were stimulated to release NF-κB by incubating with 50 ng/ml PMA for 60 minutes at 37 °C (Bork *et al.*, 1997). Cellular proteins were extracted (3.9.2.1) and this whole cell lysate was used in the TransAM™ NFκB Family Transcription Factor Assay kit. The Raji nuclear extract, a protein extract high in NF-κB, was used as a positive control to ensure that the kit was working optimally. A clear increase in the levels of NF-κB after PMA stimulation and a concentration dependent increase in NF-κB were observed (Figure 10). This demonstrates that the protocols for protein extraction and NF-κB quantification are compatible and effective for the measurement of the shifting NF-κB levels.

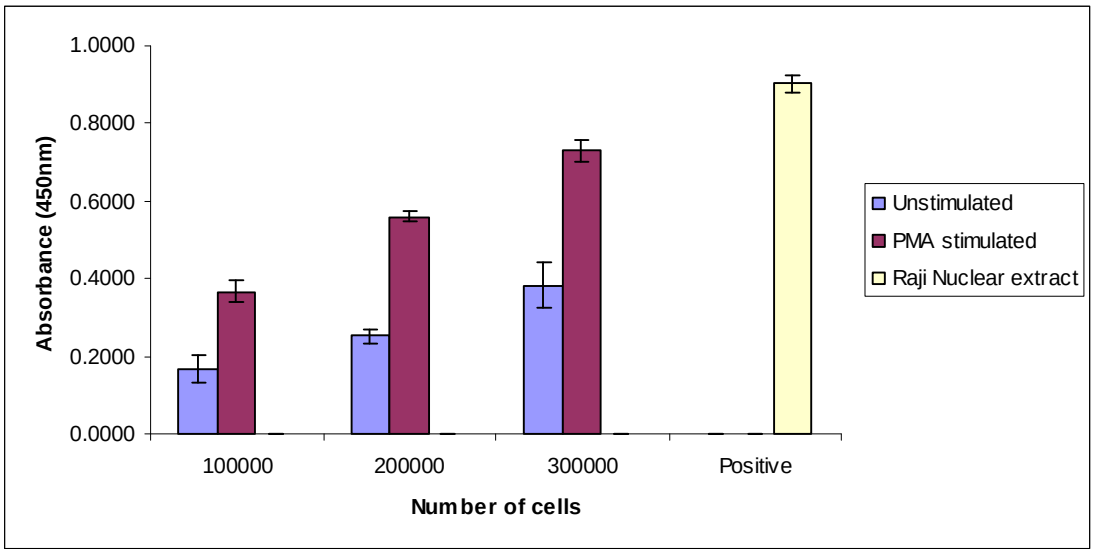


Figure 10: Quantification of NF-κB in resting and stimulated HeLa cells by ELISA-based assay. NFκB Family Transcription Factor Assay of HeLa cells showing an increase in the transcription factor after PMA stimulation as well as a cell concentration-dependant increase in NF-κB. All PMA stimulated were significantly different from their unstimulated counterparts ($p<0.05$).

4.6 Activity of plant compounds against NF-κB activation in HeLa cells

HeLa cells were seeded at 2×10^5 cells per well on a 24 well plate and allowed to adhere overnight. These were treated with 50 μM of each plant compound for 1 hour before stimulation with PMA for 60 minutes as previously described (Bork *et al.*, 1997). Controls for PMA stimulation only and unstimulated cells were also conducted. Cellular proteins were extracted and NF-κB measured using the TransAM™ NFκB Family Transcription Factor Assay as described above. The significant reduction in NF-κB that was clear with 106A and 38B ($p<0.05$ vs. unstimulated), both sesquiterpene lactones, was not evident with the flavonoids (8A, 8B and 8C) and the sesquiterpene (10B) (Figure 11). The sesquiterpene lactone 124D reduced NF-κB levels but this reduction was not significant. Absorbance values can be found in the Appendix (Figure 17).

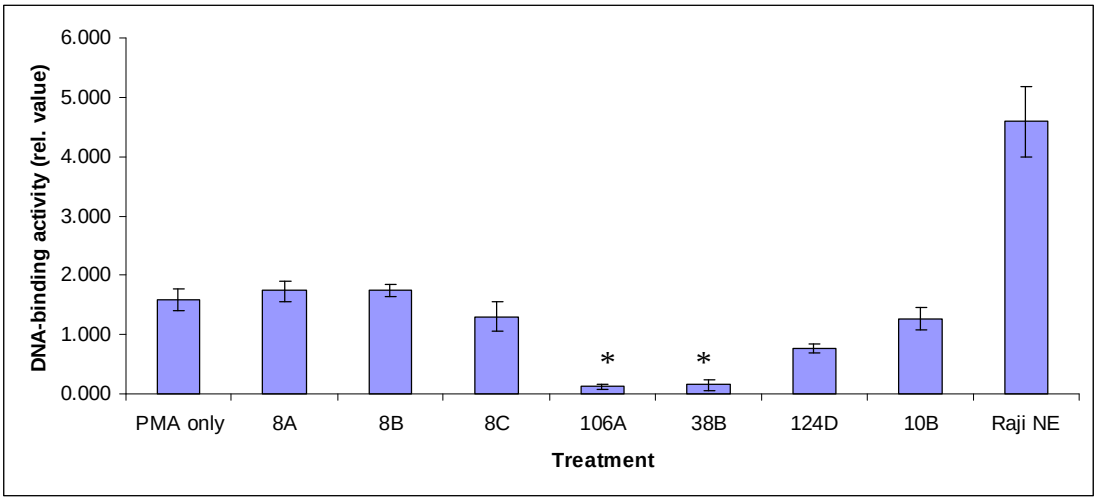


Figure 11: NF-κB DNA-binding levels in stimulated and plant compound treated HeLa cells. TransAM™ NFκB Family Transcription Factor Assay of HeLa cells showing NF-κB levels after treatment with plant compounds and stimulation with PMA. As in Figure 9 the Raji Nuclear extract was used as a positive control. (*significantly lowered, $p<0.05$)

4.7 Optimisation of NF-κB assay for gp120 stimulation of cardiomyocytes

Human cord-blood stem cell-derived cardiomyocytes were seeded at 3×10^5 cells per well on a 24 well plate and allowed to adhere overnight. These cells were treated with 4 µg/ml HIV-1_{Du151} gp120 for 2, 3 and 4 hours to induce NF-κB levels or were left untreated. Thereafter, cellular proteins were extracted and NF-κB measured using the TransAM™ NFκB Family Transcription Factor Assay as above. Absorbance values can be found in the Appendix (Figure 18). Stimulation with gp120 for two hours gave the best stimulation of 2.6-fold, although longer stimulation continued to show an elevation in NF-κB levels of at least two-fold (Figure 12). Only stimulation of cardiomyocytes with gp120 for two hours significantly raised NF-κB levels ($p<0.05$). This was used in future experiments with gp120 and HIV-1.

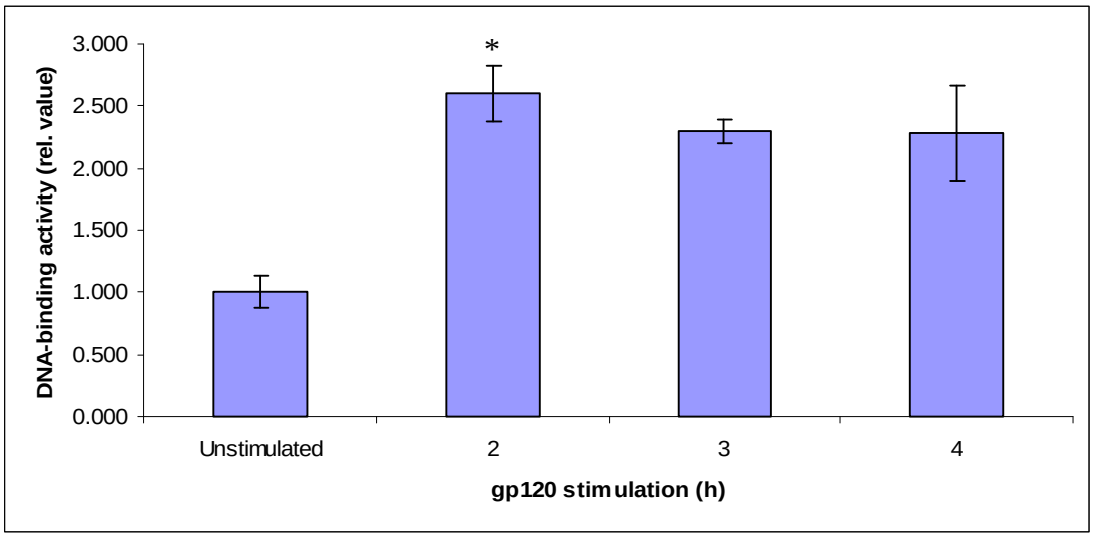


Figure 12: NF-κB DNA-binding levels in HIV-1_{Du151} gp120 treated cardiomyocytes. NFκB Family Transcription Factor Assay of human cord-blood stem cell-derived cardiomyocytes showing a stimulation of NF-κB levels after treatment with HIV-1_{Du151} gp120. Treatment with gp120 for two hours significantly elevated NF-κB levels as compared to unstimulated cells (* $p<0.05$).

4.8 Stimulation of NF-κB in cardiomyocytes by HIV-1 and gp120

Human cord-blood stem cell-derived cardiomyocytes were seeded at 3×10^5 cells per well on a 24 well plate and allowed to adhere overnight as above. These cells were treated with 4 μg/ml HIV-1_{Du151} gp120, HIV-1 CM9 (8 ng/ml of p24) or left untreated for two hours. Several controls were conducted: treatment with 10 ng/ml TNF-α for 30 minutes as a positive control for NF-κB stimulation (Osborn *et al.*, 1989); heat inactivation of gp120 to determine if denaturation affects NF-κB stimulation; and media from uninfected PBMC as a control for HIV-1 stimulation. Cellular proteins were extracted and NF-κB measured using the TransAM™ NFκB Family Transcription Factor Assay as before. All treatments elevated NF-κB levels compared to unstimulated cells (Figure 13). Treatments with TNF-α, HIV-1 and HIV-1 negative were significantly different from unstimulated cells ($p<0.05$), with gp120 resulted in higher NF-κB levels that were highly significant as compared to HIV-1 stimulation ($p<0.001$). Absorbance values can be found in the Appendix (Figure 19).

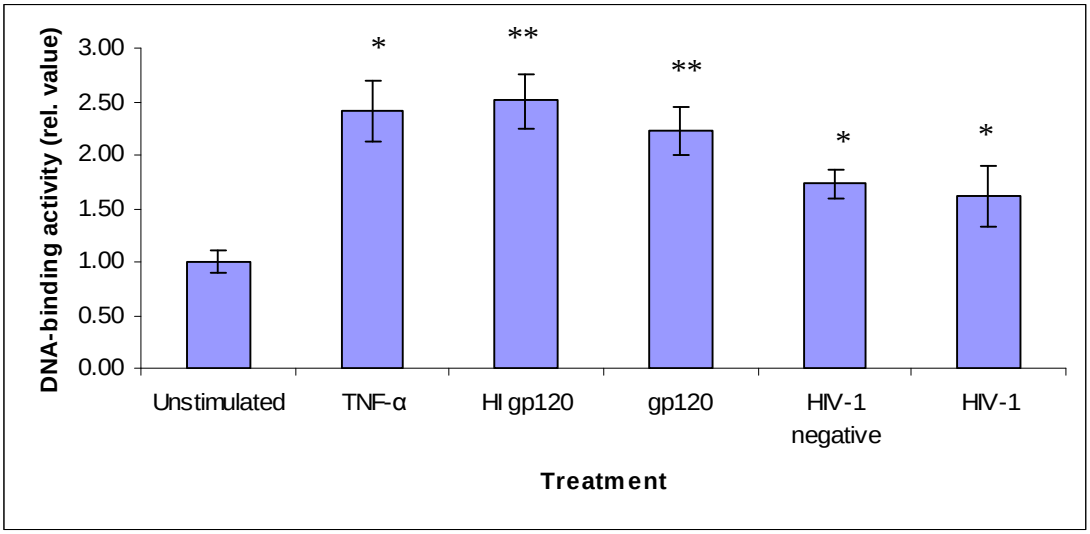
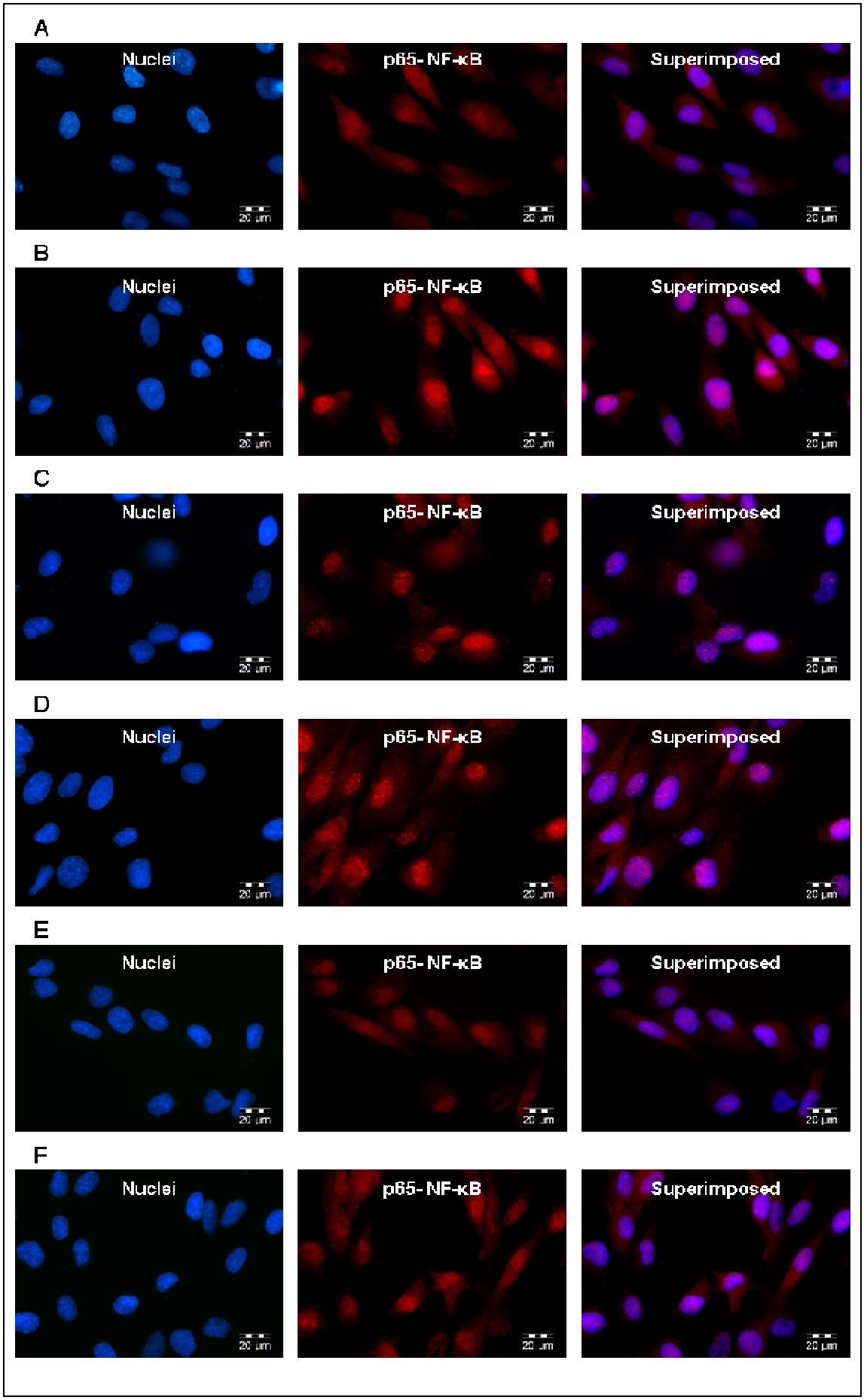


Figure 13: Quantification of NF-κB DNA-binding in cardiomyocytes by treatment with gp120 or HIV-1. NFκB Family Transcription Factor Assay of human cord-blood stem cell-derived cardiomyocytes showing a stimulation of NF-κB levels after treatment with HIV-1_{Du151} gp120 and HIV-1. All treatments including the controls for gp120 and HIV-1, as well as TNF-α, were significantly higher than unstimulated cells (* $p<0.05$; ** $p<0.001$).

These results were confirmed by immunocytochemistry. Human cord-blood stem cell-derived cardiomyocytes were seeded at 1.5×10^5 cells per well on a chamber slide and allowed to adhere overnight. These cardiomyocytes were treated with 4 µg/ml HIV-1_{Du151} gp120, HIV-1 CM9 (8 ng/ml of p24) or left untreated for two hours as above. Cells were fixed and stained as described earlier (3.7). A rabbit anti-p65 antibody was used as the primary antibody. This was visualised using an Alexa Fluor® 532-conjugated secondary antibody (Invitrogen, USA). Unstimulated cardiomyocytes exhibited low levels of NF-κB in their cytoplasm (Figure 14A). The incubation with TNF-α resulted in an increase in cytoplasmic and nuclear NF-κB after 30 minutes (Figure 14B). Both heat inactivated and functional gp120 show an increase in nuclear NF-κB (Figure 14C and D respectively), although functional gp120 shows higher cytoplasmic levels of the transcription factor than its heat inactivated counterpart. Treatment with HIV-1 negative media from PBMC and HIV-1 (Figure 14E and F

respectively) showed a marginal increase in nuclear NF-κB, with HIV-1 stimulation appearing brighter than HIV-1 negative PBMC media.


A
Nuclei
p65-NF-κB
Superimposed
B
Nuclei
p65-NF-κB
Superimposed
C
Nuclei
p65-NF-κB
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D
Nuclei
p65-NF-κB
Superimposed
E
Nuclei
p65-NF-κB
Superimposed
F
Nuclei
p65-NF-κB
Superimposed

Figure 14: Fluorescent images of NF-κB stimulation in cardiomyocytes by HIV-1_{Du151} gp120 and HIV-1. Fluorescence microscopy images of human cord-blood stem cell-derived cardiomyocytes stained with anti-p65. These images show untreated cardiomyocytes with low levels of NF-κB in the cytoplasm (*A*), cells stimulated with 10 ng/ml TNF-α for 30 minutes (*B*), cardiomyocytes treated with 4 μg/ml heat inactivated HIV-1_{Du151} gp120 (*C*), 4 μg/ml HIV-1_{Du151} gp120 (*D*), HIV-1 negative media (*E*), and HIV-1 (8 ng/ml of p24) (*F*). The red dye indicates the presence of the p65 subunit of NF-κB and the blue the DAPI-stained nuclei. Other than the TNF-α positive control, only stimulation with heat inactivated and functional gp120 clearly induced nuclear and cytoplasmic NF-κB. (Magnification, 500×).

4.9 Modulation of NF-κB by plant compounds in gp120-stimulated cardiomyocytes

Human cord-blood stem cell-derived cardiomyocytes were seeded at 3×10^5 cells per well on a 24 well plate and allowed to adhere overnight. Cardiomyocytes were pre-incubated with compounds 106A or 38B at the stated concentration in cell culture medium for one hour before washing these cells with PBS and treating with 4 μg/ml HIV-1_{Du151} gp120 for two hours (Hehner *et al.*, 1998). Controls were carried out by stimulation using gp120 without treatment by either sesquiterpene lactone, together with a control for resting cells. Cellular proteins were extracted and NF-κB measured using the TransAM™ NFκB Family Transcription Factor Assay. Treatment with 106A or 38B at the two stated concentrations in gp120 stimulated cells did not bring NF-κB levels back to those seen in unstimulated cells (Figure 15). However, treatment with 5 μM 106A caused a significant decrease in NF-κB levels as compared to gp120 only ($p<0.05$). Absorbance values can be found in the Appendix (Figure 20).

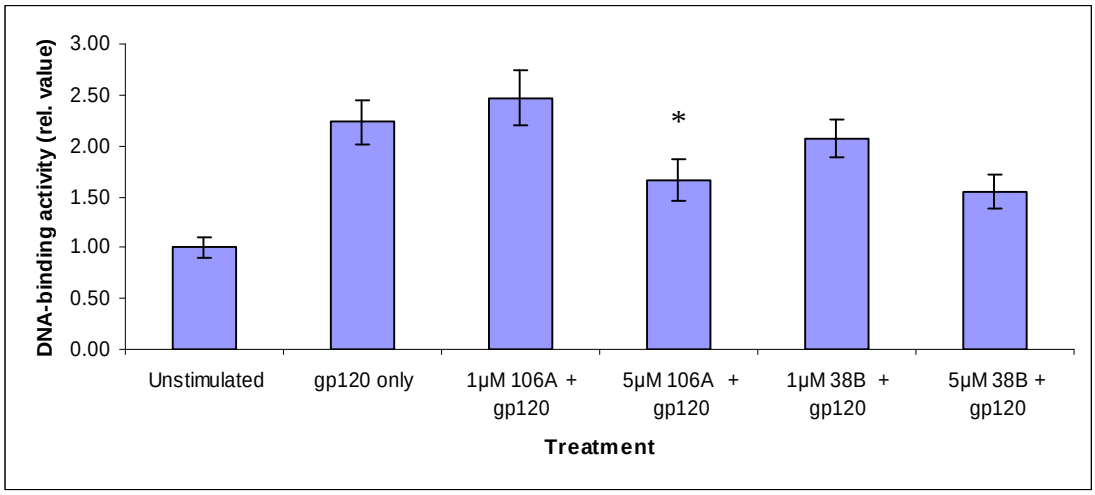

Figure 15: Mitigation of NF-κB levels in gp120-stimulated cardiomyocytes by sesquiterpene lactones. Downregulation of NF-κB DNA binding activity can be seen in both treatments at 5 µM but only treatment with 5 µM 106A significantly lowered NF-κB levels after gp120 stimulation (* $p<0.05$ vs. gp120 stimulation only).

5 DISCUSSION

5.1 HIV-1 glycoprotein gp120 expression and validation

The HIV-1 surface protein gp120 is a heavily glycosylated protein. This post-translational modification cannot be accomplished in the traditional *Escherichia coli* expression system (Gerstein, 2001). Even the baculovirus expression system, which uses the insect cell line Sf9, can only partially glycosylate the protein it produces (Fraser, 1992). Only mammalian cells appear to be able to process this protein as is seen in the infection of human $CD4^+$ cells (Gerstein, 2001). For this reason, as well as the permissibility of the cell line to transfection, the mammalian 293T cell line was used to express gp120. Once expressed and determined by western blotting that the correct protein was recovered (Figure 2), the glycoprotein needed to be validated. Surface plasmon resonance was the method chosen to do this as it shows the interaction of native proteins, with the advantage of this interaction being in real-time. The IgG1 b12 antibody was used in this experiment because it binds to the same site on the gp120 that CD4 does (Burton *et al.*, 1994). The CD4-gp120 interaction allows the virus to dock onto $CD4^+$ cells and is essential for viral entry into these cells. The IgG1 b12 antibody as it is a potent neutraliser of a broad range of HIV-1 isolates (Burton *et al.*, 1994) and recognises only functional gp120. The binding of this

antibody to the expressed HIV-1_{Du151} gp120 confirms that the gp120 is biologically active (Figure 3).

5.2 Measurement of NF-κB

Within the first year of the discovery of NF-κB as a novel transcription factor, it was found that this protein is inducible (Sen and Baltimore, 1986a). One of the first compounds found to induce the transcription factor was PMA (Sen and Baltimore, 1986a), which is still used as an NF-κB inducer today at the concentration first published by Sen and Baltimore. Tumour necrosis factor-α is also routinely employed to activate NF-κB levels (Pahl, 1999). Both of these inducers of NF-κB were used in this study to elevate NF-κB levels in the various experiments. The phorbol ester was used in the initial experiments to establish the methods. After comparison in HeLa cells (results not shown), TNF-α was found to elevate NF-κB to higher levels than PMA in a shorter space of time than the phorbol ester and was therefore used in the final experiments. Stimulation with PMA helped illustrate the activation of NF-κB in HeLa cells by immunocytochemistry, which initially resulted in a higher level of the transcription factor in the cytoplasm and appeared as brighter cytoplasmic fluorescence compared to unstimulated HeLa cells (Figure 8A and B). A longer incubation of HeLa cells with PMA showed nuclear translocation with fluorescence mostly in nuclei (Figure 8C). The immunofluorescence experiment was conducted using the same protocol as immunocytochemistry in a 96-well format and measured using a fluorometer. Unlike the clear results seen in the immunocytochemistry, the immunofluorescence test was not sensitive enough to determine the difference between resting and stimulated levels of NF-κB (Figure 9). This is possibly due to the additional washing steps which may affect the number of cells left on the plate, but the fixation and permeabilisation of the cells may also affect the readout. The TransAM™ NFκB Family Transcription Factor assay proved to be a better measure of NF-κB when it could be seen that PMA stimulation significantly raised NF-κB levels (Figure 10). The phorbol ester was also used to stimulate HeLa cells after treatment with the various plant compounds to determine their effect on NF-κB levels (Bork *et al.*, 1997). Only the plant compounds that fall into the sesquiterpene lactone category showed reduced NF-κB stimulation after incubation with PMA (Figure 11). The two sesquiterpene lactones that showed a significant decrease in NF-κB levels

after PMA stimulation (106A and 38B) were used in further experiments. In the final experiments TNF-α was used to show that the cardiomyocytes can be stimulated to release NF-κB (Figures 13, 14 and 15).

5.3 Stimulation of NF-κB in cardiomyocytes by HIV-1 and gp120

A number of groups have conducted studies on the effects of HIV-1 proteins on cardiomyocytes to determine the molecular mechanism of HIVCM (Fiala *et al.*, 2004; Kan *et al.*, 2000; Twu *et al.*, 2002). Most of these groups have had to rely on data from rat or rabbit cells due to a lack of a human cardiomyocyte cell line. Recently cord-blood stem cells were used to produce a human cardiomyocyte cell line, allowing for the testing of live human cardiomyocytes against HIV-1 and its proteins. Before conducting the experiments with HIV-1, the exposure time of the cardiomyocytes to gp120 needed to be optimised. This was to ensure that the exposure was long enough to possibly stimulate NF-κB but not cause cell death due to apoptosis (Fiala *et al.*, 2004; Twu *et al.*, 2002). Human cord-blood stem cell-derived cardiomyocytes were exposed to the expressed gp120 for two to four hours and NF-κB levels tested by the ELISA-based NF-κB assay. All time-points raised NF-κB levels to at least double that of unstimulated cardiomyocytes (Figure 12). The exposure time of two hours was chosen for gp120 and HIV-1 stimulation in future experiments as it was the only treatment that showed a significant elevation of NF-κB levels and correlates to gp120 studies on cardiomyocytes conducted by other groups (Kan *et al.*, 2000). The exposure of human cardiomyocytes to gp120 significantly increased NF-κB levels (Figures 12 and 13) as was shown previously in rat myocytes (Kan *et al.*, 2000). Heat inactivation of the protein showed almost identical results as native gp120 i.e. it did not lower the NF-κB response as compared to gp120 (Figure 13). However, immunocytochemistry of cardiomyocytes stimulated with the heat inactivated control and gp120 (Figure 14C and D respectively) showed that although the nuclear levels of the transcription factor may appear similar, there is a higher cytoplasmic component to the gp120 stimulation. This may simply mean that gp120 treatment of cardiomyocytes elicits a prolonged NF-κB response, one that may be shortened by heat inactivation of the viral protein. It is also possible that the protein remains in its native conformation due to inefficient heating of the protein as various temperatures and heating periods have been previously described (Barak *et al.*, 2002; Lee *et al.*, 2005; Zauli *et al.*, 1996). A common contaminant of protein expression is

the heat-stable *Escherichia coli* LPS (Cardoso *et al.*, 2007). It is unlikely that the gp120 stock was contaminated with LPS as a mammalian expression system was used and sterile techniques and endotoxin-free reagents employed. Incubation of HIV-1 isolate CM9 resulted in significantly elevated levels of NF-κB but this elevation was mirrored by its control of PBMC media (Figure 13). This makes it impossible to know whether any part of the elevation may be attributed to the virus. These conditions were repeated and tested using immunocytochemistry where the intensity of staining after HIV-1 stimulation was slightly higher than that seen after treatment with the PBMC medium (Figure 14E and F). Unfortunately, the software for the fluorescent microscope cannot distinguish whether there is a significant difference in the fluorescence of any two pictures. The elevation seen in the control of medium from uninfected PMBC was expected due to the presence of IL-2 (Hazan *et al.*, 1990) but it was hoped that the stimulation with HIV-1 would be higher than this. The stimulation with gp120 elicited a higher NF-κB response than stimulation with HIV-1. This is probably due to a higher concentration of gp120 in the incubation with the protein only as compared to the virus-associated protein in the HIV-1 stimulation which was quantified by p24 assay. Another consideration is that there are many more exposed epitopes on free gp120 than on the viral envelope and these epitopes may elicit a higher NF-κB response.

The choice of HIV-1 isolates used in this study was intentional. Both Du151 and CM9 strains are HIV-1 subtype C viruses, the predominant subtype found in Sub-Saharan Africa. These isolates are also both R5 viruses i.e. they use the CCR5 chemokine receptor to facilitate viral entry (Cilliers *et al.*, 2003; Williamson *et al.*, 2003). This allows for infection and replication of these viral strains in macrophages. However, it is always a challenge to replicate HIV-1 in macrophages *in vitro*, as was evident in this study. Had the infection and replication in macrophages worked, it could have provided data on the possible role of cytokines produced by HIV-1 infected macrophages on human cardiomyocytes. It may have also been possible to see whether IL-2 was solely responsible for the increase in NF-κB levels seen in the HIV-1 treatment of cardiomyocytes, as macrophages do not require IL-2 to aid infection.

5.4 Modulation of NF-κB by plant compounds in gp120-stimulated cardiomyocytes

A number of groups have shown the damaging effects of gp120 on cardiomyocytes. A study on the effects of gp120 on rat cardiac myocytes found that co-stimulation of these cells with gp120 and IL-1β raised nitric oxide levels significantly as compared to IL-1β stimulation alone (Kan *et al.*, 2000). The study also connected this nitric oxide elevation to p38-mediated stimulation of NF-κB. This group realised for the first time that gp120 has a direct effect on cytokine production in cardiomyocytes and proposed that the interaction between viral proteins and cytokines contribute to HIVCM. Another study found that gp120 inhibits the contraction of rabbit cardiomyocytes and their L-type Ca^{2+} current (Chen *et al.*, 2002). This group took note of the fact that nitric oxide has been shown to modulate Ca^{2+} currents in ventricular myocytes. This alludes to elevated nitric oxide levels causing the inhibition of contraction in gp120-stimulated cardiomyocytes. NF-κB may be the common element in all of these pathways and an NF-κB inhibitor that has shown efficacy in human cardiomyocytes against HIV-1 or gp120-induced stress could prove to be a valuable asset. Sesquiterpene lactones have been shown to be inhibitors of NF-κB activation by many studies (Bork *et al.*, 1997; Hehner *et al.*, 1998; Lopez-Franco *et al.*, 2006; Lyss *et al.*, 1998). These compounds have also been shown to have a high unspecific toxicity and to be particularly cardiotoxic in the 10^{-4} to 10^{-3} M range (Schmidt, 1999). The cytotoxicity assays conducted in this thesis is consistent with these observations, where the only cardiotoxic compounds were 106A and 38B, both sesquiterpene lactones (Figure 7C). These compounds were used at non-cardiotoxic concentrations to modulate the effect that gp120 and HIV-1 had on NF-κB levels in cardiomyocytes. These concentrations showed at least 80% cell viability in the cytotoxicity assays after a three hour incubation with the compound at the low micromolar range, a range that most sesquiterpene lactones show their bioactivity (Schmidt, 1999). An interesting note is that low concentrations of sesquiterpene lactones like those used in the present study may enhance cardiomyocyte contractility by increasing the amount of intracellular Ca^{2+} released on stimulation (Schmidt, 1999), thereby possibly negating the effect of gp120 on cardiomyocyte contractility. Of all the treatments conducted on gp120-stimulated cardiomyocytes in this thesis, only 5 μM 106A significantly lowered NF-κB levels as compared to gp120 stimulation alone (Figure 15). This did not bring NF-κB levels to within the range of

unstimulated cardiomyocytes but does show that the compound is effective against gp120 stimulation. The 106A compound was isolated from the *Vernonia staehelinoides* plant and was shown to have antiplasmodial activity *in vitro* but at a concentration that was toxic to mammalian cells (Pillay *et al.*, 2007). This plant has been reportedly used in traditional medicine but no particulars of its use have been detailed (Watt and Breyer-Brandwijk, 1932). A methanol extract from a different South African species of the same genus, *Vernonia stipulacea,* was found to stimulate the activity of the HIV-1 reverse transcriptase enzyme at a concentration of 100 μg/ml in an assay that measures activity of the expressed enzyme only and does not test the whole virus and infection (Bessong *et al.*, 2005). It is important to note that the crude extract of the plant was used in the study above while a pure compound was used in this thesis.

5.5 Future considerations

There needs to be a fine balance when it comes to the administration of sesquiterpene lactones in the clinical setting. A general administration, especially one at too high a dosage, could cause immunosuppression and lowered host defence (Ghosh *et al.*, 1998). This is further complicated by liver apoptosis if there is an elevation in TNF-α levels (Tak and Firestein, 2001), as is seen in patients with HIVCM (Barbaro *et al.*, 1999; Twu *et al.*, 2002). Oral absorption of sesquiterpene lactones has been shown to be incomplete (Schmidt, 1999), making it next to impossible to estimate the correct dosage to recommend. More advanced drug delivery systems will be needed to ensure only targeted cells receive the compound and at the correct dosage.

There are a number of issues related to this study that still need to be investigated. The effects of HIV-1 without the presence of IL-2 as well as HIV-infected macrophages on NF-κB levels in cardiomyocytes merits further study. These results could provide information on the possible role of cell-free HIV-1 and cytokines produced by HIV-1 infected macrophages on human cardiomyocytes. The elucidation of the membrane receptor that relays the presence of gp120 may also provide valuable insights into HIVCM and the signalling pathways involved. The 106A compound appears to be a candidate for further study on the modulation of cellular responses due to gp120 stimulation in human cardiomyocytes. A higher dosage or longer incubation time with this sesquiterpene lactone may lower NF-κB levels to within the range of

resting cells. Further study on the effect of gp120 and HIV-1 on nitric oxide levels and contractility of human cord-blood stem cell-derived cardiomyocytes could provide answers to the molecular mechanism of HIVCM. The effect of the 106A compound on these parameters may also provide a much needed reduction in the damage of cardiomyocytes seen in HIVCM.

6 CONCLUSION

The HIV-1 surface glycoprotein gp120 elevated NF-κB levels in human cord-blood stem cell-derived cardiomyocytes by more than 2-fold compared to unstimulated control cells. This suggests that the damage to cardiomyocytes caused by gp120 in HIVCM is mediated by upregulation of the NF-κB transcription factor.

The sesquiterpene lactone 106A, at a concentration of 5 μM, significantly lowered the NF-κB response due to gp120 stimulation in human cord-blood stem cell-derived cardiomyocytes. The 106A compound could prove valuable in further studies on the modulation of cellular responses due to HIV-1 or gp120 induced stress in human cardiomyocytes.

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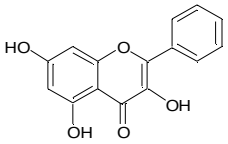
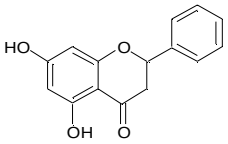
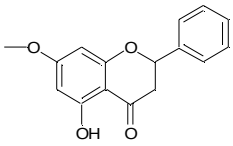
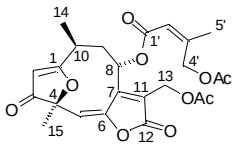
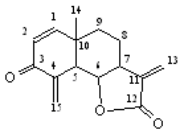
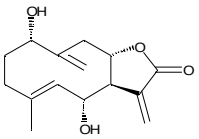
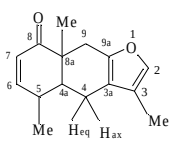
8 APPENDIX

Table 1: Panel of natural compounds derived from South African indigenous plant

Sample number	Structure	Class of compound	Origin
8A		Flavonoid	*Helichrysum aureonitens*
8B		Flavonoid	*Helichrysum aureonitens*
8C		Flavonoid	*Helichrysum aureonitens*
106A		Sesquiterpene lactone	*Vernonia staehelinoides*
38B		Sesquiterpene lactone	*Brachylaena transvaalensis*
124D		Sesquiterpene lactone	*Oneosiphon piluliferum*
10B		Sesquiterpene	*Siphonochilus aethiopicus*

SDS PAGE gel and buffer formulations

8% Resolving gel

40% Acrylamide / Bis-acrylamide (19:1)	2 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
20% (w/v) SDS	0.05 ml
10% (w/v) Ammonium persulphate	0.1 ml
Tetramethylethylenediamine	0.01 ml
H_20	5.34 ml

4% Stacking gel

40% Acrylamide / Bis-acrylamide (19:1)	0.5 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
10% (w/v) Ammonium persulphate	0.05 ml
Tetramethylethylenediamine	0.005 ml
H_20	3.17 ml

1× SDS Running buffer

25 mM Tris-HCl
200 mM Glycine
0.1% (w/v) SDS

2× SDS Sample buffer

4% (w/v) SDS
4 mM β-Mecaptoethanol
8% (w/v) Glycerol
80 mM Tris-HCl, pH 6.8
0.02% (w/v) Bromophenol Blue

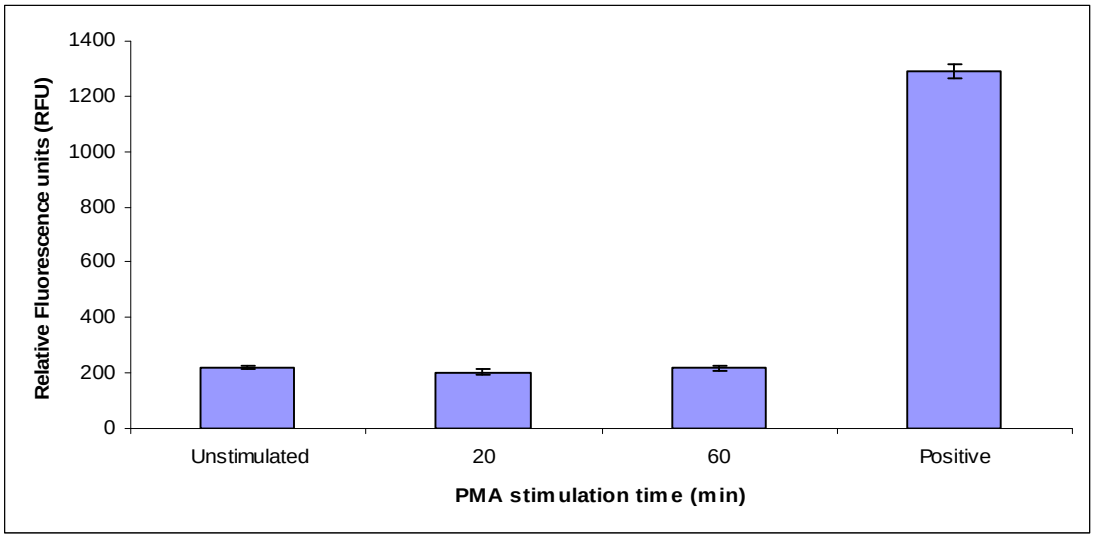


Figure 16: Quantification of NF-κB in resting and stimulated HeLa cells by immunofluorescence. NF-κB levels relative to unstimulated cells show no significant difference between unstimulated cells and those stimulated with PMA 20 and 60 minutes respectively (p>0.05). The Raji Nuclear extract was used as a positive control.

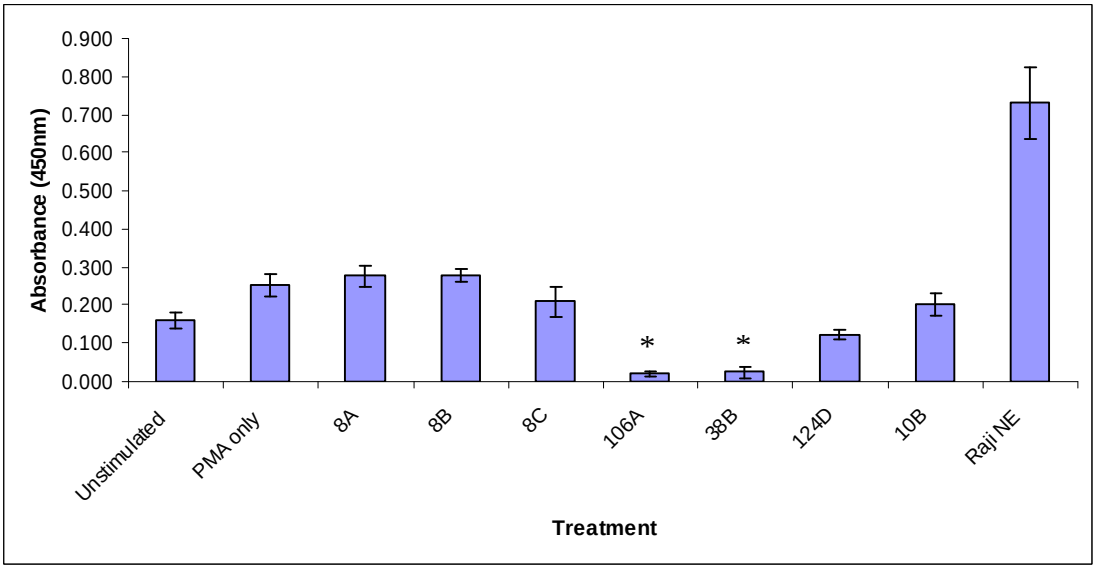


Figure 17: NF-κB levels in stimulated and plant compound treated HeLa cells. TransAM™ NFκB Family Transcription Factor Assay of HeLa cells showing absorbance values after treatment with plant compounds and stimulation with PMA. (*significantly lowered, p<0.05)

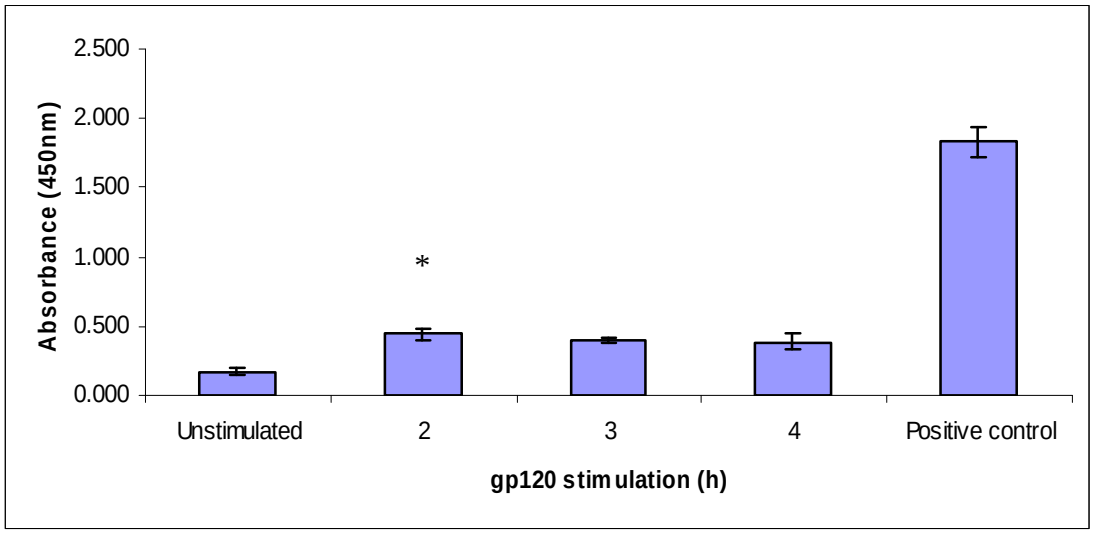


Figure 18: NF-κB levels in HIV-1_{Du151} gp120 treated cardiomyocytes. NFκB Family Transcription Factor Assay of human cord-blood stem cell-derived cardiomyocytes showing a stimulation of NF-κB levels after treatment with HIV-1_{Du151} gp120. Treatment with gp120 for two hours significantly elevated NF-κB levels as compared to unstimulated cells (* $p<0.05$).

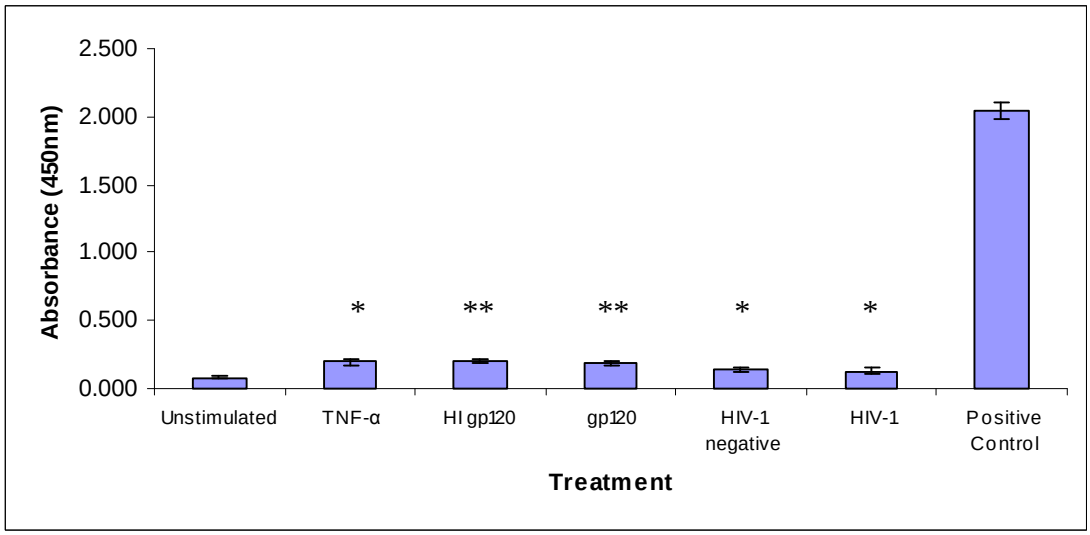


Figure 19: Quantification of NF-κB in cardiomyocytes by treatment with gp120 or HIV-1. NFκB Family Transcription Factor Assay of human cord-blood stem cell-derived cardiomyocytes showing a stimulation of NF-κB levels after treatment with HIV-1_{Du151} gp120 and HIV-1. All treatments including the controls for gp120 and HIV-1, as well as TNF-α, were significantly higher than unstimulated cells (* $p<0.05$; ** $p<0.001$).

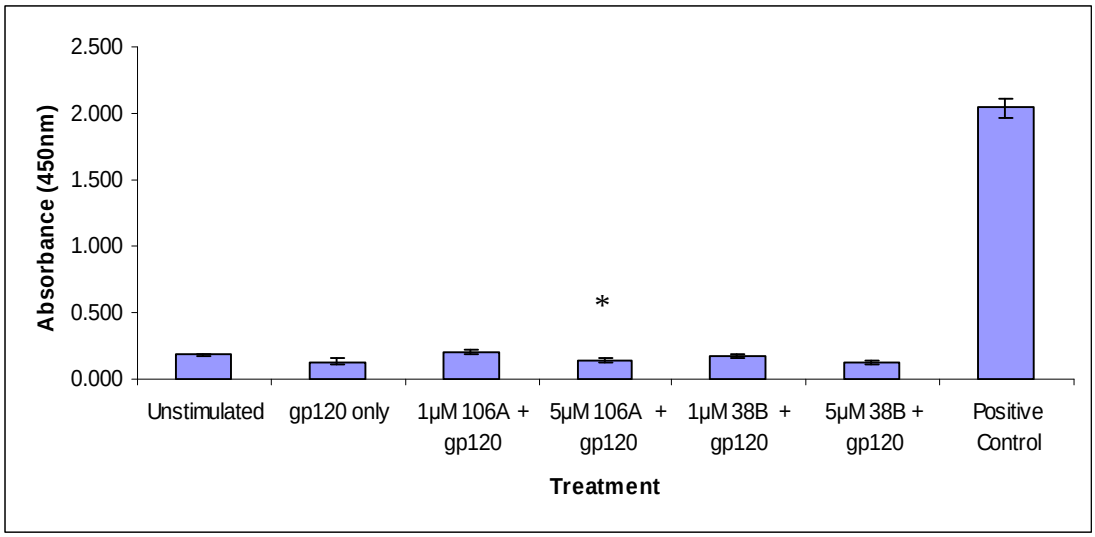


Figure 20: Mitigation of NF-κB levels in gp120-stimulated cardiomyocytes by sesquiterpene lactones. Downregulation of NF-κB levels can be seen in both treatments at 5 μM but only treatment with 5 μM 106A significantly lowered NF-κB levels after gp120 stimulation (* $p<0.05$ vs. gp120 stimulation only).