ANALYSIS OF HIV-INDUCED CARDIOMYOPATHY USING ANTI-GP120 APTAMERS

Walter Rangel Lopes de Campos

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Walter Rangel Lopes de Campos declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……..**.....**(signature of candidate)

……..day of……..(month), 2010

DEDICATION

This work is dedicated to the memory of my father, Joaquim José Lopes de Campos (1954-2006) that passed away after a brief, but violent struggle against cancer. His altruistic nature towards his loved ones was the most remarkable facet of his character. He was raised in poverty but vowed never to let his family feel such burden. He was and remains a role model. He set and lived by very high moral and ethical standards. He always believed in the power of education and was always proud of academic achievements. He was the main reason why I even studied beyond a BSc. I never dreamt of pursuing a PhD, but he rightfully reasoned with me to do so.

PUBLICATIONS

Walter Rangel Lopes de Campos, Dayaneethie Coopusamy, Lynn Morris, Bongani Mayosi, Makobetsa Khati (2009). Cytotoxicological Analysis of a gp120 binding Aptamer with Cross-Clade Human Immunodeficiency Virus Type 1 Entry Inhibition Properties: Comparison to Conventional Antiretrovirals. *Antimicrobial Agents and Chemotherapy. 53:7; 3056-3064.*

Walter Rangel Lopes de Campos, Lynn Morris, Bongani Mayosi, Makobetsa Khati (2010). Elucidation and inhibition of human cardiomyocyte apoptosis during HIV-1 infection by the broad-neutralizing, anti-gp120 RNA aptamer UCLA1. *Circulation*. **(Submitted)**.

Walter Rangel Lopes de Campos, Heidi Flick, Lynn Morris, Bongani Mayosi, Makobetsa Khati (2010). Abrogation of aberrant cytokine expression with the neutralizing aptamer UCLA-1 during HIV infection has a protective effect on human cardiac myocytes. *Antimicrobial Agents and Chemotherapy*. **(Submitted)**.

PRESENTATIONS

Walter Rangel Lopes de Campos, Makobetsa Khati.
Aptamers: A new frontier in diagnostics and therapeutics
CSIR Outcomes Conference 2008, Pretoria, South Africa (Oral).

Walter Rangel Lopes de Campos, Dayaneethe Coopusamy, Lynn Morris, Bongani Mayosi, Makobetsa Khati.
Cytotoxicological profiles cross-clade HIV-1 neutralizing aptamer in human cardiomyocytes and immune system cells: a comparative study against a panel of 17 antiretrovirals in clinical use.
AIDS Vaccine 2008, Mexico City, Mexico (Poster).

Walter Rangel Lopes de Campos, Lynn Morris, Bongani Mayosi, Makobetsa Khati.
Elucidation and inhibition of cardiomyocyte apoptosis during HIV-1 infection by a shortened derivative of gp120 binding aptamer called UCLA1.
International AIDS Society 2009, Cape Town, South Africa (Poster).

Walter Rangel Lopes de Campos, Lynn Morris, Bongani Mayosi, Makobetsa Khati.
Cardiomyocyte apoptosis during HIV-1 subtype C infection and its mitigation using a novel, non-cytotoxic anti-gp120 RNA aptamer.
UCT Department of Medicine Research Day 2009, Cape Town, South Africa (Oral).

ABSTRACT

HIV-associated cardiomyopathy is a multifactorial disease with a broad spectrum of aetiologies that arise due to chronic immunosupression during HIV infection. The intricate relationship between HIV infection and cardiac co-morbidity was investigated with the aid of HIV-neutralizing aptamers. These synthetic nucleic acid ligands with antibody-like properties are molecular tools with multifunctional applications ranging from drug discovery to diagnostics and therapeutics. The advent of the HIV/AIDS pandemic has naturally married the field of HIV therapy and diagnostics with that of aptamer technology. By employing a HIV-1 neutralizing aptamer, named UCLA1, raised against the viral surface envelope glycoprotein 120, I dissected some of the pathways leading to cardiomyocyte apoptosis in a cell culture system. In chapter one I investigated the potential cytotoxic effects of UCLA1 by comparing it against a panel of 17 antiretrovirals (ARVs) in clinical use with the goal of establishing a safety portfolio geared towards its use as a therapeutic agent. Using cultured human cardiomyocytes and primary peripheral blood mononuclear cells (PBMCs), I selected some of the major biological markers of ARV-induced cytotoxicity and found no measurable deleterious effect, especially when compared to other ARVs used in the same study. In chapter two, the permissiveness of cardiomyocytes to HIV infection as well as the relationship between virus-host interaction and caspase-mediated apoptosis were investigated. Non-productive, receptor and tropism-independent infection was observed, which was arrested after the reverse transcription stage. However, interaction between the virus gp120 and the host's CXCR-4 chemokine receptor preferentially activated caspase-9 triggering robust mitochondria-mediated apoptosis. A shift from mitochondrial-initiated, caspase-9 mediated to Fas-ligand initiated, caspase-8 mediated was observed when CM were co-cultured with HIV-infected MDM. UCLA1 protected against caspase-9 mediated

apoptosis but not caspase-8 mediated. Finally in chapter three I provided answers for the shift in caspase activation by showing that supraphysiological levels of IL-1β and IL-6 during HIV infection of MDM augment the effects of tumor necrosis factor (TNF). These observation provide new insight into the complex pathophysiology of HIVCM and highlight the potential of UCLA1 as a novel therapeutic agent to fight HIV and some of its associated diseases.

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ABBREVIATIONS

3TC	Lamivudine
µg	Microgram
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
AMD3100	Plerixafor
AMP	Amprenavir
ARVs	Antiretrovirals
AZT	Zidovudine
B40	gp120 neutralizing RNA aptamer 40
bFGF	basic Fibroblast Growth Factor
CCR3, 5	Chemokine (C-C motif) receptor 3, 5
CD 4, 8, 68	Cluster of Differentiation 4, 8, 68
CM	Cardiomyocytes
CXCR4	Chemokine (C-X-C motif) receptor 4
CYP4503A4	Cytochrome Oxidase 450 3A4
D4T	Stavudine
DC-SIGN	Dendritic cell-specific Intracellular Adhesion Molecule-3 grabbing non-integrin
ddC	Dideoxycytidine
DMEM	Dulbecco's Modified Eagle's Media
DNA	Deoxyribonucleic acid
DRV	Darunavir
EDTA	Ethylenediamine Tetra Acetic Acid
EFV	Efavirenz
ELISA	Enzyme-linked Immunoabsorbent Assay
FACS	Fluorescence-Activated Cell Sorting
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
G-CSF	Granulocyte Colony Stimulating Factor
gp120, gp41	glycoprotein 120 Kda, 41 Kda
HAART	Highly Active Antiretroviral Therapy
HIV-1, 2	Human Immunodeficiency Virus type-1, 2

HIVCM	HIV-associated Cardiomyopathy
i.e	*id est*, that is
IFN-γ	Interferon γ
IC_{50}/EC_{50}	50 % inhibitory concentration
IgG	Immunoglobulin G
IL-1β, 2, 4, 5, 6, 7 8, 9, 10, 13, 15, 17	Interleukin-1β, 2, 4, 5, 6, 7, 8, 9, 10, 13, 15, 17
IND	Indinavir
iNO	inducible Nitric oxide Synthase
IP-10	Inflammatory Protein 10
LPV	Lopinavir
LTR	Long Terminal Repeat
MAO-A, B	Monoamine Oxidase A, B
MCP-1	Monocyte Chemotactic protein 1
MDM	Monocyte-Derived Macrophages
mg	Milligram
MIP-1α, β	Macrophage Inflammatory Protein α, β
MTC	Mother-to-child
mtDNA	mitochondrial DNA
NEV	Nevirapine
NFV	Nelfinavir
nM	nanomolar
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NO	Nitric Oxide
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
p24	Gag protein 24 Kda
p38 MAPK	Mytogen Activated Protein Kinase 38
PDGF	Platelet-derived Growth Factor
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PCR	Polymerase Chain Reaction
PIs	Protease Inhibitors

RNA	Ribonucleic Acid
r/t	room temperature
RT-PCR	Real-Time PCR
SQV	Saquinavir
ROS	Reactive Oxygen Species
RTV	Ritonavir
SPR	Surface Plasmon Resonance
T-20	Enfuvirtide
TCID50	50 % Tissue Culture Infectious Dose
TDF	Tenofovir
TNF-α	Tumor Necrosis Factor α
TNF-R1	TNF soluble Receptor 1
TPV	Tipranavir
TUNEL	Terminal Deoxynucleotidyl dUTP Nick-End Labelling
VEGF	Vascular Endothelial Growth Factor
Z-LEHD-FMK	Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone
Z-IETD-FMK	Z-IIe-Glu(O-Me)-Thr-Asp(O-Me) fluoromethyl ketone

CHAPTER 1

INTRODUCTION

1.1 – The epidemiology of HIV/AIDS

Human immunodeficiency virus (HIV) is the aetiological agent of Acquired Immune Deficiency Syndrome (AIDS) which has cumulatively infected approximately 65 million people and caused an estimated 25 million deaths worldwide since it was first identified in 1983 (Barre-Sinoussi et al., 1983). According to the UNAIDS 2009 report an estimated 31.1 million - 35.8 million people are currently infected with the virus, of which approximately 25 million live in Sub-Saharan Africa accounting for 64% of all people living with HIV (Figure 1). In Africa, during 2008 alone it was estimated that 1.6 million – 2.2 million people became newly infected by HIV and 2 million lost their lives to AIDS in 2008 alone (UNAIDS, 2009).

Globally, heterosexual transmission remains the dominant mode of transmission, accounting for 85 % of all HIV infections. Outside sub-Saharan Africa, a third of all HIV infections are acquired by needle-sharing amongst intravenous drug users, mostly in Southeast Asia and Eastern Europe (UNAIDS, 2009).

A disconcerting feature of the pandemic in the last decade is the disproportional number of new infections in women and young people between the ages of 15-24 years, which currently account for half of all new infections. Women now make up about 42 % of those infected worldwide and over 70 % in sub-Saharan Africa (UNAIDS, 2009). The rising incidence and prevalence of HIV in women of child-bearing age has led to an increase in the number of children infected. An estimated 3.5 million children had vertically acquired HIV by the end of 2005, of which 3 million live in Sub-Saharan Africa alone (UNAIDS, 2009).

Despite some efforts, South Africa's AIDS epidemic is one of the worst in the world and shows no signs of decline. Approximately 6.1 million people, representing 20.7 % of the total population were estimated to be HIV positive, resulting in 360,000 deaths by the end of 2005 alone (UNAIDS, 2009). By the end of 2004, a third of pregnant women who attended public antenatal clinics were infected and trends overtime show a gradual increase in HIV prevalence. This has resulted in a mother-to-child (MTC) HIV infection of half a million children in 2008 (UNAIDS, 2009).

In spite of the rising number of new infections, there are increasing numbers of countries reporting stabilisation or declines in HIV prevalence (e.g. Zambia, Tanzania, Kenya, Ghana, Rwanda, Burkina Faso and Zimbabwe). However, increasing morbidity and mortality rates coupled with a maturing HIV epidemic must be taken into account when interpreting data (Simon, Ho, and Abdool Karim, 2006). Moreover the apparent decline in prevalence may be brought about by the number of HIV-related fatalities which is higher in some regions than those of new infections. However, behavioural change cannot be ignored and has contributed significantly to the decline in new HIV infections (Gregson et al., 2006).

The current reality is that HIV/AIDS morbidity and mortality continue to increase at a very fast pace in Sub-Saharan Africa, particularly in South Africa. Unless this increase is curtailed, the impact of HIV/AIDS is expected to more than double the burden of premature mortality by the year 2010 (Bradshaw et al., 2003).

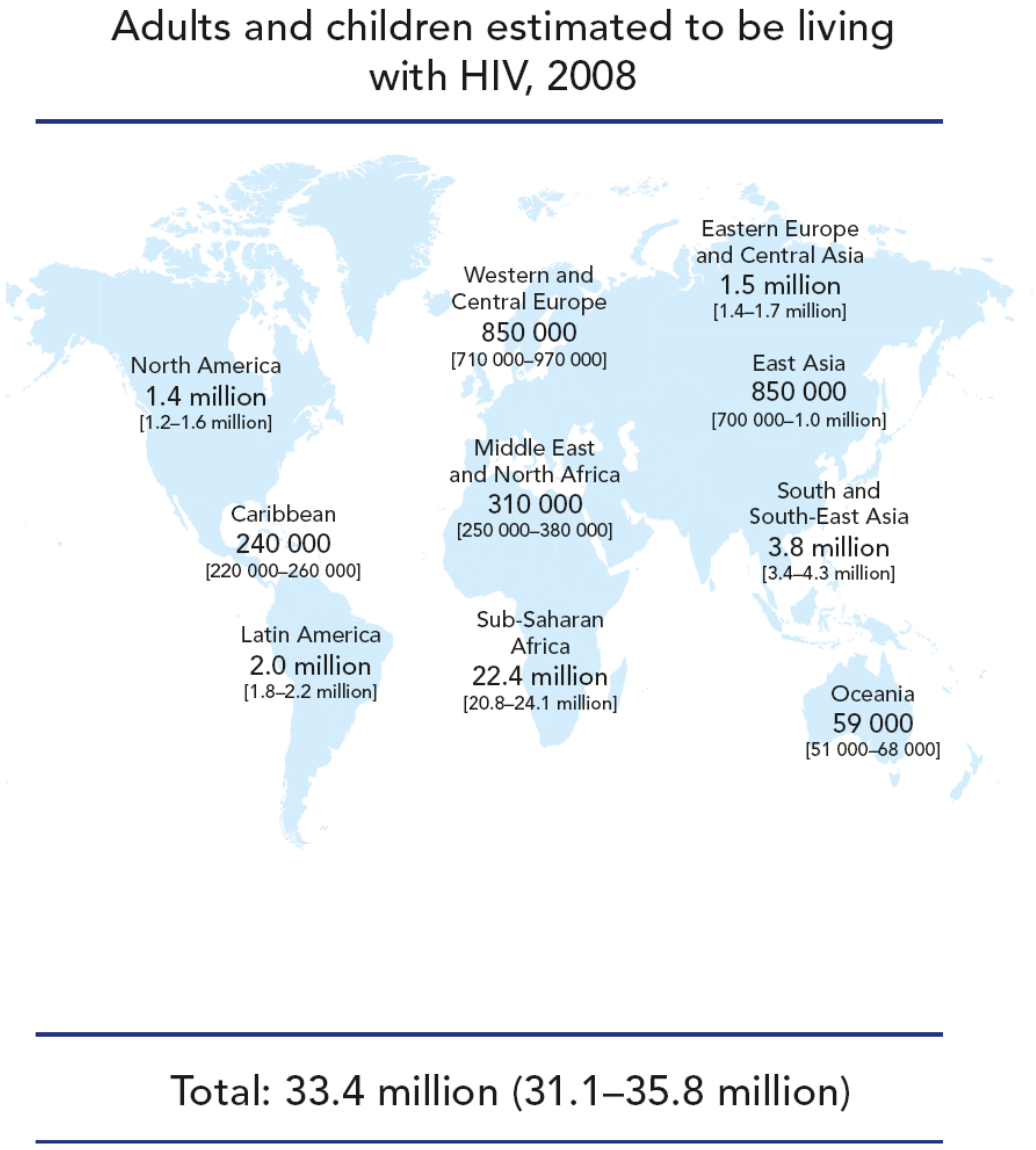

Figure 1: A global perspective of HIV infection (UNAIDS report, 2009)

1.2 – AIDS pathogenesis and HIV-associated cardiomyopathy

1.2.1 – AIDS pathogenesis

HIV-1 started as a zoonosis, originating from primates infected with the Simian Immunodeficiency Virus (SIV), although through natural selection and immunological pressure in the new host, HIV diverged from SIV (Zhu et al., 1998, Gao et al., 1999, Korber et al., 2000, Marx et al., 2001). Transmissions between humans can take place either vertically, from mother-to-child (MTC) or horizontally, from sexual contact. Heterosexual intercourse accounts for the largest number of infections worldwide and is the major drive of the HIV-pandemic (Kilmarx, 2009). Contaminated blood products, particularly contaminated blood in blood banks, re-using of needles in low-resource countries and needle-sharing amongst intravenous drug users are also important routes of transmission (Kilmarx, 2009). However, the ability of an exposed individual to became infected depends on a large number of factors, not only host and viral but also environmental. Nonetheless, when HIV-1 escapes the host's natural barriers, it establishes an infection primarily in $CD4^+$ T lymphocytes. In the first 4 to 8 weeks following exposure, the virus replicates unabatedly reaching very high viral loads while the patient remains asymptomatic (Ford, Puronen, and Sereti, 2009). It is mainly at this stage that viral reservoirs are established (Siliciano and Siliciano, 2000). These consist of quiescent, mostly $CD4^+$ cells that are infected but their dormant state, does not allow for viral replication. The virus undergoes reverse transcription and integrates into the genome but can only begin replicating when the cells are activated. Quiescent cells can remain in this state for decades, thereby perpetuating the infection.

The clinical scenario of the newly infected individual changes upon sero-conversion, when the body's adaptive branch of the immune system begins to produce neutralizing antibodies against the circulating virus resulting in a sharp decline in viral load (Ford, Puronen, and Sereti, 2009). At this stage fever, lymphadenopathy, pharyngitis and myalgia set in. Some cases are severe enough to merit hospitalization. However, this state of malaise resolves within a few days and the patient returns to normal but the struggle between the immune system and the virus has just begun. T-cell depletion occurs not only due to the budding out process of virions from infected cells but also as a result of chronic immune activation (Miura and Koyanagi, 2005). The presence of virus and viral proteins increase the expression of TNF superfamily ligands and their receptors in T-cells and macrophages which can in turn induce apoptosis in bystander cells (Campbell et al., 2004; Ma and Woolf, 1995; Zhang et al., 2001). This mechanism of cellular depletion accounts for most of the T-cell and macrophage loss. It is also likely that this process contributes to cell apoptosis in many different tissues. Recent work, including my own, has shown that an almost identical mechanism accounts for cardiomyocyte apoptosis in cultured cells (Fiala et al., 2004b; Twu et al., 2002). It is therefore likely that cardiovascular damage takes place at this early stage of HIV-1 infection.

The progressive loss of T-lymphocytes and macrophages goes unnoticed for approximately 9±2 years in adult patients according to clinical trials and cohort studies (Mellors et al., 1997; Egger et al., 2002; Jaén et al., 2008), thought the progression to AIDS and risk of death is faster in low-income countries (Braitstein et al., 2006). Generally speaking, immunosuppression becomes of clinical concern once the $CD4^+$ cell count drop from 1,500 cells/μl to below 200 cells/ μl. The onset of

immunosuppression brings an array of opportunistic diseases. The patient falls ill frequently and for long periods of time. If left untreated, the life expectancy is less than two years. Between the onset of AIDS and death, cardiomyocyte destruction becomes more pronounced as do AIDS-related neuropathies (Highleyman, 2009). Approximately 10 % of untreated patients are suspected to succumb to cardiovascular complications (Barbaro, 2003; d'Amati, di Gioia, and Gallo, 2001; Ntseke and Mayosi, 2009), although most lose their lives to upper-respiratory tract infections and tuberculosis (UNAIDS, 2009).

1.2.2 – Cardiac involvement during HIV infection

The link between HIV-infection and cardiac disease was recognized as early as 1983 (Autran et al., 1983) but its prevalence is still a matter of much debate. Nonetheless, incidence rates ranging from 28 % to 73 % have been reported prior to the introduction of highly active antiretroviral therapy (HAART) (Anderson et al., 1988; Baroldi et al., 1988; d'Amati, di Gioia, and Gallo, 2001; Reilly et al., 1988). However, cardiac involvement in AIDS patients was not clinically significant and the majority of cases were only detected at the time of autopsy. Furthermore, cardiac disease in HIV positive individuals is often clinically silent, reversible and usually overshadowed by clinical manifestations in other organs, primarily the lungs and brain. Therefore the number of significant cardiac disease during life represents a very small, albeit rapidly increasing percentage, of cardiac disease detected at necropsy (Sudano et al., 2006).

There are a number of studies that have examined the epidemiology, clinical presentation, and outcome of HIV-associated cardiovascular disease in Africa (Magula and Mayosi, 2003; Mutimura et al., 2008; Ntsekhe and Hakim, 2005; Ntsekhe and Mayosi, 2008). The studies confirmed that cardiac abnormalities are more common in HIV-infected people, compared to normal controls, and that about half of hospitalized patients and a significant proportion of patients followed up over several years develop cardiac abnormalities. The commonest HIV-related cardiac abnormalities identified to date were cardiomyopathy, pericarditis and myocarditis (Barbaro, 2003, Netseke and Mayosi, 2009). Tuberculosis was the major cause of large pericardial effusion in Africa (Cegielski et al., 1990; Taelman et al., 1990; Reynolds et al., 1992; Hsia and Ross., 1994; Pozniak et al., 1994; Maher et al., 1997; Mayosi et al., 2008). However, due to the very limited data on the pathogenesis of cardiomyopathy in Africa, particularly lack of evidence from cardiac histology, it is difficult to determine to which extent opportunistic infections other than TB lead to cardiovascular dysfunction. Viral myocarditis has been suggested as a significant cause of cardiac morbidity (Paravinci et al., 1991), but protozoan and bacterial opportunistic pathogens have been found at the time of autopsy in the African context (Anderson et al., 1988; Longo-Mbenza et al., 1998). In one study involving 16 subjects in the DRC identified toxoplasmosis and cryptococcosis in 40% of cases of HIV-associated cardiomyopathy (Longo-Mbenza et al., 1998). However, due to its sample size and restricted geography location, this data should be interpreted with caution and broad generalizations regarding the identity of cardiac pathogens should be avoided.

There is a growing body of evidence that points to a direct link between HIV infection and cardiovascular disease, independently of opportunistic infections and immunodeficiency in the clinical setting (Grody, Cheng, and Lewis, 1990; Rodriguez et al., 1991; Herskowitz et al., 1994; Lipshultz et al., 1990) and experimental models (Fiala et al., 2004; Kan et al., 2005; Kan, Xie, and Finkel, 2000; Kan, Xie, and Finkel, 2006; Twu et al., 2002).

The lack of resources has limited the diagnosis of cardiovascular dysfunction to non-invasive imaging techniques (electrocardiography, ecography and X-ray) which provide very limited information on the etiology of the disease which also decreases treatment options. Nonetheless, there are three distinct clinical presentations of HIVCM, distinguishable by the evolution of the disease in the patient from asymptomatic to hospitalized (Ntseke and Mayosi, 2009). Left ventricular dysfunction is the hallmark of HIVCM, which is usually asymptomatic in it's early stages and is associated with a range of abnormalities such as dilated ventricles with impaired systolic function, non-dilated ventricles with evidence of impaired diastolic, non-dilated ventricles with impaired diastolic function, dilated cardiac chambers with normal ejection fraction (Hakim et al., 1997; Nzuobontane et al., 2002; Twagirumukiza et al., 2007; Longo-Mbenza et al., 1997; Felker et al., 2000; Niakara et al., 2002; Longo-Mbenza et al., 1995; Longo-Mbenza et al., 1998). A slightly different clinical presentation is highlighted by heart failure with evidence of dilated ventricles and depressed systolic function, which is strongly associated with severe immunodeficiency, indicated by $CD4^+$ T-cell counts below 100 cells/µl (Currie et al., 1995; Hakim et al., 1997; Nzuobontane et al., 2002; Twagirumukiza et al., 2007). A third scenario of HIVCM is observed in hospitalized patients with advanced AIDS

and opportunistic infections, where patients develop heart failure in the absence of a clinical history of heart disease (Ntseke and Mayosi, 2009).

A well described cardiovascular complication of HIV infection is ischemic heart disease (Matetzky et al., 2003; Lohse et al., 2007; Grinspoon et al., 2008). The etiology of the disease, as with most other cardiovascular problems is poorly understood. Recently, two studies were undertaken with the aim of gaining more insight into the pathophysiology of this disease (Becker et al., 2010; Becker et al., 2010b). In these two studies, HIV-positive, treatment naïve patients with acute coronary syndrome were found to be younger, had similar traditional risk factors and a lower artherosclerotic burden but higher thrombotic burden than HIV-negative patients with the same condition. Serological data showed that HIV positive patients had higher frequencies of anticardiolipin and antiprothrombin antibodies, lower levels of protein C and higher levels of factor VIII. This data suggests a prothrombic state in the pathogenesis of acute coronary syndromes which could be related to chronic immune activation and bacterial translocation.

1.2.3 –HIV-1 infection of Cardiomyocytes and apoptotic triggers

Studies in this area are still in its infancy but there is mounting evidence to suggest that HIV infection is capable of causing cardiac myocyte apoptotic death (Chaves et al., 2003; Chen et al., 2002; Kan et al., 2005) (Fiala et al., 2004a) (Fiala et al., 2004b); (Kan, Xie, and Finkel, 2006). However, the exact aetiology remains controversial, as is the potential relationship of intramyocardial HIV-infected cells to the development of cardiac pathology (Chaves et al., 2003); (Fiala et al., 2004b). What is certain is that

HIV has been found to productively infect infiltrating inflammatory cells, macrophages and lymphocytes in heart tissue of HIV positive individuals and a correlation was established between the extent of macrophage infiltration and the risk of HIVCM (Liu et al., 2001). Infection of cardiomyocytes by HIV is relatively well documented and has been described *in vivo* (Grody, Cheng, and Lewis, 1990; Herskowitz et al., 1994). However, *in vitro* data in animal models points to a non-productive infection (Fiala et al., 2004b; Twu et al., 2002). Interestingly, Rebolledo and colleagues reported that HIV-1 does not infect but efficiently replicates in human foetal cardiac myocytes 14 to 18 weeks old (Rebolledo et al., 1998). Infection was only achieved with a VSV-G-pseudotyped HIV-1 vector.

The mechanism by which HIV induces apoptotic death in cardiac tissue is still a question of much debate and very few studies have been carried out to date to provide any definite answers. A combination of virokines, cytokines, *in situ* productive infection of infiltrating macrophages and non-productive HIV infection of cardiomyocytes all seem to play a role in pro-apoptotic signalling, involving the components of both mitochondrial and death receptor pathways (Barbaro et al., 1999; Raidel et al., 2002; Twu et al., 2002).

Cardiomyocyte apoptosis is also likely to be triggered by infiltrating, HIV-infected macrophages via the extrinsic or death-receptor pathway (Twu et al., 2002). These activated macrophages secrete an array of inflammatory mediators of which TNF-α (tumour necrosis factor) and COX-2 (cyclooxygenase) play the major role in extrinsic apoptotic signalling (Liu et al., 2001). The binding of TNF-α and Fas ligand to their respective receptors leads to caspase 8 activation and phagocytosis of the target cell.

To date, it is not yet known which mechanism plays a greater role in cardiomyopathy, the most common form of HIV-induced cardiac disease. Most argue that HIV-infected infiltrating macrophages play a much more significant role than direct (intrinsic) pathogen action (Barbaro, Fisher, and Lipshultz, 2001; Twu et al., 2002). There is indeed much evidence to support this argument. However it is important to highlight the fact that to my knowledge, all *in vitro* studies were carried out using rat tissue, either neonatal ventricular myocytes or mice embryonic stem cells induced to differentiate into cardiomyocytes. While these murine models have proved to be relatively accurate, they do not mimic human cardiac tissue.

Furthermore, all work done to date on human cardiac tissue was carried out on paraffin-embedded formalin fixed tissue using a combination of immunocytochemistry, riboprobe hybridization, PCR and apoptosis assays (Fiala et al., 2004b; Liu et al., 2001; Twu et al., 2002). The complexities of a failing heart coupled to the time lapsed between death and tissue extraction from biopsies takes a toll in the integrity of the quality of the sample. These variables would not be very relevant if the difference between intrinsic and extrinsic tissue damage was clear cut.

1.2.3.1 – TNF, IL-1β and IL-6: the deadly trio of cytokines

The vast majority of cytokines including the ones discussed here are pleitrophic in nature and for this reason, I will only focus on their role in the degeneration of the cardiovascular system and progression to HIVCM.

Cytokines belong to a superfamily of proteins, comprising more than 100 members and are secreted by most cells, playing an essential role in angiogenesis, auto-immune diseases, inflammation and tumor growth. All cytokines signal through 7-helix G-protein coupled receptors and most of these receptors exhibit heterogeneous binding properties whereby several cytokines may bind to the same receptor and elicit different responses, sometimes even antagonistic responses. The most important cytokine-producing cells during HIV-infection and progression to HIVCM are tissue MDM. HIV-infected, infiltrating tissue MDM exhibit a cytokine profile that is not only conducive and suppressive of HIV infection and replication but also act in a paracrine fashion resulting in prolonged inflammation and tissue damage (Fantuzzi, Belardelli, and Gessani, 2003; Kedzierska et al., 2003). The pleiotropic nature of many cytokines as well as their dependence on other cytokines, has made linking cytokines as well as cytokine receptors to cardiovascular morbidity a slow and arduous process. Furthermore, most of the knowledge of cytokine-linked cardiovascular complications arises from work undertaken in non-HIVCM patients. Nevertheless, the tumor necrosis family of cytokines and receptors have been identified as key modulators as have the pro-inflammatory cytokines IL-1β and IL-6 (Barbaro et al., 2000; Fiala et al., 2004b; Finkel et al., 1992; Long, 2001; Monsuez et al., 2007; Satoh et al., 1996; Wollert and Drexler, 2001). The CXCR4 chemokine receptor has been recently linked to CM apoptosis but it has been recognized as an apoptotic receptor in MDM for over a decade (Herbein et al., 1998; Yuan et al., 2008). Interestingly however, its natural ligand, the chemokine stromal-cell-derived factor 1 (SDF-1) is a strong inhibitor of HIV infection and has not been linked to apoptosis initiation in either CM or MDM (Bleul et al., 1996; Oberlin et al., 1996).

1.2.3.2 - TNF signalling and CM apoptosis

This soluble cytokine, which under physiological conditions is mostly secreted by macrophages was the first cytokine identified and was named after its properties of inducing extensive cytotoxicity in several tumor cell lines as well as necrosis in certain animal models of cancer (Carswell et al., 1975; Granger et al., 1969; Kolb and Granger, 1968). The members of this superfamily span 27 receptors and 18 ligands (Locksley, Killeen, and Lenardo, 2001), but TNF exerts its biological apoptotic role by interacting with their cognate receptors, namely the TNF-receptor 1 and 2 (Locksley, Killeen, and Lenardo, 2001). Both receptors bind membrane bound as well as soluble ligand. TNF-R1 is constitutively expressed in most cells, but TNF-R2 is very tightly regulated and almost exclusively found in the cells of the immune system. TNF-R1 is the key receptor of TNF signalling in most cells whereas TNF-R2 assumes this role in lymphoid tissue. The importance of TNF-R2 in paracrine signal transduction is still poorly understood, but its likely to be minor since it can only be activated by membrane-bound and not soluble TNF (Grell et al., 1999). However, the extracellular domains of both receptors can be cleaved, resulting in soluble fragments with bimodal roles (Xanthoulea et al., 2004). These soluble fragments can either neutralize TNF or they can extend its plasma half-life. Furthermore, the proteolytic shedding of these receptors also function as a rapid way of down-regulating the effects of TNF by reducing receptor density on the cell surface.

The molecular mechanisms of TNF-induced cell death are relatively well understood and progresses through activation of NF-κB family of inflammatory transcriptional activators (Wajant, Pfizenmaier, and Scheurich, 2003). Nonetheless, TNF-initiated

apoptosis plays a comparatively minor role when compared to its central role in inflammation and immune-system development. From a pathophysiological perspective, high concentrations induce shock-like symptoms and it thus plays a pivotal role in septic shock (Mannel and Echtenacher, 2000). On the other hand, prolonged exposure to low concentrations can result in cachexia, which is also the origin of one of the several names for TNF, cachectin (Beutler et al., 1985). However, the systemic toxicity brought about by TNF, as well as most pro-inflammatory cytokines involved in HIVCM, is mostly due to cellular mediators such as nitric oxide (NO) and not necessarily related to its capability to initiate cell death (Monsuez et al., 2007).

The death-signalling cascade is initiated upon TNF binding to its death receptor TNF-R1. The silencer of death domain (SODD) protein which is bound to the cytosolic portion of the receptor and is responsible for relaying the death signal thought its death domain (DD), dissociates. The now exposed death domain recruits the death-domain containing adaptor protein (TRADD) through homophilic interactions. At this point, depending whether signal is apoptotic or necrotic in nature, the there is a divergence in the downstream event. During necrosis, the TNRF-1-TRADD complex serves as a platform for the binding of the death domain-containing serine-threonine kinase RIP and the TNF receptor-associated factor 2 (TRAF2). The way in which RIP is involved in the production of reactive oxygen species (ROS) is yet unknown, but this is the final step in necrotic signalling as ROS lead to extensive and irreversible DNA damage, leading to the activation of poly ADP-ribose polymerase 1 (PARP-1). This enzyme uses up large quantities of NAD^+ through the synthesis of poly-ADP ribose, leading to ATP depletion, creating conditions favourable to necrosis (Eguchi, Shimizu, and Tsujimoto, 1997; Leist et al., 1997).

Under apoptotic conditions, the death domain adaptor protein FADD and not RIP is recruited to the TNF-R1 cytoplasmic domain. This complex then recruits caspase-8 through its death-effector domains residing in the amino-terminal region of both molecules (Sprick et al., 2000; Yeh et al., 1998; Zhang et al., 1998). The downstream effects of this complex are discussed in detail below (section 1.2.5).

There is some contradicting evidence that the purported non-death domain containing TNF-R1 also has the capability to induce apoptosis, since studies using TNF-R2-specific antibodies have shown that this receptor alone is sufficient to initiate cell death (Grell et al., 1993; Vandenabeele et al., 1995). However, stimulation of TNF-R2 by exogenous, soluble ligand is not enough to trigger apoptosis (Grell et al., 1999). The signal must be generated by endogenous, membrane-bound TNF. Furthermore, the effect of endogenously produced TNF on TNF-R1 is greatly increased by the existing stimulation of TNF-R2.

There is a contrasting relationship between TNF-α expression and HIV infection. Through activation of NF-κB, TNF-α significantly enhances HIV replication in infected cells (Griffin et al., 1991; Naif et al., 1994), but on the other hand, it suppresses viral replication in bystander cells, through signalling via the TNF-R2 (Herbein and Gordon, 1997).

1.2.3.3 - IL-1β and IL-6: complementing TNF in cardiac morbidity

IL-1β and IL-6 will be discussed together because although they do not share structural and sequence homology, they are always co-secreted and exert near

identical effects in the progression and augmentation of cardiac pathology. Moreover, it has been demonstrated that IL-6 induction can be used as an alternative, indirect assay system for IL-1β production (Van Damme and Van Snick, 1988).

IL-1β and IL-6 are key mediators of inflammation and are produced by many cell types, but mostly by monocytes, macrophages, fibroblasts and endothelial cells upon stimulation by pathogens and other pro-inflammatory cytokines including TNF (Kishimoto, 1989). High levels of IL-1β and IL-6 are strongly correlated with rheumatoid arthritis (de Benedetti et al., 1991; Hermann et al., 1989; Houssiau et al., 1988; Mayet et al., 1990) and congestive heart failure (Long, 2001; Wollert and Drexler, 2001). High concentrations have also been detected in cardiac myxomas and cervical and bladder carcinomas (Scheller, Ohnesorge, and Rose-John, 2006). Recently, in a 3 year follow up study of patients with acute myocardial infarction IL-6 (IL-1β was not measured) was identified as a key marker in mortality prediction (Tan et al., 2009). In *ex-vivo* and murine models, IL-1β and IL-6 have been associated with decreased cardiac contraction, extensive cardiac remodelling and increased mortality (Finkel et al., 1992; Givertz and Colucci, 1998; Li et al., 2005). Cardiac pathology caused by these cytokines is further compounded by their ability to enhance HIV replication in chronically infected $CD4^+$ T cells as well as in MDM (Kedzierska et al., 2003), since there is a strong link between productive viral replication and cardiovascular morbidity.

The mechanism of signal transduction of IL-6 is mediated by the signal transducing protein gp130 (Taga and Kishimoto, 1997). IL-6 first interacts with the membrane associated IL-6 receptor (IL-6R), forming an IL-6/IL-6R complex. This complex then

binds to gp130, which initiated the signal cascade (Rose-John and Heinrich, 1994). IL-6R binding is vital for gp130 interaction and yet some cells that do not express IL-6R are still responsive to IL-6. This can be explained by the secretion of limited quantities of IL-6R. The soluble IL-6R complexes with IL-6 thus enabling gp130 activation in IL-6R$^-$ cells (Mackiewicz et al., 1992; Taga et al., 1989).

IL-1β can also be simply called IL-1, although there are two functional isoforms, α and β, the former is more predominant in mice whereas the later in humans and they seem to share identical structure and function (Priestle, Schar, and Grutter, 1988; Van Damme and Van Snick, 1988), despite sharing less than 30 % sequence homology (March et al., 1985). There are minor functional difference between the two forms, mostly related to T-cell maturation and differentiation (Fibbe et al., 1989). Their signal is relayed through the IL-1 type 1 receptor (IL-1RI), although there is a second receptor, IL-1 type II receptor (IL-2RI), but since downstream events cannot be detected upon ligand binding, it is considered a decoy receptor.

The signalling cascade of both cytokines that lead to CM morbidity and apoptosis are relayed via the p38 MAP kinase pathway inducing the transcription of inducible nitric oxide synthase (iNOS) via NF-κB activation (Finkel et al., 1992; Givertz and Colucci, 1998; Li et al., 2005). The resulting NO exerts its cardiotoxic effects by activating guanylate cyclise and increasing cyclic guanosine monophosphate (Vila-Petroff et al., 1999) resulting in cardiomyocyte shortening velocity. Furthermore, it also impairs the function of the Ca ATPase, resulting in an increase in intra-cytoplasmic calcium due to ineffective sarcolemmal calcium release as well as inadequate calcium sequestration and release from the sarcoplasmic reticulum (Marks, 2003). This

prolonged negative ionotropic effect results in caspase-mediated apoptosis (Fiala et al., 2004b; Finkel et al., 1992; Twu et al., 2002).

1.2.4 – The role of caspases in cardiomyocyte apoptosis during HIV infection

Caspases are an evolutionarily conserved family of cysteinyl proteinases that play key roles in cell survival, proliferation and differentiation (Lamkanfi et al., 2007). More specifically, the caspase gene family comprises 15 members that can be loosely grouped in two major sub-families, regulating two signalling pathways: apoptosis and inflammation. The apoptotic sub-group can be further divided into two types: initiator (caspases-2, 8, 9 and 10) and executioner (caspases-3 and to a lesser extent 6 and 7). For the purpose of this thesis, only the role of caspases in apoptosis will be discussed.

Almost all healthy cells contain several caspases as inactive latent pro-forms called precursor zymogens, synthesized as catalytically-*quasi*-dormant tripartite pro-enzymes. Initiator caspases contain large N-terminal prodomains consisting of long structural motifs containing either a pair of death effector domains (DED, caspase-8 and 10) or a caspase recruitment domain (CARD, caspases-1, 2, 4, 5, 9, 11 and 12). The presence of these prodomains allows for the formation of large multimeric complexes resulting in proximity-induced, catalytic auto-activation. There currently are four well studied multimeric complexes, namely: the apoptosome, the death-inducing signalling complex (DISC), the PIDDosome and the caspase-1-containing inflammasomes. The executioner caspases, on the other hand, lack these large

prodomains and have to be activated by the initiator caspases. In this fashion, initiator caspases provide a link between cell signalling and apoptotic execution.

1.2.4.1 – Caspase-9: the canonical caspase in the intrinsic mitochondrial death pathway

A broad range of stimuli, some well characterized (such as cytotoxic and oxidative stress; heat shock, DNA damage, radiation and growth factor deprivation) and others yet to be identified culminate in caspase-9 activation and subsequent apoptosis (**Figure 1.2**). During HIV infection, it is hypothesized that the trigger is either HIV entry into cardiomyocytes via endocytosis through the ganglioside GM1 and/or Trojan transport system or by direct interaction between cell-free virus and viral pro-apoptotic proteins with the CXCR4 chemokine receptor (Herbein et al., 1998; Yuan et al., 2008). These viral pro-apoptotic factors activate the mytogen activated protein kinase (MAPK) pathway by Erk1/2 phosphorylation via the p38 MAPK which results in a prolonged negative inotropic effect (change in mitochondrial membrane potential, $\Delta\Psi$) on cardiac myocytes leading to mitochondrial leakage of cytochrome *c* into the cell cytosol (**Figure 1.2**) (Fiala et al., 2004b; Kan, Xie, and Finkel, 2006). Cytochrome *c*, together with dATP assemble into the septameric apoptosome with the aid of Apaf-1 (apoptotic protease activating factor-1), which forms the central scaffold of the complex. Procaspase-9 is then recruited into this complex via the CARD domain. The entire complex undergoes dimerization which then results in the proteolytic activation of procaspase-9 into caspase-9. The now active, but not free caspase-9, cleaves and activates the executioner caspase-3, which in turn, cleaves a broad range of key cellular proteins as well as nuclear DNA, resulting in apoptosis.

The process leading up to caspase-9 and -3 activation is very tightly regulated by the cell and is often the target of viral proteins, since cell survival is imperative for viral replication and propagation.

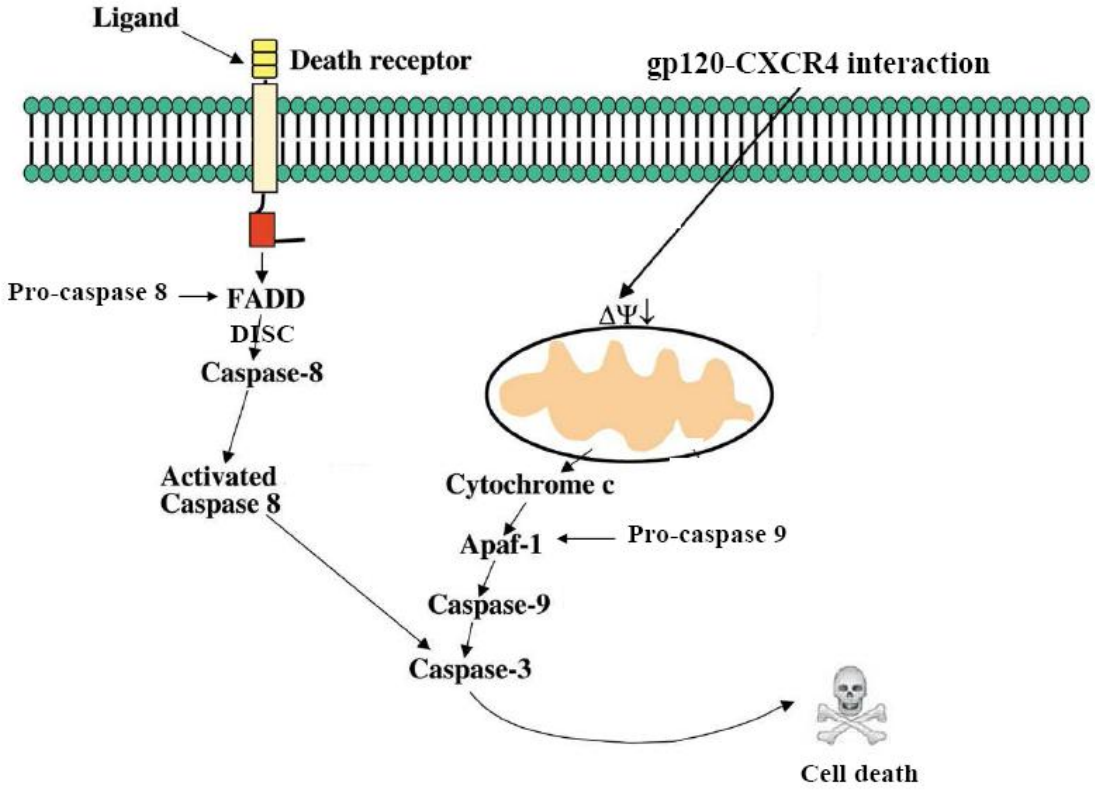

Figure 1.2. Extrinsic and intrinsic apoptotic pathways. Extrinsic cardiomyocyte apoptosis occurs when soluble ligand (TNF, Fas-ligand) activates the cell-surface death receptor. Pro-caspase 8 is then recruited into the DISC via binding to the FADD adaptor protein, where it is proteolitically activated. Mitochondrial mediated apoptosis is triggered by binding of gp120 to the CXCR4 chemokine receptor. The resulting prolonged ionotropic effect results in Cytochrome *c* leakage, which together with Apaf1, activate caspase-9. Both pathways converge at the activation of caspase-3 resulting in apoptosis.

1.2.4.2 – Caspase-8 and the extrinsic death receptor apoptotic pathway

Caspase 8 signalling begins with the interaction of ligand with a cell surface death receptor of the TNF superfamily, usually Fas, CD95 or TNF-R1 (**Figure 1.2**) (Boldin et al., 1996; Muzio et al., 1996). These receptors have death domains in their cytosolic domains and recruit two isoforms of procaspase-8 to the DISC via binding to the FADD adaptor protein (**Figure 1.2**). The interactions of the proteins recruited to the DISC are dependent on homotypic contacts. The receptor DD interacts with the DD of FADD, while the DED of FADD interacts with the N-terminal tandem DEDs of procaspase-8. Therefore, the formation of the DISC complex serves as a platform to bring the two procaspase-8 isoforms close to each other in favourable mutual orientation. These procaspase-8 dimers are now proteolytically active and auto-activate each other to mature caspase-8. Once the active caspase-8 heterodimer is formed at the DISC, the mature caspase-8 is released from the complex into the cytosol where it triggers apoptosis (Rupinder, Gurpreet, and Manjeet, 2007; Varfolomeev et al., 1998).

1.3 – HAART to heart: Implication of ARVs in HIVCM – a paradigm of AIDS pathogenesis

Antiretrovirals are the backbone of HIV therapy. These agents target the virus at different stages of its life cycle (**Figure 1.2**). Currently there are 22 FDA-approved antiretroviral agents as well as an array of novel drugs in various stages of clinical trials (Temesgen *et al.*, 2006). The current drugs can be sub-divided into four categories based on their mode of action. There are nucleotide/nucleoside analogues

(NRTIs) that block the viral enzyme reverse transcriptase by competing with the natural substrate and causing chain termination in the synthesis of pro-viral DNA (Mitsuya *et al*., 1986). There are also antiretrovirals that function as non-nucleoside analogues (NNRTIs), binding to a pocket adjacent to the catalytic pocket of the reverse transcriptase enzyme and induce a conformational change that disrupts the catalytic site (Merluzzi *et al*., 1990).

A decade ago a novel antiretroviral was added to the growing arsenal of drugs. These were protease inhibitors (PIs) and their introduction had a profound impact on mortality and morbidity rates as well as in the quality of life of many HIV positive patients (UNAIDS, 2009). The combination of NRTIs, NNRTIs and PIs was coined as HAART. PIs work by interfering with the last step of the virus life cycle: maturation (**Figure 1.2**) (Cimarelli and Darlix., 2002). Upon budding, the virion-encapsulated protease cleaves a number of proteins giving rise to mature, infectious viral particles. The PIs are encapsulated within the virion and block this step by acting as suicide inhibitors in the catalytic domain (Temesgen *et al*., 2006). The inhibition of the protease enzyme prevents maturation of the virions giving rise to non-infections particles.

1.3.1 – Molecular mechanisms of HAART-induced cardiovascular disease

The therapeutic benefits of HAART cannot be overstated, however, there is a strong correlation between therapy length and the risk of cardiovascular complications (Barbaro, 2005). Moreover antiretrovirals belonging to the NRTI, NNRTI and especially the protease inhibitor classes are particularly toxic to the cardiovascular

tissue (**Table 1**). This, in part, has to do with their target, the catalytic site of HIV-1 protease. This binding pocket is homologous to that of host proteins involved in lipid metabolism and glucose disposal (Carr et al., 1998a). This allows PIs to interfere with cytoplasmic retinoic acid binding protein 1 (CRABP-1) as well as low density lipoprotein receptor-related protein (LRP) leading to a decrease in synthesis of *cis*-9-retinoic acid and peroxisome proliferator-activated receptor type-γ (PPAR-γ) herterodimer (Carr et al., 1998a; Carr et al., 1998c). Under this conditions, an increase in adipocite apoptosis coupled with a decreased rate of pre-adipocity maturation into adipocites results in a significant increase in lipid release and decrease in triglyceride storage. This effect is further enhanced by the inhibition of proteosome activity and concomitant accumulation in the intracellular levels of the active subunit of sterol regulatory element-binding protein-1c in hepatocytes and adipocites (Mooser and Carr, 2001). The well documented impairment of hepatic LRP further interferes with chylomicron uptake and endothelial triglyceride clearance (Carr et al., 1998a; Carr et al., 1998b). The effects of these metabolic interferences are further compounded by an increase in apo C-III (Bonnet et al., 2001) and lipoprotein (a) (Periard et al., 1999). Apo C-III was found to be strongly associated with lipodistrophy, whereas lipoprotein (a) has been associated with premature atherosclerosis in HIV-negative patients (Assmann, Schulte, and von Eckardstein, 1996). Inhibition of the glucose transporter Glu4, further impairs glucose uptake in insulin-sensitive tissues thus precipitating the emergence of insulin resistance and dyslipidemia (Murata, Hruz, and Mueckler, 2000).

Table 1. Cardiovascular complications of FDA approved antiretrovirals

ARVS			
Class	**FDA approval date**	**Side-effects**	**References**
NNRTIs			
Delavirdine	April, 1997	Cardiomyopathy, hypertension, dyslipidemia	(Gill and Feinberg, 2001)
Efavirenz	September, 1998	Tachycardia, dyslipidemia	(Lennox et al., 2009)
Etravirine	January, 2008	Myocardial infarction, angina pectoris, dyslipidemia	(Katlama et al., 2009)
Nevirapine	June, 1996	Dyslipidemia	(Rodriguez-Arrondo et al., 2009)
NRTIs			
Abacavir	December 1998	Myocardial infarction? lipodystrophy	(Albrecht, 2009)
Didanosine		Portal hypertension, lipodistrophy	(Ziagen and Videx, 2008)
Emtricitabine	July 2003	Lipodistrophy, dyslipidemia	(Molina et al., 2007)
Lamivudine	November 1995	Lipodistrophy, dyslipidemia	(Kumar and Patel)
Stavudine	June 1994	Lipodistrophy, dyslipidemia	(Calmy et al., 2009)
Tenofovir	October 2001	Lipodistrophy, dyslipidemia	(Stephan, 2008)
Zidovudine	March 1987	Cardiomyopathy, syncope, lipodistrophy, dyslipidemia	(Camsonne, Hurrault de Ligny, and Letellier, 1987)
Protease Inhibitors			
Amprenavir	April 1999	Lipodistrophy, dyslipidemia	(Schooley et al., 2001)
Atazanavir sulphate	June 1999	Cardiac conduction abnormalities, lipodistrophy	(Feldt et al., 2005)
Darunavir	June 2006	Lipodistrophy, dyslipidemia	(Hughes et al., 2009)
Indinavir	March 1996	Lipodistrophy, dyslipidemia	(MacDougall, 1996)
Fosamprenavir	October 2003	Lipodistrophy, dyslipidemia	(Hester, Chandler, and Sims, 2006)
Nelfinavir	March 1997	Lipodistrophy, dyslipidemia	(Calmy et al., 2009)
Ritonavir	March 1996	Hypertension, hypotension, myocardial infarct	(Calmy et al., 2009)
Saquinavir	December 1995	Lipodistrophy, dyslipidemia	(Calmy et al., 2009)
Tipranavir	June 2005	Lipodistrophy, dyslipidemia	(Hughes et al., 2009)
Fusion Inhibitors			
Enfuvirtide	March 2003	None	(Wright et al., 2008)
Entry Inhibitors			
Maraviroc	August 2007	Myocardial infarction, hypotension	(Abel et al., 2008)
Integrase Inhibitors			
Raltegravir	October 2007	None	(Lennox et al., 2009)

* Supplementary information was obtained from the Food and Drug Administration's web site at www.fda.org/oashi/aids/virals.html

1.3.2 – Decreased cardiovascular risk of novel classes of antiretrovirals

In the past eight years, three novel drug classes have entered the market. Either by coincidence or more strict approval criteria, the incidence of cardiovascular complications when compared to other ARVs has decreased significantly (Hughes et al., 2009).

The first novel class of drug, Enfuvirtide entered the market in 2003 as a complement to HAART in drug-experienced, HIV infected patients (Manfredi and Sabbatani, 2006). It is marketed under the brand name Fuzeon® and belongs to the class of fusion inhibitors, consisting of a linear 36 amino acid peptide homologous to a segment of a region of the gp41 coat protein that is crucial for entry (Doms and Trono, 2000) (**Figure 1.3**). It therefore inhibits viral infection of target cells by competing with gp41 for the chemokine co-receptors. No negative cardiovascular events have been observed in the latest safety and efficacy trials (Wright et al., 2008).

In August of 2007 Maraviroc (Selzentry®), a CCR5 co-receptor antagonist was approved by the FDA for therapy of treatment-experienced adults with multi-drug resistance HIV. This drug is classed as an entry inhibitor and works by blocking the interaction of the virion's gp41 coat glycoprotein with the CCR5 chemokine co-receptor of the host (Hunt and Romanelli, 2009) (**Figure 1.3**). A recently published review of the clinical literature reported no significant negative cardiovascular complications (Abel et al., 2008).

The latest addition to the arsenal of new classes of ARVs was Raltegravir (Isentress®), FDA approved in October 2007. This drug belongs to the class of HIV integrase

strand transfer inhibitors blocking the integration of pro-viral DNA into the human genome (Cocohoba and Dong, 2008). It blocks the strand-transfer step of the integration cycle by binding to the active site of the viral integrase enzyme (Espeseth et al., 2000; Hazuda et al., 2000) (**Figure 1.3**). The incidence of cardiovascular complications was significantly lower than that reported for NNRTIs, NRTIs and PIs (Cocohoba and Dong, 2008). However, this drug has been on the market for less than 3 years, which is not long enough time to fully evaluate its cardiovascular safety.

Not long ago, the desperation brought about by the failure of the available antiretroviral agents made most long-term side effects acceptable. By and large, all antiretrovirals have a degree of toxicity that is exacerbated by their prolonged use (Temesgen, 2006). Nowadays in the developed world, HIV infection went from a lethal disease to a chronic, manageable ailment further accentuating the side effects brought about by chemotherapy. In fact, most complications that arise in HIV positive patients on chemotherapy can be directly traced back to the chronic, uninterrupted use of antiretrovirals (Temesgen, 2006). Furthermore, conditions like cardiomyopathy that were once silent and clinically irrelevant have come under the spotlight as a potentially debilitating and lethal complication. Therefore, there is an urgent need for novel, affordable antiretrovirals with decreased toxicity and increased specificity, which are directed at sites already targeted by the current drugs, but also novel sites.

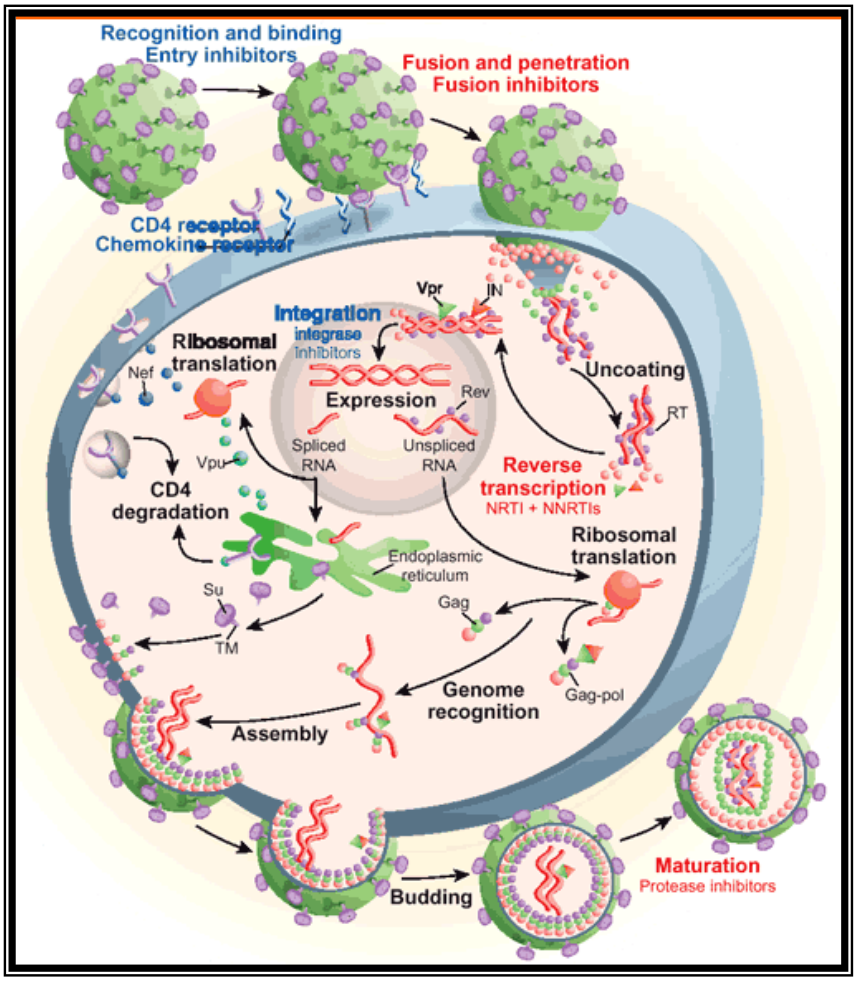

Figure 1.3. Schematic representation of possible and current drug-targeting sites during the various stages of the virus life cycle, from entry to virion maturation (Pomerantz and Horn, 2003).

1.4 – HIV neutralizing aptamers as tools for HIV research and therapy

1.4.1 – Aptamers and the SELEX process

Aptamers are oligonucleotides ligands that are selected for high-affinity and specificity for binding to molecular targets that rival those of monoclonal antibodies. They are selected using an *in vitro* process in a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX), (**Figure 1.4**) from massively complex combinatorial libraries (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990).

Using the SELEX protocol, aptamers with therapeutic potential have been raised against a wide variety of targets, from small human molecules to autoantibodies, viral proteins and even whole organisms (Lee, Stovall, and Ellington, 2006). Pegaptanib sodium, marketed under the brand name Macugen® and developed by OSI Pharmaceuticals, was the first aptamer with a therapeutic application, used for the treatment of neovascular age-related macular degeneration (EYE001, 2002; EYE001: phase II study, 2003).

Aptamers are poorly immunogenic (2003), non-toxic in therapeutic applications (2003; Lopes de Campos et al., 2009) and can be post-transcriptionally modified to increase their stability under physiological conditions (Chelliserrykattil and Ellington, 2004; Pagratis et al., 1997). Their small size when compared to antibodies (**Figure 1.5**), allows access to sites that are obscured and only transiently exposed, such as the

conserved region in gp120 crucial for virus entry. Another advantage of aptamers over antibodies is that they are made *in vitro* and can be re-folded if denatured. All these properties, coupled with their high affinity and specificity for their cognitive target (Green et al., 1995; Jellinek et al., 1995; Jellinek et al., 1993), make aptamers attractive therapeutic agents.

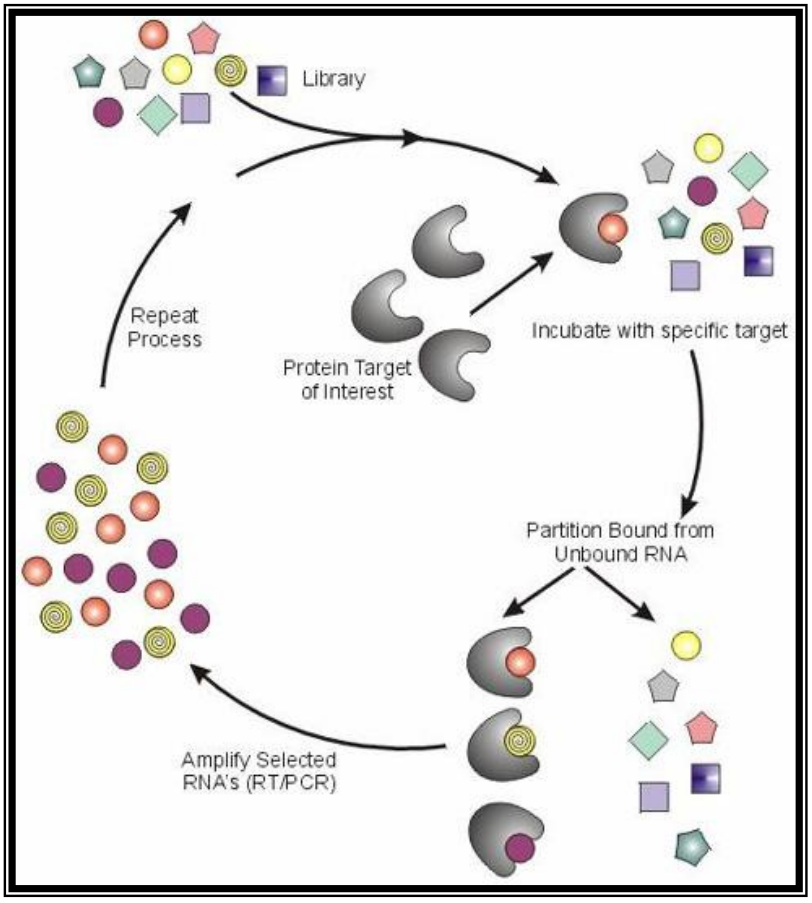

Figure 1.4. Diagrammatic representation of the SELEX process. Starting with a DNA library containing 10^{15} randomised sequences, the resulting RNA is incubated with the ligand of interest and the resulting, enriched ligand-bound RNA is amplified and reverse transcribed into DNA. The process is repeated 10 to 20 times on average until a highly enriched population of aptamers with strong binding affinity is obtained from which those with the highest affinity and specificity are selected (Tuerk and Gold, 1990).

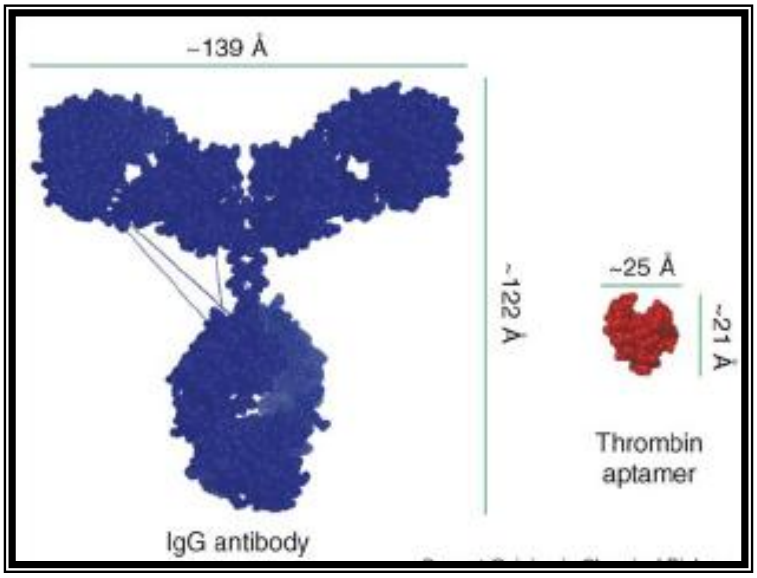

Figure 1.5. Space-filling models of an estimated comparison between a human IgG antibody and an anti-thrombin aptamer (Lee, Stovall, and Ellington, 2006).

1.4.2 – HIV-1 neutralizing aptamers as drug candidates against AIDS pathogenesis and HIVCM

Antiviral aptamers have been raised against several clinically important viruses (Gopinath, 2007): HIV (Held et al., 2006), Human Hepatitis C Virus (Kikuchi et al., 2009; Konno et al., 2008; Nishikawa et al., 2004), and Influenza (Jeon et al., 2004; Misono and Kumar, 2005) to name a few. The core of our study revolves around the work carried out by Khati *et al.*, 2003, Dey *et al.*, 2005 and Cohen *et al*., 2008 where a series of aptamers were raised against the gp120 viral coat protein of HIV-1 using surface plasmon resonance and subsequently characterized. These aptamers showed high affinity and specificity for gp120 and were capable of broad-range cross clade neutralization of HIV clinical isolates at concentrations in the low nanomolar range.

Structural studies to elucidate it's mode of action as well as to reduce its size without affecting functionality, have focused on a single neutralizing aptamer chosen based on its cross-clade HIV-1 neutralizing properties. This aptamer, B40 (later chemically modified and coined UCLA1) was shown to bind to the CCR5 binding pocket of gp120 and thereby inhibit gp120-CCR5 interaction (Cohen et al., 2008; Dey et al., 2005; Khati et al., 2003). The occlusion of this binding pocket interferes with viral fusion with the host cell membrane, a vital event that precedes entry. In this fashion, B40 effectively interferes with the viral life cycle before infection. This represents a milestone in HIV chemotherapy since all other ARVs in current clinical use only block post-entry events by targeting reverse-transcription, integration and viral maturation (**Figure 1.2**). It is important to mention that aptamers have been raised against other HIV-1 proteins, namely RT (DeStefano and Nair, 2008; Kensch et al., 2000; Li et al., 2008; Michalowski et al., 2008), REV (Symensma et al., 1996; Tuerk and MacDougal-Waugh, 1993), Tat (Yamamoto et al., 1995) and integrase (Allen, Worland, and Gold, 1995). However, these proteins take part in the virus life cycle post infection and thus are presented intracellularly. Although aptamers can achieve their correct conformation in the intracellular environment, those targeting extracellular proteins have been shown to have greater therapeutic value (EYE001, 2002; EYE001, 2003; Lupold et al., 2002; Nobile et al., 1998; Rusconi et al., 2002). In the context of HIVCM therapy, UCLA1 would be a good drug candidate since it inhibits viral replication without the cardiotoxic effects of ARVs. Of increasing concern in HIVCM pathogenesis is the role played by cell-free virions. The apoptotic potential of these virions in CM is clearly demonstrated in the results section of this dissertation as is the protective role played by UCLA1. Since cell-free virus is not targeted by ARVs, UCLA1 could prove to be of valuable therapeutic use.

1.4.3 – The future of aptamers in HIV therapy

Therapeutic aptamers for HIV-1 infections are still in its infancy. The *in vitro* neutralization data obtained with HIV-1 neutralizing aptamers targeting the gp120 surface envelope glycoprotein has been very encouraging, with aptamers showing cross-clade neutralizing activity in the low nanomolar range (Khati et al., 2003)(Mufhandu, unpublished data). They have also shown good potential in targeted delivery of siRNAs-aptamer chimeras (Zhou et al., 2008; Zhou et al., 2009). In this scenario, B40 and other aptamer with similar properties selectively delivered a 27 mer siRNA targeting the *tat/rev* region of HIV-1 to gp120 expressing cells, which efficiently blocked viral replication. Therefore, there is more than one application for neutralizing aptamers in HIV therapeutics and their potential is yet to be fully explored. However, all we have to date is *in vitro* data and there is a very large knowledge gap of the effectiveness of HIV-1 neutralizing aptamers under physiological conditions. Having good neutralizing properties is one of many variables required for the therapeutic use of aptamers. For instances, there is the risk that these aptamers may not have favourable pharmacokinetic properties, such as short plasma-half life, excessive binding to serum proteins and low conformational stability under physiological conditions. The only robust clinical data we have so far comes from Pegaptanib sodium, but the conditions under which this aptamer is used differ from that in which HIV-1 neutralizing aptamers would be used (2002; 2003). Firstly, the aptamer is administered via intraocular injection to the retina. The retina has limited irrigation, therefore it is easier to achieve higher serum concentrations of the drug under localized conditions. On the other hand, neutralizing aptamers would need to act systemically to fight HIV-1 infection and have to be evenly distributed

throughout the body. Furthermore, a delivery mechanism other than the oral route would not be well received in the HIV/AIDS community facing the reality that there currently are 23 orally-delivered and highly effective antiretrovirals in clinical use (De Clercq, 2009). However, advances in oral delivery technologies could soon overcome these hurdles (Werle, Makhlof, and Takeuchi, 2009). Several groups are working on matrices that allow the oral administration of recombinant, functional proteins, the main focus being insulin, with some very promising results recently emerging (Wood, Stone, and Peppas, 2008). This technology could be used to deliver any gastric-labile compound, including aptamers. The use of aptamers as microbicidals should be considered with caution since 73 pre-clinical and 45 clinical trials have not demonstrated efficacy in preventing HIV-1 infection with several promising microbicidal agents with potent *in vitro* activity (Cutler and Justman, 2008).

The cost factor is a major point for concern as well, since HIV-1 primarily affects resource-starved countries and since it requires life-long therapy, it must be affordable. Currently, there are a large number of highly effective ARVs that are not used in many countries due to cost considerations (Bartlett and Shao, 2009).

Aptamers do however have properties that make them attractive ARVs. The nature of their chemistry makes them virtually non-toxic (EYE001, 2002; EYE001, 2003; Lopes de Campos et al., 2009), a property that no other ARV to date can claim. Furthermore, high affinity ligands are much easier and quicker to select for than with conventional, small-molecules. The technology simply needs to be developed until it can and maybe will revolutionize the field of therapeutics.

1.5 - Objectives of this study

This work had two central objectives. The first was to examine the potential cytotoxic and cardiotoxic effects of UCLA1 in cultured human cardiomyocytes and peripheral blood mononuclear cells with the goal of using UCLA1 as a therapeutic tool. Key cytotoxic markers were examined, namely cell death, caspase activation, monoamine oxidase 1 and 2 activity, Cytochrome P450 3A4 activity and mitochondrial DNA depletion.

The second was to dissect the mechanisms through which HIV causes cardiomyocyte destruction *in-vivo* by extrapolation from a simplified *ex-vivo* model using tissue-culture adapted cardiomyocytes, primary macrophages, clinical isolates of HIV-1 subtype C and the UCLA1 aptamer.

CHAPTER TWO

Cytotoxicological Analysis of a gp120 binding Aptamer with Cross-Clade Human Immunodeficiency Virus Type 1 Entry Inhibition Properties: Comparison to Conventional Antiretrovirals

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Chapter Summary

The long-term cytotoxicity of antiretrovirals (ARVs) is among the major causes of treatment failure in HIV/AIDS patients. This calls for the development of novel ARVs with less or no cytotoxicity. Here, we compared the cytotoxic effects of a cross-clade HIV-1 neutralizing aptamer called B40, against a panel of non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs) and the entry inhibitor (EI), T20, in human cardiomyocytes and peripheral blood mononuclear cells (PBMCs). An initial screen using cell death as the end-point measurement revealed that B40 aptamer and T20 were the only test molecules that had insignificant ($0.61 < P < 0.92$) effect on viability of both cell types at the maximum concentration used. PIs were the most toxic class ($0.001 < P < 0.00001$), followed by NNRTIs and NRTIs ($0.1 < P < 0.00001$). Further studies revealed that B40 and T20 did not interfere with the cellular activity of cytochrome P450 3A4 enzyme ($0.78 < P < 0.24$) or monoamine oxidase (MAO) A and B ($0.83 < P < 0.56$) when compared to untreated controls of both cell types. Mitochondrial-initiated cellular toxicity is closely associated with use of ARVs. Therefore we used real-time PCR to quantify the relative ratio of mitochondrial to nuclear DNA as a marker of toxicity. The levels of mitochondrial DNA when compared to untreated controls, remained unchanged in cells exposed to B40 aptamer ($0.5 > P > 0.06$). These data support the development of B40 and related EI aptamers as new ARVs with no cytotoxicity at the estimated potential therapeutic dose.

1.0 – Introduction

The introduction of highly active antiretroviral therapy (HAART) has significantly reduced morbidity and mortality in HIV/AIDS patients. This however requires life-long treatment and results in toxicity in up to 50% of patients following 6-12 months of therapy (Bongiovanni et al., 2005; Mocroft et al., 1998). Treatment failure arising from toxicity has highlighted the need for close medical supervision and, ultimately, the development of novel, less toxic ARVs. This is of even greater concern in the resource-poor African continent where, unlike in developed countries, monitoring, diagnosis and management of ARV-associated toxicity is not routinely conducted (Mutimura et al., 2008). This problem is likely to increase as use of ARVs becomes more widespread in resource-poor settings (Beck et al., 2006).

Mitochondrial toxicity is one of the major complications associated with long term use of HAART (Brinkman, 2001; Brinkman and Kakuda, 2000; Kakuda, 2000; Kakuda et al., 1999; Walker and Brinkman, 2001a; Walker and Brinkman, 2001b). NRTIs used in HAART inhibit DNA polymerase γ, which is solely responsible for mitochondrial DNA (mtDNA) replication (Lewis and Dalakas, 1995). Through this mechanism, NRTIs induce depletion of mtDNA as well as mtDNA-encoded enzymes (Brinkman et al., 1999). This results in mitochondrial dysfunction and eventually leads to a range of complications such as bone marrow suppression and cardiomyopathy (DiMauro and Schon, 2003).

In addition to mitochondrial toxicity, other markers of cytotoxicity caused by ARVs are cell death, modulation of cytochrome P450 (CYP450) and monoamine oxidase MAO A and B enzymes. CYP450 enzymes are mostly amine oxidases and key

metabolizers with regard to catalytic versatility and the broad spectrum of oxidative transformation of both exogenous and endogenous molecules (Guengerich, 2001). This enzyme superfamily plays a vital role in tissue and cardiovascular health (Elbekai and El-Kadi, 2006) On the other hand, MAO enzymes are flavoenzymes that catalyse the oxidative deamination of a large number of biogenic and xenobiotic amines (Edmondson et al., 2004; Kalgutkar et al., 2001). Therefore any drug that interacts with MAO, regardless of its function, can lead to a decrease in normal MAO cellular activity. This in turn can result in a potentially lethal intracellular accumulation in the levels of its natural substrates such as serotonin (Dams et al., 2001).

For reasons of toxicity associated with use of ARVs, here we studied the cytotoxic effects of one of the potent and cross-clade HIV-1 neutralizing RNA aptamers called B40, which was recently isolated and described (Cohen et al., 2008; Dey et al., 2005b; Khati et al., 2003). B40 aptamer blocks viral entry by binding to core conserved residues on gp120 at the heart of the CCR5 binding site (Cohen et al., 2008; Dey et al., 2005a). While this is the first study that describes the *in vitro* toxicity of the experimental HIV-1 gp120 binding RNA aptamer, previous studies have shown that the vascular endothelial growth factor (VEGF) binding aptamer did not have any intrinsic toxicity in preclinical assessment (2002; Drolet et al., 2000). Single dose and repeated dosing toxicity studies with anti-VEGF aptamer conducted in rats, rabbits, and Rhesus monkeys showed that there was no-observable-adverse-effect-level (NOAEL) or dose limiting toxicity (2002; 2003; Drolet et al., 2000).

In this study, we evaluated toxicity of anti-gp120 aptamer called B40 using cell viability, CYP450 3A4 (CYP3A4), MAO A and B and mtDNA levels as markers of cytotoxicity in human cardiomyocytes and PBMCs. Toxicity was evaluated in human cardiomyocytes due to the association of cardiomyopathy with use of ARVs (Herskowitz et al., 1992; Lewis, Simpson, and Meyer, 1994) and PBMCs because $CD4^+$ T-cells and macrophages found in PBMCs are the backbone of the immune system. HIV-1 predominantly infects and replicates in these cells.

2.0 - Materials and methods

2.1 – ARVs

All ARVs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. Four classes of ARVs were tested in this study. NNRTIs: Nevirapine (NVP) and Efavirenz (EFV); NRTIs: Abacavir (ABC), Dideoxycytidine (ddC), Zidovudine (AZT), Stavudine (d4T), Tenofovir (TDF) and Lamivudine (3TC); PIs: Saquinavir (SQV), Ritonavir (RTV), Nelfinavir (NFV), Lopinavir (LPV), Tipranavir (TPV), Indinavir (IDV), Amprenavir (APV) and Darunavir (DRV) and the Entry Inhibitor (EI): Enfuvirtide (T20).

2.2 - In vitro transcription and validation of monoclonal aptamers

Plasmid DNA containing the B40 aptamer was amplified by PCR as previously described (Khati et al., 2003). The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and *in vitro* transcribed to RNA using 2′-fluoro-pyrimidines (TriLink, USA) as previously described (Khati et al., 2003). The resulting 2'-F-RNA aptamer was quantified using the NanoDrop® ND-100 spectrophotometer (Thermo Fisher Scientific, USA). Prior to use the RNA aptamer was refolded by heating in water to 95°C for 5 minutes and then cooling to room temperature for 5 minutes. This was followed by the addition of 1/20 volume of 20× refolding buffer (final concentration:10mM HEPES pH 7.4; 150mM NaCl; 1mM $CaCl_2$; 1mM $MgCl_2$; 2.7mM KCl) and incubated at room temperature for an additional 10 minutes. The refolded aptamer was validated by binding to recombinant

gp120 derived from HIV-1_{Ba-L} using BIAcore® 3000 (GE Healthcare, USA) as previously described (Khati et al., 2003).

2.3 - Recombinant HIV-1Ba-L gp120

HIV-1_{Ba-L} gp120 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.4 - Culture and phenotyping of human cord-blood stem cell-derived cardiomyocytes

The human cord-blood stem cell-derived cardiomyocytes were purchased from Celprogen (California, USA). The cardiomyocytes were cultured in Human Cardiomyocyte Expansion Media (a proprietary media containing, serum, growth factors and antibiotics) and in tissue culture flasks containing a proprietary growth matrix at 37°C, 5% CO_2. The media and flasks were also purchased from Celprogen, USA.

The cardiomyocytes were phenotyped by immunocytochemistry using a cardiac specific monoclonal antibody that targets the first 32 amino acids of the c-terminus of the human cardiac protein troponin I (R&D Biosystems, USA) and detected using an anti-goat Rhodamine labelled antibody produced in chickens (Santa Cruz, USA). Briefly, a cell monolayer growing on a chamber slide (Lab-Tek™, Nunc, USA) were fixed and permeabilized in 2% PFA and 0.1 % Triton X-100 (Sigma, Germany) for 30 min. Following this, primary antibody was added at a concentration of 10 μg/ml in the presence of 1% normal goat serum (Invitrogen, USA) and incubated for 1 hour on ice. The cells were then washed 4 times with ice-cold PBS prior to the addition of the anti-

goat rhodamine-conjugated secondary antibody, at a concentration of 5 μg/ml. Following incubation for 1 hour, the cardiomyocytes monolayer was covered with a glass coverslip in the presence of Mounting Medium (UltraCruz™,Santa Cruz Biotechnology, USA) for immunofluorescence analysis.

2.5 - Isolation and cultivation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinised human buffy coats of normal, HIV-negative donors as previously described (Khati et al., 2003). Buffy coats were obtained from the South African National Blood Services. Briefly, buffy coats were layered into Ficoll-Paque™ PLUS (GE Healthcare, USA) and PBMCs isolated by density-gradient centrifugation. Following a 2000 rpm centrifugation for 30 min at 20 ºC, the PBMC layer was harvested and washed once with ice cold PBS (Lonza, USA). The cell pellet was then suspended in 30 ml of a hypotonic solution of ice-cold ammonium chloride and incubated at room-temperature for 20 min to lyse the remaining red-blood corpuscles. The cells were harvested by centrifugation and seeded on tissue culture flasks (Corning, USA) at a density of 2×10^6 cells/ml in RPMI medium containing 20% fetal bovine serum (Sigma-Aldrich, Germany), followed by PHA stimulation and IL-2 treatment as previously described (Khati et al., 2003). For comparison we also cultured PBMCs without mitogen (PHA) and without IL-2 in X-VIVO-10 media (BioWhittaker) supplemented with 2% autologous serum because we previously showed that this system produces a slowly proliferating mixed culture of $CD4^+$ T lymphocytes and macrophages that supports a higher level of replication of HIV-1 primary isolates (Khati et al., 2003).

2.6 - Cell viability assay

PHA stimulated, IL-2 treated PBMCs and cardiomyocytes in the log-phase growth were seeded at a density of 20,000 cells/well in 96-well white opaque wall, tissue culture plates (Nunc™, Thermo Fisher Scientific, USA) in a volume of 100 µl growth media. Unstimulated PBMCs cultured in X-VIVO-10 media (BioWhittaker, USA) supplemented with 2% autologous were also seeded as above. The overlay media in either system was replaced with the relevant culture media containing the drug at a concentration equivalent to the maximum plasma concentration in patients at a pre-determined therapeutic dose (C_{max}) or an equivalent amount of the aptamer (Table 1). Following 24 hour incubation, the overlay media was removed and replaced with fresh media. The overlay media was replaced every 2 days for the duration of the experiment. The assay was done in triplicates and independently repeated twice. Following 7 days of incubation the cell viability was determined using the ATP-based assay CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA). Both the plates and the reagent were allowed to equilibrate at room temperature. Then an equal volume of reagent was added to the wells and further incubated at room temperature for 20 minutes to allow for the luminescent signal to stabilize. Following the incubation, the luminescent signal was measured in a Modulus® Microplate Reader luminometer (Promega, USA) using a count integration time of 1 second. The raw data was measured in relative light units (Merluzzi et al.) and the final results were normalized (i.e. the total number of viable cells in the treated samples were respectively subtracted by the total number of viable cells in the cell control–which was not treated with any drug or test molecule) and expressed as percentage cell death. The cell controls, which were not treated, were on average more than 95%

viable. Wells containing media alone were used as controls for background luminescence and subtracted from the test values. DMSO at a concentration of 0.1% (v/v) was used as a solvent for most ARVs; hence a control for DMSO-initiated toxicity was included whereby cells were treated with 0.1 % DMSO for the duration of the study. The general formula to calculate percentage toxicity was: % of viable cells = [(RLU of drug treated cells - RLU of medium control) ÷ (RLU of untreated cell controls - RLU of medium control)] × 100. The % of dead cells = 100% - % of viable cells.

2.7 - Caspase 3/7 activity

Representatives from each class of ARVs were selected for apoptosis screening in cardiomyocytes and PBMCs, namely: EFZ (NNRTI), ABC and TDF (NRTI), RTV and IND (PIs), T20 and B40 aptamer (EI). Cells were seeded at a density of 50,000 cells/well in 24- well tissue culture plates (Corning, USA) and incubated with ARVs at the C_{max} for 1 and 7 days respectively. After each time point elapsed the cells were immediately frozen at -80 ºC. Day 7 cells were frozen for 24 h prior to assay. The cells were then equilibrated to room temperature and an equal volume of Caspase-Glo 3/7® detection reagent was added (Promega, USA). Following 2 h of incubation the resulting luminescent signal was measured in a Modulus® Microplate Reader luminometer (Promega, USA) using a count integration time of 1 second. The results were expressed as relative light units, blank subtracted (RLU, blank subtracted) against ARVs on the x-axis.

2.8 - Monoamine oxidase A & B enzyme activity assay

This assay was carried out using the MAO-Glo™ Assay kit (Promega, USA). A 96-well white opaque wall, tissue culture plate was seeded with either cardiomyocytes or PBMCs and incubated in triplicate with the test ARV or aptamer at a maximum starting concentration of 2 μM followed by ½ log serial dilutions. The overlay media was removed and the cell monolayer was washed with PBS (Lonza, USA) and lysed with 50 μl proprietary lysis buffer (Promega, USA). The cell debris was removed by centrifugation at 2000 rpm and the supernatant was recovered and incubated with MAO A or B enzyme substrate for 3 hours at room temperature. This was followed by the addition of the detection reagent. After 1 hour of incubation the luminescent signal was measured in a Modulus® Microplate reader (Promega, USA) luminometer using a count integration time of 1 second. The raw data was measured in RLU and the final results were normalized as in the cell viability assay above and expressed as a percentage of enzyme activity. A no-substrate control sample was included as control for background luminescence and subtracted from the test values. Clorgyline (Sigma-Aldrich, Germany) was used as a positive control for MAO A inhibition and Deprenyl (Sigma-Aldrich, Germany) for MAO B. The general formula used to calculate percentage enzyme activity was: % of enzyme activity = [(RLU of drug treated cells - RLU of medium control) ÷ (RLU of untreated cell controls - RLU of medium control)] × 100.

2.9 - Cytochrome P450 3A4 activity

The P450 Glo™ Assay kit was used (Promega, USA). Cells were seeded as described above in the presence of the aptamer or ARVs and incubated for 2 days. The overlay

media was then removed and 60 µl of fresh media was added which contained 50 µM of the luciferin substrate. After 4 hours of incubation, 50 µl culture supernatant from each well was transferred to a 96-well white opaque wall, tissue culture plates (Nunc™, Thermo Fisher Scientific, USA) and an equal volume of detection reagent was added. The luminescent signal was measured after 1 minute of incubation, in a Modulus® Microplate reader (Promega, USA) luminometer as described above. A no-substrate control sample was included as control for background luminescence and subtracted from the test values. The protease inhibitor Ritonavir was used as a positive control for CYP3A4 inhibition. Cell culture supernatant from untreated cells was used as the baseline for CYP4503A4 activity.

2.10 - Real-time PCR for quantification of mitochondrial DNA toxicity

Cells were exposed to the B40 aptamer, T20 or ddC at 2 µM for 7 days. Following exposure to the test molecule for 7 days, the cell monolayer was washed with warm PBS and the cells were lysed in 50 µl lysis buffer (Promega, USA), which was compatible with the PCR mix. A volume of 2 µl lysate was subjected to PCR for both genomic and mitochondrial DNA in a LightCycler® (Roche, Switzerland). The target nuclear gene was the human polymerase γ accessory subunit (*ASPOLG*) and the mitochondrial gene was cytochrome-*c* oxidase I (*CCOI*). For *ASPOLG* the forward primer 5′ GAC CTG TTG ACG GAA AGG AG 3' and the reverse primer 3' CAG AAG AGA ATC CCG GCT AAG 5' were used for PCR amplification. The *CCOI* primer pair consisted of the forward primer sequence 5' TTC GCC GAC CGT TGA CTA TT 3' and reverse primer 3' AAG ATT ATT ACA AAT GCA TGG GC 5'. PCR reactions were carried out with the master mix Power SYBR® Green 5 (Applied Biosystems, USA) in the presence of 5 mM $MgCl_2$ (Promega, USA). Cycling

conditions consisted of a single enzyme-activation step of 10 min at 95 ºC, followed by 45 cycles of denaturing at 95 ºC for 0 s, annealing at 60 ºC for 10 s, elongation at 72 ºC for 5 s with a temperature transition rate of 20 ºC/s. The relative amount of mitochondrial and nuclear DNA was extrapolated from a standard curve and the results were calculated as the mean ratio of mitochondrial to nuclear DNA. Each experiment was carried out in triplicate and the results represent the average between two independent experiments. The mtDNA:nDNA ratio of the untreated controls was arbitrarily set at 1.0 and was used as the baseline measurement.

TABLE 1: Maximum blood plasma concentration (C_{max}) in patients and *in vitro* data of ARVs

ARVs		**C_{max}(µM)[a]**	**In vitro data**				
Class			**Cell[b]**	**Strain[c]**	**IC_{50}(µM)[d]**	**TI[e]**	**Reference**
NNRTIs	NEV	16.9	C8166	IIIB	0.04	8000	(Merluzzi et al., 1990)
	EFV	15.7	PBMC	Primary isolates	IC_{95}=0.0015-0.003*	>50000	(Young et al., 1995)
NRTIs	ABC	14.7	PBL	IIIB	4	40	(Melroy and Nair, 2005)
	ddC	0.12	PBMC	LAV	0.011	>9000	(Chu et al., 1988)
	AZT	48.8	PBMC	IIIB	0.05	1000	(Mitsuya et al., 1985)
	D4T	5.3	PBMC	LAV	0.009	7778	(Chu et al., 1988)
	TDF	1.0	MT-2	IIIB	0.003	16667	(Robbins et al., 1998)
	3TC	8.8	CEM	RF	0.18	>2000	(Coates et al., 1992)
PIs	SQV	3.7	C8166	RF	0.0002	>50000	(Roberts et al., 1990)
	RTV	21.0	PBL	Primary isolates	0.045	1089	(Kempf et al., 1995)
	NFV	7.0	MT-2	RF	0.043	651	(Patick et al., 1996)
	LPV	15.7	MT-4	NL4-3	0.005	>20000	(Carrillo et al., 1998)
	TPV	0.2	MT-4	NL4-3	0.016	>2000	(De Meyer et al., 2005)
	IND	15.4	MT-4	IIB	IC_{95}=0.1*	>4000	(Vacca et al., 1994)
	AMP	15.1	MT-4	IIIB	0.054	>1600	(Baba et al., 1997)
	DRV	13.6	MT-2	LAI	0.003	24667	(Koh et al., 2003)
EIs	T20	1.0	PBMC	LAI		-	(Wild et al., 1994)
	B40[f]	-	PBMC	Primary isolates	IC_{50}<0.01, IC_{95}<0.1*	>5000	(Cohen et al., 2008; Dey et al., 2005b; Khati et al., 2003)

[a]The C_{max} of all registered ARVs were taken from package inserts of the respective drugs.
[b]Cell type used to obtain respective *in vitro* data
[c]Strain of HIV-1 used to obtain respective *in vitro* data
[d]IC_{50} (inhibitory concentration) is the effective concentration of the drug that inhibits HIV-1 in infected cells by 50%

*IC_{95} is the concentration of drug that inhibits HIV-1 in infected cells by 95%.
[e]TI (Therapeutic Index) is the relation between the CC_{50} (inhibitory concentration that reduces cellular growth or viability of uninfected cells by 50%, data not shown) and IC_{50} and it is obtained using the general formula: $TI = CC_{50}/IC_{50}$
[f]B40 aptamer is an experimental anti-HIV-1 molecule and not yet registered for clinical use. For this reason its C_{max} in blood plasma of patient has not been established. It was used at 2 µM, which is twice the C_{max} of T20 and more than 200-fold its IC_{50}. The CC_{50} of B40 could not be obtained even after using the aptamer at 50 µM, which had similar effect as 2 µM.

3.0 – Results

3.1 - Effect of B40 aptamer in comparison to ARVs on viability of human PBMCs and cardiomyocytes

We evaluated toxicity of a novel gp120-binding and HIV-1 neutralising RNA aptamer called B40, in comparison to conventional ARVs, in human cord-blood stem cell-derived cardiomyocytes (**Figure 1**) and in human PBMCs. The strong staining for cTnI positively identified the phenotype of these poorly-reported cells as being human cardiomyocytes (**Figure 1C** and **D**). An initial screen using an ATP cell-based assay to measure cell death as the end point measurement of cell toxicity showed that B40 aptamer, which is a novel EI, did not cause death of either human cardiomyocytes (1±0%; $P = 0.74$) or PBMCs (0±5%; $P = 0.92$) at a maximum concentration of 2 μM used (**Figure 2**). The maximum concentration of 2 μM used for B40 in this study is more than 200-fold the B40 concentration (<10 nM) required to inhibit 50% (IC_{50}) of HIV-1 in PBMCs (Cohen et al., 2008; Dey et al., 2005b; Khati et al., 2003). The property of the B40 aptamer to cause no cell death at the maximum concentration used compared favourably to the registered EI called T20, which also did not cause cell death in cardiomyocytes (1±3%; $P = 0.74$) and PBMCs (0±6%; $P = 0.61$). All other classes of ARVs tested caused significant cell death in either or both cardiomyocytes and PBMCs (**Figure 2**). PIs were generally the most toxic class of ARVs in both cardiomyocytes and PBMCs causing up to almost 100% ($0.0002 < P < 0.00001$) cell death at the maximum concentration used (**Figure 2**). The two NNRTIs tested, NEV and EFV, respectively caused 80% ($0.1 < P < 0.002$) and 60% ($0.1 < P < 0.002$) cell death in PBMCs, while they both caused about 20% ($0.004 < P$

<0.002) cell death in cardiomyocytes (**Figure 2**). NRTIs were generally the second least toxic class of ARVs after EIs, causing less than 20% cell death in cardiomyocytes and between 20% and 72% toxicity in PBMCs (**Figure 2**).

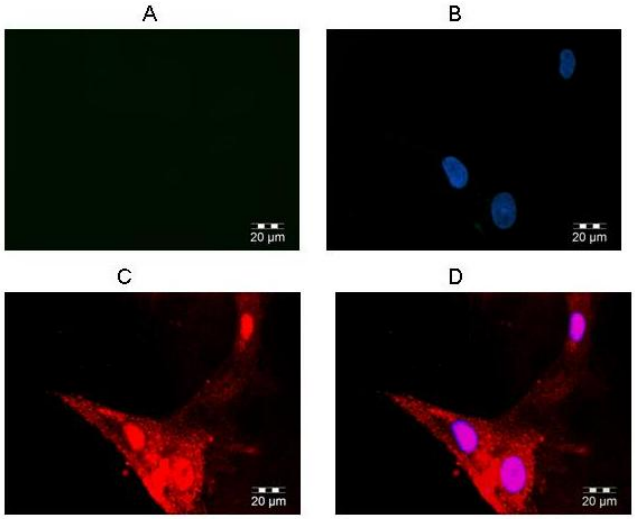

Figure 1: Phenotyping of human cord-blood stem-cell derived cardiomyocytes. Cardiomyocytes were cultured in chamber slides, permeabilized and treated with (A) rhodamine-labelled secondary antibody only to exclude non-specific binding (B) DAPI nuclear stain; (C) cTnI primary and rhodamine-labelled secondary antibody (D) a composite image was created by superimposing images B and C.

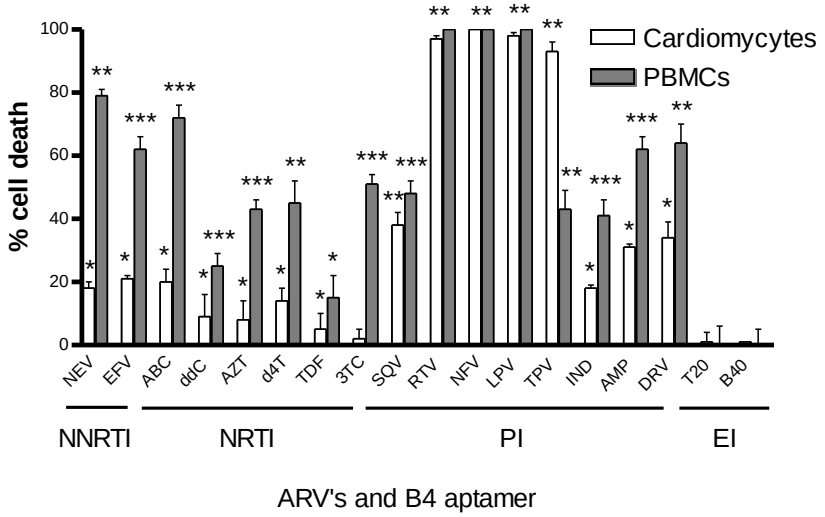

Figure 2: Effect of B40 aptamer in comparison to a panel of 17 ARVs on viability of human cardiomyocytes and PBMCs. Human cord-blood stem cell-derived cardiomyocytes or freshly isolated PBMCs were seeded at a density of 20 000 cells/well in 96 well plates and exposed to respective ARVs at a concentration equivalent to the maximum blood plasma concentration (C_{max}) shown in Table 1 or the B40 aptamer at 2 µM. All experiments were done in triplicates and independently repeated twice. ATP levels were measured as a correlate of cell viability after 7 days of exposure by relating the RLU of treated to untreated cells. The results were normalised and expressed as percentage cell death in the treated samples as compared to untreated cells. Data on the graph shows the mean and standard deviation bars of three replicates in two sets of independent experiments. ARVs whose toxicity was statistically difference from that of the untreated controls as determined by the t-test are indicated by asterisks, where, *, **, *** indicate *P* values of less than 0.01, 0.001 and 0, 0001 respectively.

3.2 - Cell death was caspase independent

We probed further into the mechanism through which ARVs were inducing cell death by measuring the activity of two executioner caspases, caspase 3 and 7 respectively. In spite of robust cell death induced by most ARVs in this study (**Figure 2**), no increase in caspase activity was observed in all ARVs tested, when compared to untreated control cells (**Figure 3 A** and **B**). A sharp and statistically significant decrease in caspase activity was observed at day 1 for ABC, RTV and IND treated PBMCs only and days 4 and 7 in both cell types treated with EFV, ABC, TDF (day 7 in PBMCs only), RTV and IND treatments, which directly correlated with the observed decrease in cell viability (**Figure 2**). T20 and B40 treated cells were the only cells that had no significant changes in caspase activity levels at the measured time points.

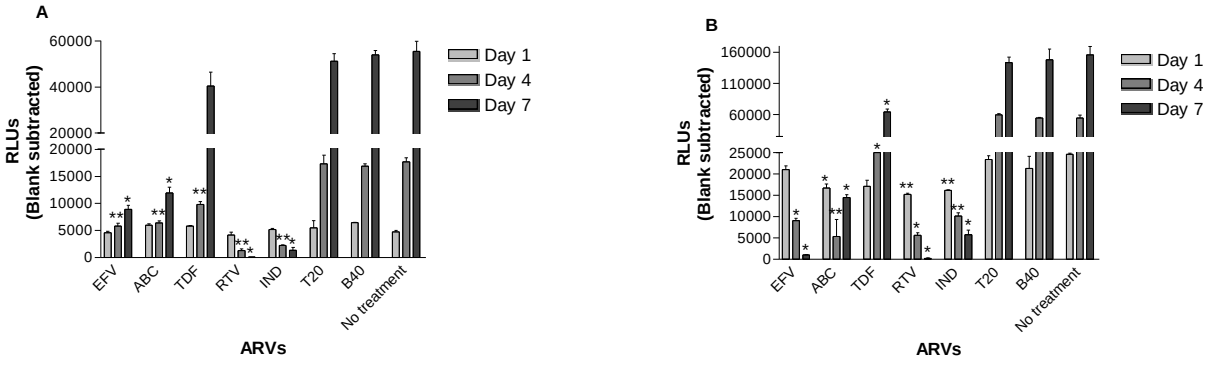

Figure 3: Caspase 3/7 activation upon ARV exposure. Cells seeded at a density of 50,000 cells/well were exposed to EFZ, ABC, TDF, RTV, IND, T20 and B40 aptamer at the C_{max} for 1 and 7 days respectively. No statistically significant increases were observed for any ARV, including the B40 aptamer in either cardiomyocytes (A) or PBMCs (B) when compared to the untreated controls. A statistically significant

decrease in caspase activity was observed in day 1 in PBMCs only for ABC, RTV and IND treated cells; at day 4 and 7 for EFV, ABC, TDF (day 7 only in PBMCs), RTV and IND treated cells. The asterisks * and ** denote *P* values of less then 0.002 and 0.0002 respectively. The data was presented in bar graphs relating RLUs to ARVs and shows the mean and error bars of three replicates.

3.3 - B40 aptamers did not affect levels of MAO enzymes A and B

Since the B40 aptamer and T20 (both EIs) were the only test molecules that had no effect on cell viability and caused insignificant cell death–an end point marker of cytotoxicity; we selected them for more in-depth cytotoxic analysis at the enzyme and cell metabolism level. We measured the interaction of B40 aptamer with MAO A and B enzymes, because any drug that interacts with MAO A or B, regardless of its function, can lead to a decrease in normal MAO cellular activity. This in turn can result in a potentially lethal intracellular accumulation in the levels of its natural substrates such as serotonin. It is therefore important to screen potential drugs for possible interaction with MAO enzymes. In this study, we measured the levels of both MAO A and B enzymes in the presence of B40 aptamer and for comparison, T20. The results clearly indicated that neither of the MAO isozyme was inhibited or induced by B40 aptamer or T20 in both cardiomyocytes and PBMCs as compared to untreated control cells (**Figure 4A-D**). However, MAO A was inhibited in a concentration-dependent manner by clorgyline (**Figure 4A** and **B**) and MAO B in a similar manner by deprenyl (**Figure 4B** and **D**). Clorgyline and deprenyl, which were used as inhibitors of MAO A and MAO B respectively, served as good controls and confirmed the presence and activity of both isozymes in cardiomyocytes and PBMCs,

and that these isozymes were amenable to interference by certain drugs or specific inhibitors.

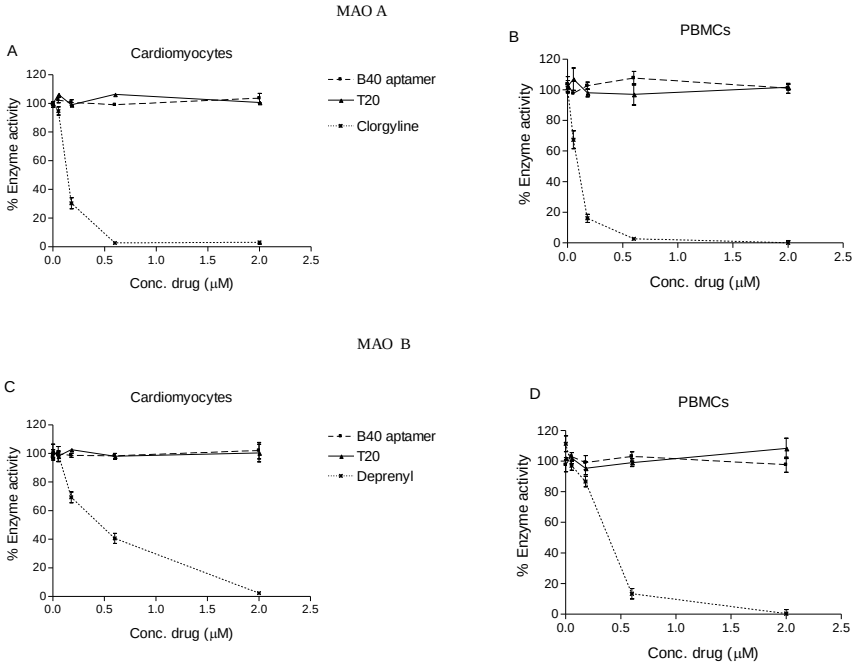

Figure 4: MAO A and B enzyme modulation by B40 aptamer and T20 in cardiomyocytes (**A, C**) and PBMCs (**B, D**). Cells were incubated in triplicates with a serially diluted test molecule at a maximum concentration of 2 μM for 2 hours. A cell lysate was incubated with MAO A or B substrate for 1 hour followed by the addition of detection reagent and the resulting luminescence signal was quantified in a luminometer. The no-treatment control was used as the baseline for maximum enzyme activity. No change in enzyme activity was detected for both MAO A and B in the presence of B40 aptamer and T20 for both cardiomyocytes and PBMCs. However, there was a dose-dependent inhibition of MAO A by clorgyline and MAO B by deprenyl, which are respectively well documented inhibitors of MAO A and MAO B indicating the presence and activity of both enzymes in human

cardiomyocytes and PBMCs. Enzyme activity was calculated by relating the RLUs of untreated control cells to that of the treated cells as a percentage. Data on the graphs represent the mean and error bars of three replicates in two sets of independent experiments.

3.4 - B40 aptamer did not affect the level of CYP450 3A4 enzyme

Cytochrome P450 3A4 is another key enzyme involved in cell metabolism and commonly affected by the PI class of ARVs. For this reason, we tested if B40 aptamer modulates this enzyme in human cardiomyocytes and PBMCs. B40 aptamer favourably compared to T20 and did not significantly affect the level of CYP3A4 compared to untreated negative controls in both cardiomyocytes (P = 0.78 for T20 and P = 0.37 for B40 aptamer) and PBMC (P = 0.62 for T20 and P = 0.24 for B40 aptamer) even at the highest concentration used (**Figure 5A** and **B**). However, as a positive control, CYP3A4 was significantly inhibited in a concentration-dependent manner by the protease inhibitor Ritonavir in human cardiomyocytes (P = 0.0001) and PBMCs (P = 0.0008).

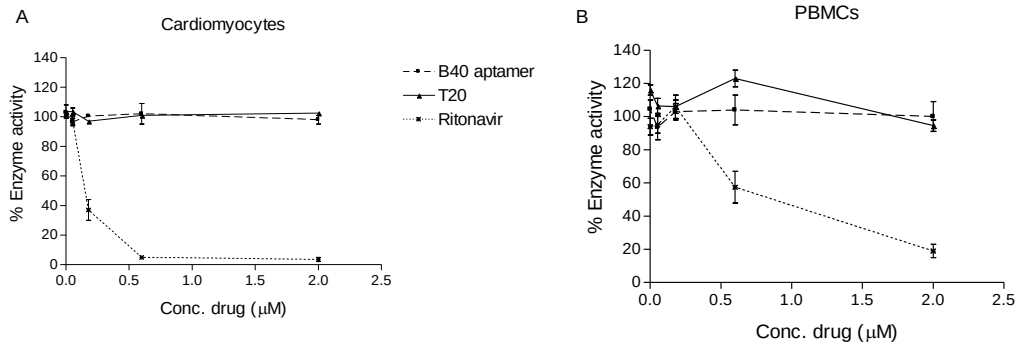

Figure 5: Effect of B40 aptamer and T20 in CYP3A4 enzyme activity in cardiomyocytes (**A**) and PBMCs (**B**). Cells were seeded at a density of 20 000

cells/well and exposed to the respective test molecule in triplicate for 2 days at serially diluted concentrations. The overlay media was removed and fresh media was added containing a luciferin luminogenic substrate for CYP3A4 enzyme. After four hours of incubation the luciferin detection reagent was added and the resulting luminescence signal was quantified in a luminometer. The no-treatment control was used as the baseline for maximum enzyme activity and Ritonavir, a known inhibitor of CYP3A4 (Ikezoe et al., 2004; Patel et al., 2004), as a positive control. Data on the graphs represent the mean and error bars of three replicates in two sets of independent experiments.

3.5 - B40 aptamer did not cause mitochondrial DNA toxicity

Mitochondrial toxicity is one of the major complications associated with long term use of ARVs, specifically NRTIs. To complete the circle, we further tested if B40 aptamer will cause mitochondrial DNA depletion in human cardiomyocytes or PBMC. Similar to T20, the B40 aptamer did not cause a significant decrease in the mtDNA levels when compared to the untreated control in both cardiomyocytes and PBMCs (**Figure 6**). The mtDNA: nDNA for B40 aptamer in cardiomyocytes was 1.17±0.19, P = 0.3 and in PBMCs was 1.16±0.11, P = 0.06, while that for T20 in cardiomyocytes was 1.01±0.17, P = 0.5 and for PBMCs was 1.08±0.10, P = 0.3. The NRTI ddC was used as a positive control for mtDNA depletion and resulted in an mtDNA: nDNA ratio of 0.72±0.12, P = 0.006 in cardiomyocytes and 0.41±0.18, P = 0.0004 in PBMCs (**Figure 6**).

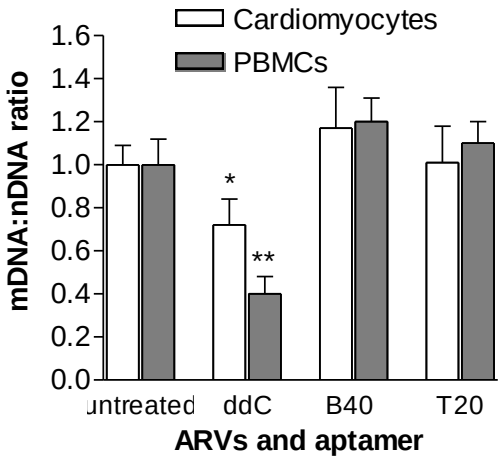

Figure 6: Effect of B40 aptamer and T20 on mitochondrial toxicity as measured by mtDNA:nDNA ratio in cardiomyocytes and PBMCs. Cardiomyocytes and PBMCs were respectively exposed to 2 μM of B40 aptamer, T20 or ddC in triplicates for 7 days. Relative amounts of nuclear and mitochondrial DNA were quantified by real time quantitative PCR amplification of the *CCOI* nuclear and the *ASPOLG* mitochondrial genes respectively. The data was extrapolated from a standard curve relating cycle numbers to relative DNA concentration. The mtDNA:nDNA ratio of untreated sample was arbitrarily set at 1.0. Only the ddC treated cells showed a statistically significant decrease in mtDNA:nDNA ratio compared to untreated control as determined by a t-test and indicated by asterisk, where * and ** indicate p values of less than 0.001 and 0.0001 respectively. Data on the graph represent the mean and error bars of three replicates in two independent experiments.

4.0- Discussion

This study describes the cytotoxic effects of a novel, cross-clade HIV-1 neutralising RNA aptamer called B40 in comparison to conventional ARVs. We used cell death, caspase 3/7 activation, MAO A and B, CYP450 3A4 and mitochondrial DNA levels as markers of toxicity in human cardiomyocytes and PBMCs. First, in contrast to most registered ARVs tested, the B40 aptamer did not cause death of cardiomyocytes and PBMCs even at the highest concentration tested of 2 µM. This is encouraging, particularly because the IC_{50} of HIV-1_{Ba-L} and several HIV-1 primary isolates by the B40 aptamer in PBMCs is less than 10 nM (Cohen et al., 2008; Dey et al., 2005b; Khati et al., 2003). We have also observed that cell death is independent from caspase 3 and 7 activation. These are executioner caspases that are involved in all caspase-mediated apoptotic pathways (Chowdhury, Tharakan, and Bhat, 2008) and our data clearly demonstrated that NRTI and NNRTIs induced apoptosis is caspase independent. These observations are in general agreement with the literature where cell death was frequently reported as being the result of mitochondrial injury (Kohler and Lewis, 2007). Mitochondrial toxicity is one of the major side effects and causes of treatment failures associated with mid to long-term use of ARVs, especially NRTIs (Kohler and Lewis, 2007). The severity of mitochondrial toxicity can range from clinically silent to life-threatening conditions such as lactic acidosis (Carr et al., 1998b). One of the most accepted hypotheses of mitochondrial toxicity is that ARVs cause a depletion of mitochondrial DNA due to inhibition of the mitochondrial DNA polymerase γ (Lewis and Dalakas, 1995; Lewis, Day, and Copeland, 2003). This results in impaired production of mitochondrial enzymes, which participate in oxidative phosphorylation, ultimately leading to diverse organ-specific and/or

systemic pathological changes. It was for this reason that we examined the B40 aptamer for mitochondrial toxicity and we have unequivocally shown that B40 does not interfere with mitochondrial DNA synthesis, ruling out potentially deleterious mitochondrial interactions associated with the therapeutic use of this compound, via the known pathways of ARV-induced mitochondrial toxicity. Due to the high incidence of these cases, the NRTI ddC is used with extreme caution in the clinical setting. Furthermore, PI-related toxicity was also caspase-independent as cell-death was not preceded by an increase in caspase-3 and -7 activity and the sharp decrease in caspase activity for most ARVs (**Figure 3**) was the result of a decrease in the total number of viable cells (**Figure 2**). The exact mechanism by which PIs induce cell death have not been fully dissected (Badley, 2005), but cell cultures treated with Ritonavir showed an accumulation of growth-arrested cells in the G1 phase and high levels of expression of the universal cyclin-Cdk kinase inhibitor p21 (Gaedicke et al., 2002). It has been speculated that accumulation of p21 could be due to inhibition of cellular proteosome resulting in cell death (Sekiguchi and Hunter, 1998). Therefore necrosis due to chemical insult is the most likely cause of cell death although caspase-independent apoptosis cannot be ruled out (Badley, 2005).

Another favorable property of B40 aptamer shown in this study is that the aptamer did not interact with MAO A and B enzymes. The two MAO isoforms are present in most tissues, including peripheral tissues and the myocardium (Grimsby et al., 1990; O'Carroll et al., 1989; Rodriguez et al., 2000; Saura et al., 1996; Shih, Grimsby, and Chen, 1990; Thorpe et al., 1987). Drugs interacting with MAO A or B can cause either up-regulation or down-regulation of the enzymes, leading to concomitant accumulation or depletion of neurotransmitters and resulting in serious clinical

effects. Clorgyline and deprenyl, which we used in this study as positive controls, are well documented inhibitors of MAO A (Hewton, Salem, and Irvine, 2007; Kitanaka et al., 2005) and MAO B (Braestrup, Andersen, and Randrup, 1975; Elsworth et al., 1978), respectively. No ARV in current clinical use has been shown to interact with either MAO A or MAO B. Notwithstanding, given the metabolic importance of MAO A and MAO B and its putative interaction with other drugs, it was imperative and prudent to look at its potential interaction with the B40 aptamer.

The B40 aptamer, favorably compared to T20, also did not affect CYP3A4 activity in both PBMCs and cardiomyocytes. Most ARVs, notably PIs are substrates and potent competitive inhibitors of CYP3A4 (Ernest, Hall, and Jones, 2005). The CYP450 superfamily of haeme-containing enzymes is the major catalyst for the oxidative transformation of most therapeutic drugs and xenobiotics in general, as well as a vast array of endogenous substances (Kalgutkar, Obach, and Maurer, 2007). The interaction of ARVs with members of the CYP450 enzyme superfamily often causes inactivation of the enzyme leading to decreased plasma levels of protease inhibitors (Kalgutkar, Obach, and Maurer, 2007). This effect is often counteracted in the clinical setting by the co-administration of Ritonavir, which is a potent inhibitor of CYP3A4 (Ikezoe et al., 2004; Patel et al., 2004), with other protease inhibitors (Youle, 2007). In this case, Ritonavir plays a dual role of both antiretroviral activity and CYP450 3A4 enzyme inhibition to boost plasma levels of other PIs. However, this is closely associated with an increased incidence of both metabolic and somatic cardiac changes, which are often silent diseases because they are not readily diagnosed. Lipodystrophy, hyperlipidaemia and insulin resistance are some of the most common such disorders (Carr and Cooper, 2000; Carr et al., 2000; Carr et al., 1998a; Carr et

al., 1998b). Furthermore, the potent inhibition of CYP3A4 by most PIs, especially Ritonavir (Ikezoe et al., 2004; Patel et al., 2004) seriously restricts the use of other auxiliary drugs that are also metabolized by the same enzyme as this could lead to life-threatening cardiac events such as arrhythmias. The property of B40 aptamer to potently and broadly neutralize HIV-1 (Dey et al., 2005b; Khati et al., 2003) without inhibiting CYP3A4 therefore makes it an ideal candidate drug to complement existing ARVs.

Taken together, these data show that B40 aptamer does not interfere with any of the major pathways of ARV-related cytotoxicity. These data agree with toxicological observations made with Pegaptanib sodium (Macugen®), which is the first and only aptamer-based therapeutic agent in clinical use (Kourlas and Schiller, 2006). Macugen® was approved for clinical use in 2005 and it did not exhibit any intrinsic toxicity when evaluated in preclinical studies (2002; 2003; Drolet et al., 2000). The only side effect reported arises almost exclusively from the injection procedure rather than the drug itself (Gragoudas et al., 2004).

The cytotoxicological data observed in this study further suggests that B40 in particular and aptamers in general may be safer than most ARVs. This argues for the development of B40 aptamer and related anti-gp120, HIV-1 neutralizing aptamers (Khati et al., 2003) as new ARVs (entry inhibitors) with no cytotoxicity. While entry inhibitors such as the B40 aptamer are expected to be less or not toxic because they act extracellularly, the results of this study should be treated with caution because there may be *in vitro* artifact. A key challenge now is to evaluate the efficacy,

pharmacokinetics and toxicity of B40 and related aptamers in a more dynamic *in vivo* animal model in preclinical studies.

CHAPTER THREE

HIV-1 unproductively infects human cardiomyocyte and induces apoptosis that can be mitigated by anti-gp120 aptamer

Submitted to: Circulation, February 2010

Chapter Summary

HIV-associated cardiomyopathy (HIVCM) is of clinical concern in developing countries were access to highly active antiretroviral therapy (HAART) is limited. Here we further elucidated the aetiology and pathophysiology of the disease. Novel approaches to improve its clinical outcomes based on a previously reported HIV-1 neutralizing anti-gp120 aptamer called UCLA-1were also evaluated. Furthermore, we investigated the direct and indirect effects of HIV-1 infection on cultured human cardiomyocytes and the mechanisms leading to cardiomyocytes damage and ways to mitigate the damage. We established a cell-based model of HIVCM. We discovered that HIV-1 unproductively (i.e. its life cycle is arrested after reverse transcription) infects cardiomyocytes. Furthermore, we found that cell-free virus initiates apoptosis through caspase-9 activation, preferentially via the intrinsic or mitochondrial initiated pathway. CXCR4 receptor-using viruses were stronger inducers of apoptosis than CCR5 (R5) utilizing variants. HIV-1 induced apoptosis of cardiomyocytes was mitigated by the UCLA1 aptamer. However, UCLA1 had no protective effective on cardiomyocytes when apoptosis was triggered by HIV-infected monocyte-derived macrophages (MDMs). Moreover, when HIV-1 was treated with UCLA1 prior to infection of MDMs, it failed to induce apoptosis of cardiomyocytes. Taken together, this data suggest that cell-free virus causes a mitochondrial initiated apoptotic cascade, which signal through caspase-9, whereas MDM-associated HIV-1 causes apoptosis predominantly via the death-receptor pathway, mediated by caspase-8. UCLA1 protects cardiomyocytes from caspase-mediated apoptosis, directly by binding to HIV-1 and indirectly by preventing infection of MDMs.

1.0 - Introduction

Chronic, long-term HIV-1 infection causes a broad range of clinical complications, some of which remain poorly elucidated. One such disease is HIV-associated cardiomyopathy (HIVCM). This disease affects the cardiovascular system and is brought about by the gradual deterioration of cardiomyocytes (CM) due to chronic HIV-1 infection. This pathology has a broad spectrum of aetiologies ranging from secondary infections in immunocompromised patients, mostly in third-world countries (Magula and Mayosi, 2003; Ntsekhe and Hakim, 2005; Ntsekhe and Mayosi, 2008), to side effects associated with HAART (Barbaro, 2003). There is however an increasing body of clinical and experimental data that points to a direct role of HIV-1 and its associated proteins as important causes of HIVCM (Fiala et al., 2004; Kan et al., 2005; Kan, Xie, and Finkel, 2000; Kan, Xie, and Finkel, 2006; Twu et al., 2002). HIV-1 infection of human CM has been observed in cardiac biopsies of HIV positive patients (Grody, Cheng, and Lewis, 1990; Herskowitz et al., 1994; Lipshultz et al., 1990), although its outcome is controversial. More clear is the role played by its structural, surface envelop glycoprotein called gp120, which has been reported to cause apoptosis of murine CM (Fiala et al., 2004; Kan et al., 2005; Kan, Xie, and Finkel, 2000; Kan, Xie, and Finkel, 2006; Twu et al., 2002). The cardio toxic effects are believed to be triggered in a caspase-dependent, mitochondria-initiated fashion, when gp120 interacts with the CXCR4 receptor on the surface of CM (Yuan et al., 2008). This in turn results in the induction of a negative ionotropic effect through increase in nitric oxide (NO) production via p38 mitogen activated protein kinase (p38 MAPK)-iPLA2-troponin I initiated NF-κB activation (Kan et al., 2005; Kan, Xie, and Finkel, 2000; Kan, Xie, and Finkel, 2006). The end result of NO production

is free-radical insult through the formation of reactive oxygen species (ROS), leading to the loss of mitochondrial membrane potential and membrane permeabilization, in a Bcl-2-inhibitable manner (Green and Reed, 1998; Kroemer, Dallaporta, and Resche-Rigon, 1998). The exact pathway is not fully understood but leakage of cytochrome *c* into the cell cytosol following permeabilization of the mitochondrial membrane triggers the activation of the caspase 9/3 complex eventually culminating in nuclear DNA fragmentation (Budihardjo et al., 1999).

The apoptotic signal is not restricted to host's cell interaction with viral proteins. Cytokines secreted by HIV-infected cells, primarily infiltrating macrophages, also play a role in the development of HIVCM. Furthermore, evidence suggests that tumour necrosis factor (TNF) might be the main cause in murine cells, by interacting with the its respective death receptor, TNF-R1, leading to extrinsic or death-receptor mediated apoptosis (Finkel et al., 1992; Monsuez et al., 2007). However, *in vitro* apoptosis triggered by gp120 is initiated preferentially through the CXCR4 receptor than the death-receptor. Nonetheless, analysis of cardiac biopsies revealed strong expression of both caspase-9 and TNF, which was detected in infiltrating inflammatory cells of HIVCM patients, but not in HIV-infected patients without HICVM, making a strong argument towards death-receptor initiated apoptosis *in vivo* (Twu et al., 2002). Therefore, there is a growing body of evidence suggesting that both pathways are at play, but why and under which conditions each pathway is favoured remains to be investigated. In this chapter, we report the HIV-1 infection kinetics of human CM as well as the associated apoptotic pathways and receptors involved during HIV-CM interaction. We also show that a shortened and synthetic derivative of gp120 binding and HIV-1 broadly neutralizing aptamer called UCLA1 mitigated HIV-initiated apoptosis in CM (Mufhandu *et al*., 2010 unpublished data).

2.0 – Materials and methods

2.1 - Cell cultures

The human cord-blood stem cell-derived cardiomycocytes (CM) were purchased from Celprogen (USA) and cultured in Human Cardiomyocyte Expansion Media as previously described (Lopes de Campos et al., 2009). Briefly, the cells were cultured at 37°C, 5% CO2 in tissue culture flasks and 96 well plates containing a proprietary growth matrix purchased from Celprogene.

Peripheral-blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque™ PLUS (GE Healthcare, USA) density gradient centrifugation from heparinised human buffy coats of normal, HIV-negative donors and cultured as previously described(Khati et al., 2003). Briefly, PBMCs were harvested by centrifugation and seeded on tissue culture flasks (Corning) at a density of 2×10^6 cells/ml in RPMI medium containing 20% fetal bovine serum (Sigma-Aldrich) followed by stimulation with phytohemagglutinin (PHA) and treatment with interleukin-2 (IL-2) as described(Khati et al., 2003). Monocyte derived macrophages (MDMs) were isolated from PBMCs by adherence and cultured on chamber slides (Lab-Tek™, Nunc, USA) in X-VIVO 10 medium (BioWhittaker) supplemented with 2 % autologous for 7 days. After 7 days incubation, differentiated MDMs were typed by flow cytometry using an antibody directed against the CD68 cell surface receptor (R&D Systems, USA) and detected with a secondary FITC-conjugated (KPL, USA).

2.2 - HIV-1 isolates

A panel of 7 previously reported(Cilliers et al., 2005; Coetzer et al., 2006) clinical isolates of HIV-1 subtype C were used in this study. They were grouped according to co-receptor utilization. A total of two R5 tropic viruses (SW2, SW4), two X4 viruses (SW12 and TM46b) and three R5/X4 viruses (CM9, DU179-05-99, RP1 and SW30) were respectively used to infect cells. Virus conditioned media (cond media) was prepared from day 5 culture supernatants of HIV-infected PHA-stimulated IL-2 treated PBMCs.

2.3 - Antiretrovirals

Zidovudine (AZT) and AMD3100 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA.

2.4 - UCLA1 anti-gp120 monoclonal aptamer

UCLA1 is a shortened derivative of B40t77 RNA aptamer(Dey et al., 2005) produced using solid-phase synthesis as described(Cohen et al., 2008). The UCLA1 aptamer is capped and stabilized by addition of an inverted thymidine at the 3′-end and a dimethoxyltrityloxy-$(CH_2)_6$-SS-$(CH_2)_6$-phospho linker at the 5′-end as described (Cohen et al., 2008).

2.5 - PCR detection of proviral DNA in CM

A total of 50,000 CM or PBMCs cultured in 96-well plates (Corning, USA) were exposed to HIV-1 at a concentration of 200 ng/ml p24. Virus-containing supernatant we pre-treated with 400 U/ml of DNase I in 5 mM MgCl2 for 1 h at room temperature, to remove any pro-viral DNA that may interfere with the PCR detection. Cultures we done in the presence or absence of 500 nM of the entry inhibitor AMD3100, 100 nM RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) or 100 nM of UCLA1 aptamer. The infection was facilitated by spinoculation at 1220 *g*, 32 ºC for 1 h. The virus-infected cells were further incubated at 37 ºC, 5 % CO_2 and harvested at 0, 2, 4, 6, 24 h and 6 days respectively. Prior to harvesting, the cell monolayer was washed 6 times with 150 µl of warm PBS (Lonza, USA). The cells were incubated for 2 h at 55 ºC in 200 µl of lysis buffer containing 2 mg/ml of protein kinase, 0.45 % NP-40 (Roche, USA) and Tween-20 (Sigma-Aldrich, USA), 5 mM $MgCl_2$ and PCR buffer (Promega, USA). Following protein digestion and inactivation of proteinase K (10 min at 95 ºC) the cell lysate was subjected to PCR amplification as previously described(Schmidtmayerova et al., 1998).

2.6 - ***Detection and modulation of HIV-1 initiated apoptosis in cultured CM by TUNEL***

Cardiomyocytes seeded at a density of 20,000 cells/well were treated either with culture medium (mock-treatment), 500 nM of AMD3100, 100nM RANTES, 100 nM of Z-IETD-FMK, a preferential caspase 8 inhibitor (casp8i, Promega, USA), 100 nM

Z-LEHD-FMK, a preferential caspase 9 inhibitor (casp9i, Promega, USA) or 100 nM UCLA-1 for 1 hour at 37^{o}C in the CO_2 incubator. Cells were then exposed to HIV-1 subtype C clinical isolates at 30 ng/ml p24. After 3 days of incubation, the cell monolayers cultured in flat-bottom 96-well plates (Corning, USA) were fixed in 1% (w/v) paraformaldehyde and washed twice with 200 µl of a proprietary wash buffer (Guava Technologies, USA). The cells were then resuspended in 25 µl of a Brd-UTP labelling mix (Guava Technologies, USA) and incubated for 60 min at 37 ºC. At the end of the incubation a volume of 200 µl of a proprietary wash buffer from the same manufacturer was added and the cells were harvested by centrifugation. The cells were then resuspended in 50 µl of the anti-BrdU-TRITC staining mix for 30 min at room temperature. A volume of 150 µl of rinsing buffer was added and the cells were analysed in a Guava EasyCyte Plus flow cytometer (Guava Technologies, USA). Data was analysed using the TUNEL program in the Cytosoft data acquisition and analysis software, Version 5.2. A total of 2000 events were acquired at a time and each well was acquired in triplicate. The results were displayed in a dot plot relating forward scatter (FSC) over fluorescence intensity (PM1) as well as a histogram (PM1 fluorescence intensity vs. number of events). The general apoptosis inducer staurosporin (Sigma-Aldrich, USA) was used as a positive control for TUNEL staining.

2.7 - *Cardiomyocyte apoptosis in HIV-infected MDM co-cultures*

Day 7 monocyte derived macrophages (MDMs) seeded at a density of 50,000 cell/well in a 24 well tissue culture plate (Corning, USA) were infected with R5 and dual-tropic viruses (30 ng/ml, p24) for 24 h in a total volume of 200 µl of X-VIVO 10

medium (BioWhittaker) supplemented with 2 % autologous serum. The cells were then washed 3 times with an equal volume of PBS followed by the addition of 50,000 uninfected CM. To investigate apoptotic triggers and modulators, cells were cultured in the presence or absence of a TNF-α inhibitor (TNF-Ra, R&D Systems, USA), AMD3100, casp8i, casp9i and UCLA-1. Controls included mock-infected and infected CM and MDM alone and uninfected CM-MDM co-cultures. Following two days of exposure the cell monolayer was washed twice with warm PBS prior to trypsin (Lonza, USA) treatment. The strong adhesion and resistance properties of MDM were exploited to recover the less adherent CM. A 2 min trypsin treatment at 37 ºC was enough to recover over 95 % of the CM but less than 5 % of the macrophages, thereby minimizing cross-contamination. MDM contamination of CM cultures was determined by flow cytometry using a 1:200 dilution of a FITC-conjugated antibody directed against the CD4 cell surface receptor, which is absent on CM. The CM were then stained for TUNEL detection as described above.

2.8 - Indirect immuno-fluorescence for Cytochrome c release detection in HIV-exposed CM

Cardiomyocytes were either mock-infected or infected with the HIV-1_{CM9} dual tropic strain for 2 days in the presence of either 100 nM of casp8i; 100 nM of casp9i or 100 nM of UCLA1. For all casp8i and casp9i, the inhibitors were added to the cell monolayer 2 h prior to infection. The UCLA1 aptamer was first incubated with the virus for 1 h before infection. Following 2 days of incubation the cells were harvested, fixed, stored and washed as above. A total of 20,000 cells/well in 96-well, U-bottom plates, were treated with 5 μg/ml of mouse-anti human cytochrome *c* monoclonal

antibody (Santa Cruz Biotechnology, Inc., USA) in a volume of 20 µl for 1 h at 37 ºC. The cells were then harvested by centrifugation, washed twice in an equal volume of PBS and counter stained with 1 µg/ml of Alexa Fluor 514 goat anti-mouse polyclonal antibody (Invitrogen, USA) in a 50 µl volume for 30 min at room temperature. At the end of the incubation, rinsing buffer was added up to 200 µl and the cells were analysed in the Guava EasyCyte Plus flow cytometer (Guava Technologies, USA).

2.9 – Caspase-8 and -9 enzyme activity and cell viability

CM seeded in 96-well tissue culture plates (Corning, USA) at a density of 10,000 cells/well in a volume of 150 µl of proprietary growth media (Celprogen, USA). The cells were then exposed to eleven different conditions, namely 1 - media alone, 2 - conditioned media, 3 - HIV, 4 - 100 µM of preferential caspase 8 inhibitor Z-IETD-FMK (casp8i) (Promega, USA), 5 - 100 µM of preferential caspase 9 inhibitor Z-LEHD-FMK (casp9i) (Promega, USA), 6 - conditioned media and 100 µM Z-LEHD-FMK, 7 - conditioned media and 100 µM Z-IETD-FMK, 8 - conditioned media and 100 nM UCLA1 aptamer, 9 - HIV pre-incubated for 1 h with 100 nM of UCLA1 aptamer, 10 - HIV and 100 µM Z-LEHD-FMK, 11 - HIV and 100 µM Z-IETD-FMK and harvested daily for 7 days. The harvesting involved the storage of the 96-well plates at -80 ºC. On day 7, the plates were allowed to equilibrate to room temperature followed by quantification of caspase 8 and 9 activity as well as cellular ATP levels, using the Caspase-Glo®8, Caspase-Glo®9 and the CellTiter-Glo® luminescence-based detection kits as instructed by the supplier (Promega, USA). Wells containing media alone were used as controls for background luminescence and subtracted from the test

values. The results were expressed as relative light units (RLUs) and plotted in a graph relating RLU to number of day.

3.0 - Results

3.1 - HIV-1 infection of CM is co-receptor independent and abortive

The ability of HIV to invade cultured cardiomyocytes based on co-receptor tropism was accessed as was the virus life cycle once inside the host.

Cultured human CM were infected by all the R5, X4 and R5/X4 subtype C HIV-1 isolates tested in the presence or absence of respective co-receptor inhibitors as evidenced by the presence of proviral DNA (**Table 1**). This contrasted with the successful inhibition of infection of PBMCs by all subtype C HIV-1 isolates in the presence of specific inhibitors of the relevant co-receptors (**Table 1**). Furthermore, the synthesis of proviral DNA and its dose-dependent inhibition by AZT (**Figure 1**), suggested that HIV-1 infects CM, undergoes reverse transcription but does not integrate into the host's genome. It also showed that the pro-viral DNA is synthesised inside the host by the viral reverse transcriptase and is not an artefact caused by the presence of residual pro-viral DNA in the viral supernatant even after DNase treatment. Strengthening this evidenced was the time-dependent decrease in proviral DNA as observed by the peak in pro-viral DNA load 2 h post infection and subsequent decrease to almost undetectable levels after 24 h and complete absence after 6 days (**Figure 1**). In contrast with the infection kinetics observed in PBMCs, there is a time-associated increase of pro-viral DNA, indicative of pro-viral DNA amplification and hence productive infection (**Figure 1**).

Table 1: HIV-1 infection of cultured human CM and primary PBMCs. The presence of pro-viral DNA as indicated by the presence of a 539 bp fragment amplified from the U3/U5 region was indicative of viral entry and is shown as +. PCR negative samples were considered entry negative and are shown as -.

		Entry Inhibitors							
		Untreated		AMD3100 (500 nM)		RANTES (100 nM)		UCLA1 (100 nM)	
HIV Strain	Tropism	CM	PBMCs	CM	PBMCs	CM	PBMCs	CM	PBMCs
SW2	R 5	+	+	+	+	+	-	+	-
SW4	R 5	+	+	+	+	+	-	+	-
SW12	X4	+	+	+	-	+	+	+	-
TM46b	X4	+	+	+	-	+	+	+	-
cm9	R5X4	+	+	+	-	+	+	+	-
DU179 (05-99)	R5X4	+	+	+	+	+	+	+	-
RP1	R5X4	+	+	+	+	+	+	+	-

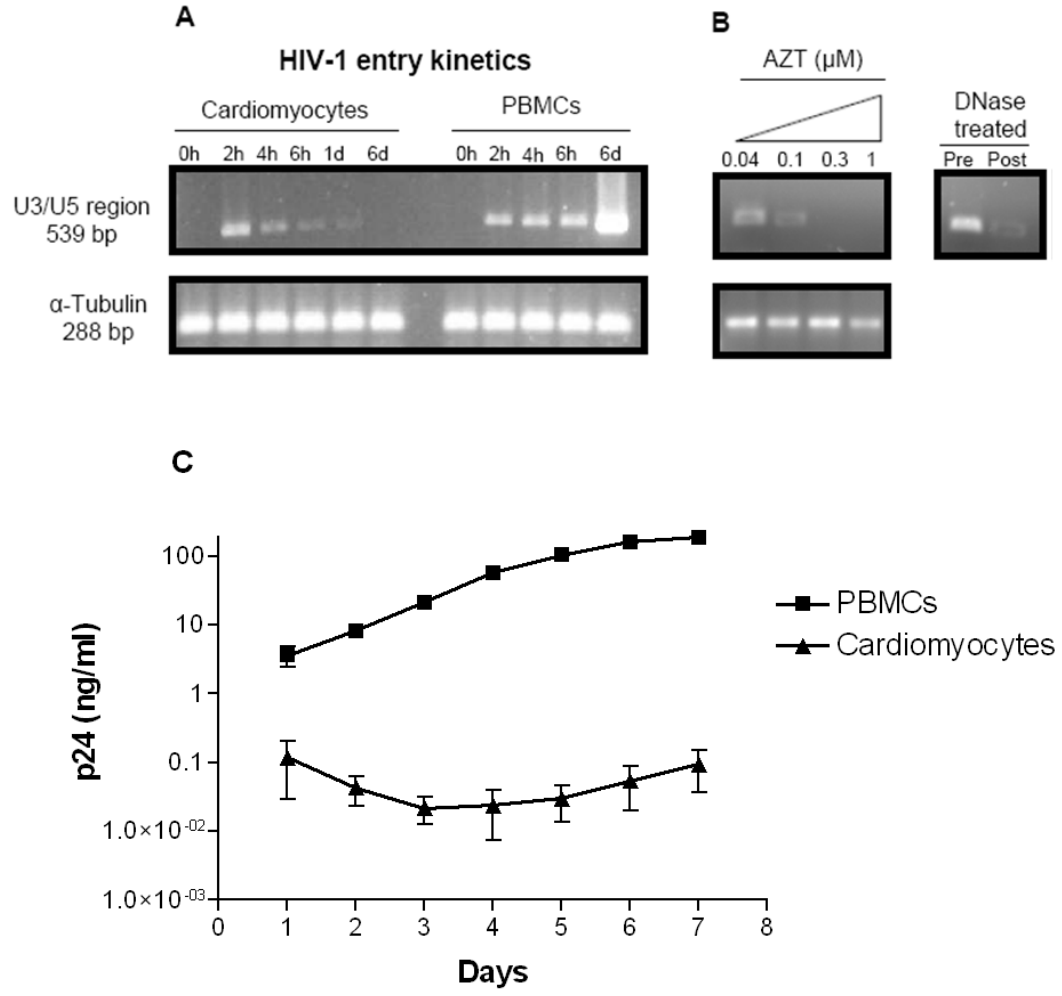

Figure 1: Kinetics of HIV infection of CM and PBMC. (A) Agarose-gel electrophoresis of the PCR-amplified U3/U5 region of CM9-infected culture lysates. The 539 bp fragment indicates the presence of proviral DNA. Cell lysates were analysed at 0, 2, 4, 6, 24 h and 6 days post infection. A 288 bp region of the gene coding for α-tubulin was amplified and resolved at the same time to ensure equal loading of the wells. (B) CM were exposed to serial dilutions of AZT at a maximum concentration of 1 μM for 2 h prior to infection. Two hours post-infection, cell lysates were analysed for the presence of proviral DNA as above. Agarose-gel electrophoresis of cell-free virus culture lysates were digested with DNaseI for 1 h prior to infection.

3.2 - *Apoptosis of CM is HIV-1 tropism-dependent and is triggered predominantly by the interaction of HIV-1 with the CXCR4*

The consequences of the interaction between HIV-1 of different co-receptor tropisms and the chemokine receptor CXCR4 on CM was investigated. Induction of apoptosis in CM by X4 viruses was on average 3 times higher than that of R5 viruses (54 % ± 6.6 % TUNEL positive for X4 viruses vs. 18 % ± 5.3 % for R5 viruses, **Figure** 2). Dual-tropic (R5/X4) viruses also induced considerably more apoptosis in CM than R5-tropic viruses by a magnitude of 2.7 (**Figure 2**). Almost complete inhibition of apoptosis induced by R5 viruses was observed in the presence of the highly selective CXCR4 antagonist, AMD3100 as well as the UCLA1 aptamer resulting in an average cellular protection against apoptosis from 82 ± 5.0 % to 97 ± 2.0 % (P = 0.024) (**Figure 2**). Apoptosis was even more pronounced in CM infected with X4 and dual tropic viruses where, on average, the TUNEL positive population decreased 7.4 fold, from 52 ± 6 % to 5 ± 3 % ($P < 0.001$) in the presence of AMD3100 only (**Figure 2**). The UCLA1 aptamer did not show as strong a protective effect as AMD3100 but cell survival still decreased nearly 4 fold, from 52 ± 6 % to 13 ± 6 % ($P < 0.001$) when compared to untreated controls (**Figure 2**). On the other hand, RANTES, the physiological ligand for the CCR5 chemokine receptor, had no statistically significant effect in protecting CM from HIV-1 associated apoptosis when compared to untreated controls ($P = 0.62$) (**Figure 2**). Therefore apoptosis is triggered through the CXCR4 receptor and is stronger when initiated by X4 utilizing viruses. The synthetic CXCR4 receptor antagonist protects CM from apoptosis as does UCLA1, albeit to a lesser extent.

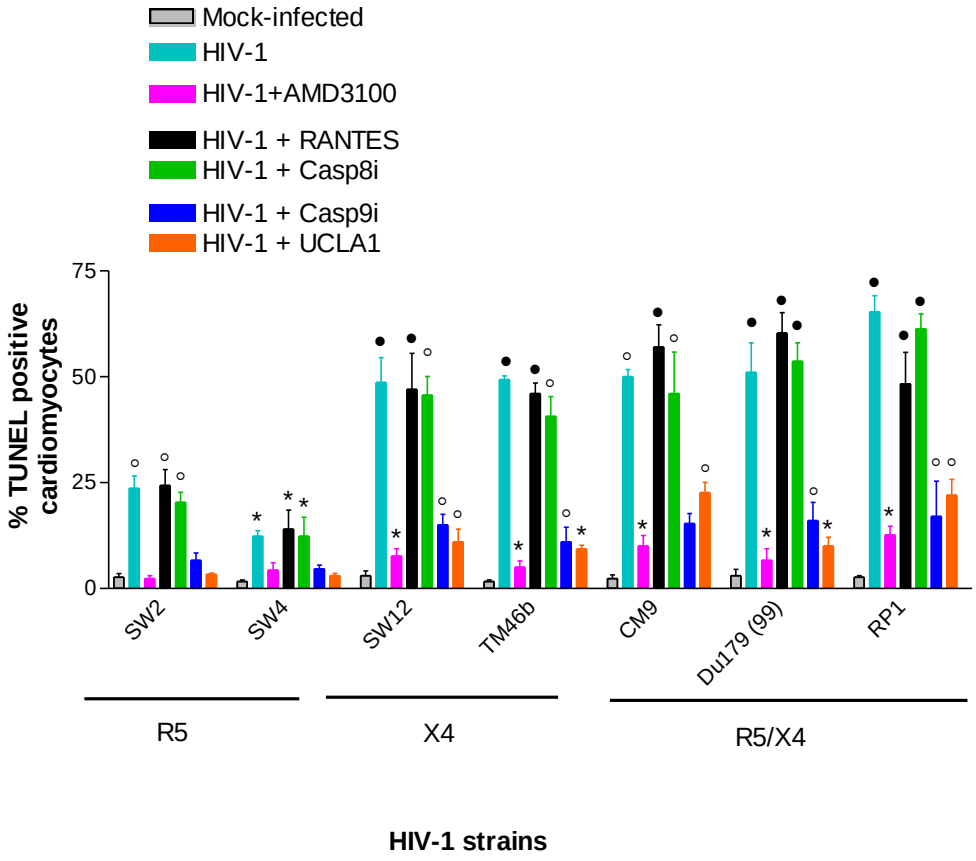

Figure 2: Quantification of the relationship between chemokine co-receptor utilization and apoptosis initiation by TUNEL staining of CM, 3 days post infection with HIV-1. CM were exposed for 3 days to: mock infection; HIV; HIV and AMD3100; HIV and RANTES; HIV and casp8i; HIV and casp9i; HIV andUCLA1. After 3 days he cells were stained for TUNEL detection with a TRITC conjugated antibody directed against Brd-UTP Fluorescent labelled cells were analysed in the Guava EasyCyte Plus flow cytometer (Guava Technologies, USA) using the Cytosoft software, Version 5.2. A total of 2000 events were acquired for both assays and the data is representative of 3 independent experiments. The data was acquired in dot plots relating fluorescent intensity to forward scatter and presented as bar graphs as percentage of the total cell population that was TUNEL positive. Statistically

significant TUNEL positive results, as indicated by the *t*-test, are marked as: *, $P < 0.05$; •, $P < 0.005$; º, $P < 0.0005$.

3.3 - The extrinsic or death-receptor pathway is the preferred apoptotic mechanism in CM co-cultured with HIV-infected MDM

There is evidence suggesting that HIV-1 infected, infiltrating macrophages in cardiac tissue are strongly associated with caspase-dependent CM apoptosis.

There was no statistically significant difference in apoptosis modulation between untreated CM exposed to HIV-1 infected MDMs with all the 5 strains in the presence of AMD3100 ($P > 0.09$), except for DU179-99, where there was a decrease in TUNEL positive cells from 24.33 ± 3.05 % to 15.66 ± 3.21 % ($P = 0.027$) (**Figure 3**). No significant apoptosis modulation was achieved in the presence of the UCLA1 aptamer ($P > 0.07$) (**Figure 3**). Strong protection against apoptosis was achieved in the presence of TNF-R1 (4.6 fold ± 2.6, $P < 0.02$) and casp8i (5.3 fold ± 2.2, $P < 0.02$), two key components of the extrinsic apoptotic pathway (**Figure 3**). The X4-tropic viruses induced apoptosis 1.35 times more efficiently than the dual tropic viruses, although this difference was not statistically significant ($P = 0.36$) (**Figure 3**). Thus, HIV-1 infected macrophages induce apoptosis in uninfected CM via the death receptor pathway and can only be mitigated by soluble TNF-R1. Neither UCLA1 nor AMD3100 had any protective effect.

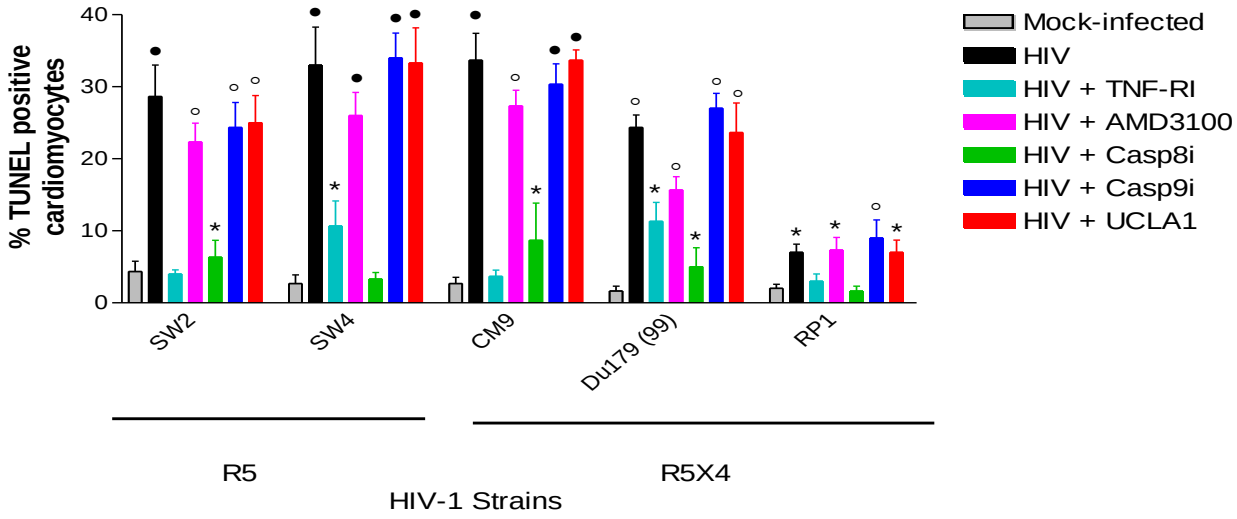

Figure 3: CM apoptosis in a co-culture model with HIV-infected MDM. MDM were infected with the R5-tropic viruses SW2, SW4 or the dual tropic viruses CM9, DU179 (99) and RP1 for 24 h. Uninfected CM were added and the co-cultures were then incubated for a further 24 h. Following incubation, the cell monolayers were stained for TUNEL detection. Statistically significant decreases in TUNEL positive cells, as indicated by the *t*-test, are marked as: *, $P < 0.05$ and •, $P < 0.005$ º, $P < 0.0005$ respectively.

3.4 - The intrinsic or mitochondrion-initiated apoptosis is the preferred death-signal pathway mediated by caspase 9 activation

Cytochrome *c* leakage from the mitochondria as well as caspase-9 activation are markers of the intrinsic mitochondrial-initiated pathway and caspase-8 activation is the canonical caspase of the extrinsic apoptotic pathway.

Untreated control cells showed a viable, healthy population with only 2.4 ± 0.7 % staining positive for Cytochrome *c* release (**Figure 4 A**). This contrasted with the strong staining in HIV-infected cells, accounting for 41.77 ± 3.0 % of the total (**Figure 4 B**). The preferential caspase-8 inhibitor conferred a minor but significant protection against apoptosis (32.2 ± 5.3 % apoptotic cells) (**Figure 4 C**). Conversely, very strong apoptosis inhibition was achieved in the presence of the preferential caspase-9 inhibitor, which effectively reduced the number of apoptotic cells to 14.3 ± 3.6 % of the total cell population (**Figure 4 D**). UCLA1 also had a significant protective effect whereby 22.4 ± 4.4 % of cells stained positive (**Figure 4 E**).

There was an inversely proportional link between total cellular ATP, which was a measure of cellular viability, and caspase-9 activation during HIV-1 infection (**Figure 5 A**). A drop in ATP levels was observed 1 day after peak caspase-9 activity and subsequent recovery at day 5 correlated with a decrease in caspase-9 levels. Both caspase-9i and UCLA1 had a protective effect in cell viability as indicated by an exponential increase in cellular ATP from day 3 to 7.

Caspase 8 activation in HIV infection did not rise significantly from the untreated control cells or any other test condition (**Figure 5 B**). Furthermore there was no statistical difference between cells treated with casp8i or UCLA1 and untreated, infected cells up to day 3 ($P = 0.5$). There was however an exponential increase in enzyme activity levels in infected, UCLA1 treated cells, very similar to untreated controls from day 3 to 7. The opposite was observed with casp8i treated cells, where there was a decline in enzyme activity up to day 4 followed by an increase up to day 7.

Peak caspase-9 enzyme activity was observed two days post-exposure to HIV-1, resulting in a 9.3 fold increase in enzyme levels, when compared to the untreated controls (P = 0.01) (**Figure 5 C**). This was followed by a sharp decrease in enzyme activity relative to control cells. The preferential caspase-9 inhibitor only allowed for a 2.62 fold increase in enzyme activity in the presence of HIV-1 after two days of treatment (P = 0.02). UCLA1 also had a modulatory effect in caspase-9 activity during infection (RLU = 2410, P = 0.001), albeit weaker than that of casp9i (RLU = 1515, P = 0.02). All other test conditions did not significantly affect caspase 9 levels (P > 0.07) during the entire course of the experiment. Therefore both intrinsic and extrinsic apoptosis are triggered by cell-free HIV in CM, however the extrinsic pathway is dominant as the high levels of caspase-9 activity indicated. Caspase 9 is also a key player in CM apoptosis since its inhibition resulted in almost complete abrogation of apoptosis and inhibition of caspase-8 had a much less pronounced effect. UCLA1 obstruction of the CXCR4 binding site of gp120 also blocked apoptosis. Therefore apoptosis is initiated by interaction of cell-free virus with the CXCR4 chemokine receptor the signal is transduced via the preferential activation of caspase-9.

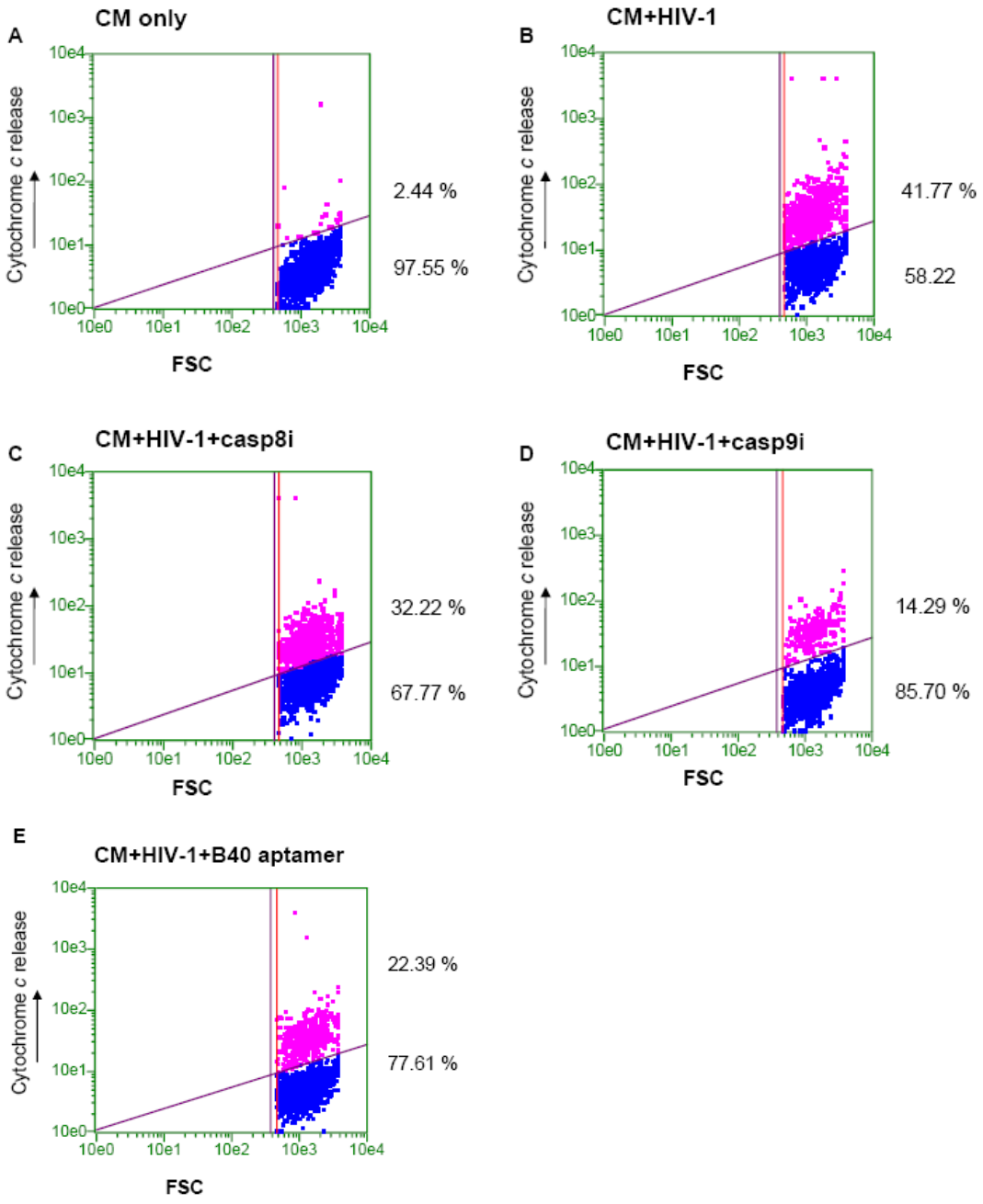

Figure 4: Cytochrome *c* release in HIV-infected CM. CM were either: (A) mock infected; (B) HIV-1 infected; (C) infected in the presence of casp8i; (D) infected in the presence of casp9i and (E) infected with virus pre-incubated with UCLA1. Data were presented in dot plots relating fluorescent intensity as a measure of Cytochrome *c* release against forward scatter.

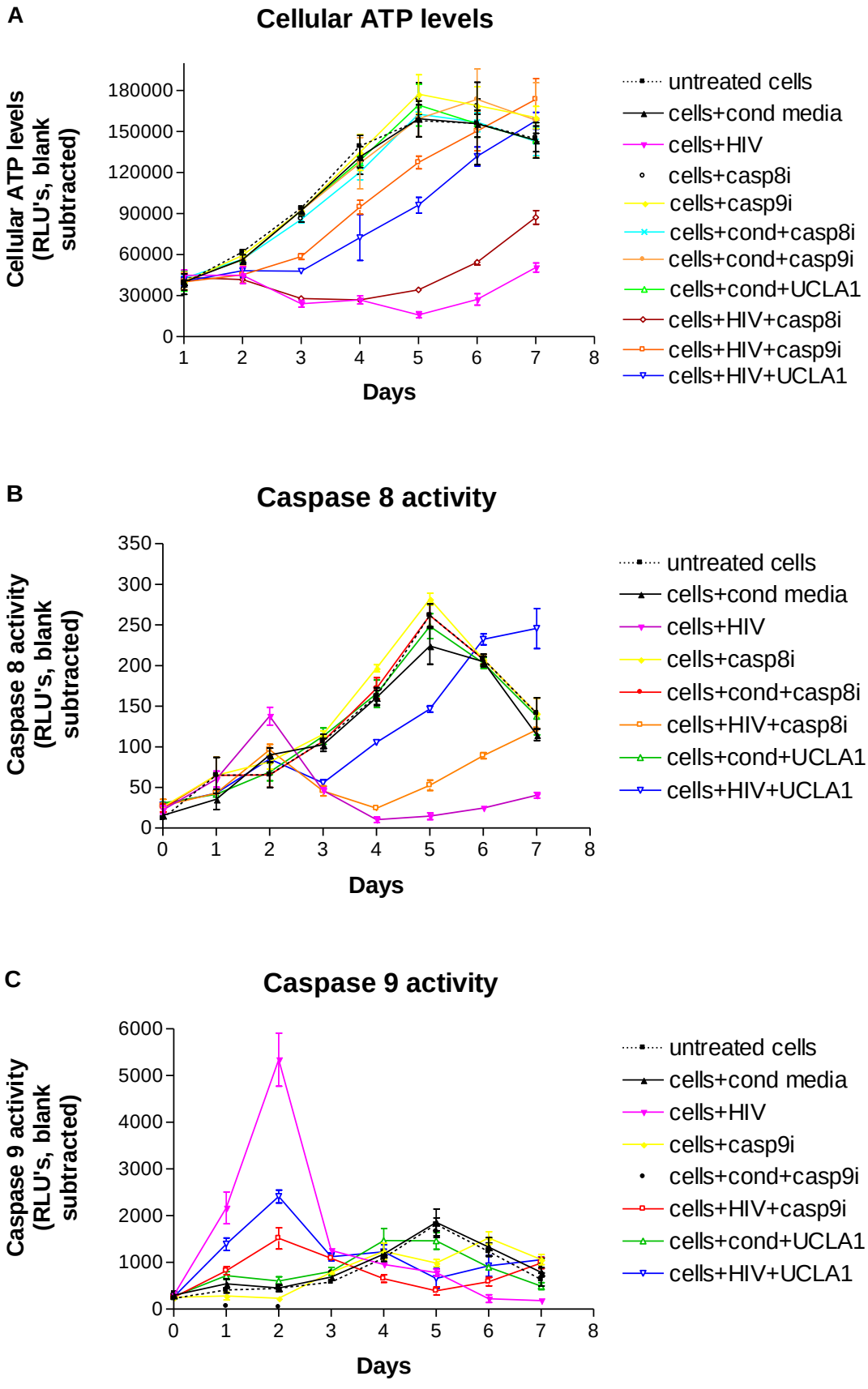
A
Cellular ATP levels
Cellular ATP levels (RLU's, blank subtracted)
180000
150000
120000
90000
60000
30000
0
1
2
3
4
5
6
7
8
Days
untreated cells
cells+cond media
cells+HIV
cells+casp8i
cells+casp9i
cells+cond+casp8i
cells+cond+casp9i
cells+cond+UCLA1
cells+HIV+casp8i
cells+HIV+casp9i
cells+HIV+UCLA1
B
Caspase 8 activity
Caspase 8 activity (RLU's, blank subtracted)
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
Days
untreated cells
cells+cond media
cells+HIV
cells+casp8i
cells+cond+casp8i
cells+HIV+casp8i
cells+cond+UCLA1
cells+HIV+UCLA1
C
Caspase 9 activity
Caspase 9 activity (RLU's, blank subtracted)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
Days
untreated cells
cells+cond media
cells+HIV
cells+casp9i
cells+cond+casp9i
cells+HIV+casp9i
cells+cond+UCLA1
cells+HIV+UCLA1

Figure 5: caspase-8 and -9 activity in CM infected with HIV-1. CM were exposed to: media alone; conditioned media; HIV; 50 μM of casp8i; 50 μM of casp9i; conditioned media and 50 μM of casp8i; conditioned media and 50 μM of casp9i; conditioned media and 100 nM UCLA1 aptamer; HIV and 50 μM of casp8i; HIV and 50 μM of casp9i; HIV pre-incubated for 1 h with 100 nM of UCLA1 aptamer. Cells were harvested daily for 7 days. (A) ATP levels were measured as a correlate of cell viability; (B) caspase-8 and (C) caspase-9 activity as indicators of extrinsic and intrinsic apoptosis respectively. All assays were luminescence-based and presented as graphs relating RLU to number of days.

4.0 – Discussion

Apoptosis has been observed during HIV infection but its aetiology remains poorly understood. In the current study we elucidated some key mechanisms of CM apoptosis during HIV-1 infection by employing a range of specific apoptosis inhibitors as well as the HIV-1 neutralizing aptamer UCLA1. Firstly, we accessed the ability of HIV-1 to infect CM. We used two R5, two X4 and three R5X4 subtype C HIV-1 and observed a tropism-independent infection (**Table 1**). The formation of pro-viral DNA (**Figure 1A**), further suggests that HIV-1 can enter and infect human cardiomyocytes; albeit unproductively. Entry of HIV-1 into cardiomyocytes is thought to be mediated by binding of the virus to ganglioside GM1, inducing a re-modelling of the cell membrane and formation of microvilli thereby entangling the virions and subsequent internalization via macropinocytosis (Fiala et al., 2004; Liu et al., 2002; Twu et al., 2002). The lack of entry inhibition in the presence of known coreceptor antagonists (**Table 1**) further strengthens the argument for a receptor-independent mode of entry. Nonetheless the virus was unable to complete its life cycle, as indicated by the time-dependent decrease in pro-viral DNA upon infection (**Figure 1A**) as well as by the absence of p24 antigen in culture supernatants (**Figure 1.C**). It is likely that the pro-viral pre-integration, nuclear-localization complex is never formed resulting in a time-dependent degradation of the pro-viral DNA in the cytoplasm. There is also *in vivo*, clinical evidence for direct infection of CM in AIDS patients (Grody, Cheng, and Lewis, 1990; Herskowitz et al., 1994) but these studies were limited to detecting viral nucleic acid and proteins in cardiomyocyte biopsies. No studies were undertaken to probe into the viral replication cycle in the infected tissue.

Experimental data has also revealed that although the virus cannot complete its life cycle, it was still capable of inducing significant caspase-dependent apoptosis. Furthermore, entry was not required for apoptosis since the apoptotic signal was initiated by interaction with the cell-surface receptors, the CXCR4 chemokine receptor and the TNF-R1 death receptor. The predominant apoptotic pathway varied from CXCR4-triggered, mitochondrion-initiated to CD95/Fas-ligand initiated, depending on the culture conditions as previously reported in T cells (Bottarel et al., 1999; Roggero et al., 2001). In the absence of MDM, HIV-1 directly interacts with the CXCR4 chemokine receptor on CM, resulting in cell death. This death-inducing signalling however is significantly stronger in X4 and dual tropic viruses, possibly because these viruses interact with higher affinity with the CXCR4 receptor than R5 tropic viruses. The UCLA1 aptamer binding site maps to the CCR5/CXCR4 binding region of gp120 on HIV-1 (Cohen et al., 2008; Dey et al., 2005; Khati et al., 2003), which satisfactory explains its strong anti-apoptotic properties, very similar to that brought about by the highly selective CXCR4 receptor ligand AMD3100. This observation, together with Cytochrome *c* leakage into the cell cytosol points to a CXCR4/HIV interaction as the key apoptosis trigger as recently reported in murine CM (Yuan et al., 2008) and earlier in primary human macrophages (Herbein et al., 1998), resulting in mitochondrial initiated apoptosis (Roggero et al., 2001; Twu et al., 2002) independent of death-receptor mediated signalling (Adachi, Gottlieb, and Babior, 1998). It has been reported that the CXCR4 receptor is involved in both caspase-dependent and caspase-independent apoptosis induction. There is however some controversy regarding the circumstances under which each pathway is favoured. Some groups have reported that caspase-dependent apoptosis requires interaction with both CD4 and CXCR4 receptors, and in the absence of CD4, the apoptotic signal

initiated by CXCR4 is caspase-independent (Bottarel et al., 1999; Nardelli et al., 1995; Vlahakis et al., 2001). On the other hand, an elegant and more recent study conducted by Roggero and co-workers revealed that CXCR4 cooperation with a CD4 signal was not required for the induction of mitochondrial-mediated apoptosis (Roggero et al., 2001). Furthermore, deletion of the cytoplasmic tail of CD4 in cells co-transfected with both CD4 and CXCR4 did not shift the apopotic pathway from caspase-dependent to caspase-independent (Biard-Piechaczyk et al., 2000; Jacotot et al., 1997). A strong argument in this favour can also be made in our study, in that we detected caspase activity in the absence of CD4 transcription and expression (results not shown).

Furthermore, we observed that both apoptotic pathways were intimately related with caspase activity, an observation not supported in a previous study using T-cells (Vlahakis et al., 2001). This study argues that X4 tropic HIV-1 env glycoprotein initiates a caspase-independent apoptosis signal. The 9.3 fold increase in cellular caspase 9 levels, and the improved cell survival brought about by its inhibition, further strengthens the argument for a caspase-dependent apoptotic pathway. Comparing these two studies may not be possible at all not only because different cell types were investigated but also due to the fact that we made use of fully infectious, cell-free virions and not pure preparations of recombinant, monomeric gp120, which in its native form, is trimeric and could elicit different apoptotic responses. Beyond this we found evidence for a second, caspase-dependent apoptotic pathway, initiated at the TNF-R1 death receptor in the presence of HIV. Although the peak in caspase 8 activity was not statistically significant, there was a significant degree of cellular protection when HIV-exposed CM were treated with the preferential caspase 8

inhibitor Z-IETD-FMK. Caspase 8 is regarded as being a key initiator of death receptor mediated apoptosis and under physiological conditions, it is primed by the TNF superfamily member of related apoptosis triggers (Boldin et al., 1996; Muzio et al., 1996). Our case for extrinsic apoptosis rests on the merit of its inhibition rather than on upregulation of its downstream effector caspase.

The MDM-cardiomyocyte co-culture model shows a drastic shift in the preferential apoptotic trigger, towards the extrinsic pathway, initiated by interaction with TNF-R1 by secreted death-signalling molecules produced by infected MDMs. Under these conditions apoptosis progressed with much more rapid kinetics, measured in hours as opposed to days when initiated by the CXCR4 receptor. The almost complete abrogation of apoptosis in the presence of TNF-R1 as well as casp8i sheds further light into preferential apoptotic pathways and receptor trigger during HIV-1 infection/exposure of CM. In this scenario, the UCLA1 aptamer had no impact in apoptosis modulation, which was expected since the extrinsic or death-receptor initiated apoptosis is triggered by host, rather than viral factors. Nonetheless, by preventing macrophage infection, UCLA1 can strongly modulate both apoptotic pathways. A further advantage of UCLA1 over conventional antiretrovirals is that it directly neutralizes the deleterious effects of cell-free virus by preventing it from interacting with the CXCR4 chemokine receptor. In fact, protease-inhibitors used in HAART give rise to non-infectious virions with fully functional envelope glycoproteins that have been observed to lead to apoptosis of non-infected $CD4^+$ and $CD8^+$ T-cells (Esser et al., 2001). Since the ratio of circulating non-infectious to infectious virions is exponentially greater (Dimitrov et al., 1993; Piatak et al., 1993), the role played by these non-infectious virus particles in the progression to

cardiovascular disease cannot be ignored, particularly in cell expressing the CXCR4 chemokine receptor. Therefore, it is perhaps erroneous to assume that because these viral particles are not capable of productive infection, that they play no role in HIV pathogenesis. This argues for the development of a novel class of ARVs, such as UCLA1, that can target both cell-free and cell associated virus as a strategy to preventive the deleterious consequences of non-productive infection of CM and broaden the effectiveness of HAART.

CHAPTER FOUR

Aberrant production of TNF, IL-1β and IL-6 cytokines by HIV-infected macrophages triggers cardiomyocyte apoptosis and is restored by UCLA-1

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Chapter Summary

The aetiology of HIV-associated cardiomyopathy (HIVCM) is poorly understood due to its multifactorial nature as well as the difficulties in generating good cellular and animal models. Here, we created a simple cellular model of HIVCM using cultured human cardiomyocytes (CM) and primary human macrophages. We successfully mitigated CM apoptosis with a non-toxic, previously described RNA aptamer called UCLA1, which binds gp120 and broadly neutralizes HIV-1 infectivity. When infectious virions were pre-treated with UCLA1, the expression levels of 27 different cytokines remained unaltered when compared to infected controls. During co-culture with HIV-infected monocytes derived macrophages (MDM), extensive apoptosis was observed in CM. However, when cultures were pre-treated with a soluble death receptor of the tumor necrosis factor superfamily (TNF-R1) and with neutralizing antibodies against the cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6), singly or in combination, there was a significant improvement in CM survival. Recombinant IL-6 and IL-1β had no apoptotic effect directly but when combined with TNF, CM death was significantly greater than TNF treatment alone. Together, these data show that IL-1β, IL-6 and TNF homeostasis are crucial to preserve CM integrity. This was achieved in the presence of UCLA-1, which further highlights the utility of the aptamer as a potentially safe and effective therapeutic agent to complement current ARVs.

1.0 - Introduction

Human immunodeficiency virus-associated cardiomyopathy (HIVCM) is a growing health concern and is fast emerging as a disease with significant morbidity in long-term infected HIV-positive patients (Barbaro, 2005b; Lewis, 2000; Magula and Mayosi, 2003; Ntsekhe and Hakim, 2005; Ntsekhe and Mayosi, 2008). To date, the aetiology and outcome of this AIDS-related pathology remains poorly understood (Barbaro, 2003a). The difficulties in dissecting the events that precede the onset of HIVCM are increased by the multifactorial nature of the disease. It has been shown that virions as well as viral proteins secreted by HIV-infected cells can result in significant cardiac damage (Fiala et al., 2004; Jacotot et al., 1997; Lewis, 2001; Lipshultz et al., 1990; Twu et al., 2002). Furthermore, cardiotrophic viruses that arise due to immunodeficiency (Hajjar et al., 2005; Herskowitz et al., 1994) as well as antiretrovirals (Carr and Ory, 2006) have been identified as being important triggers of HIVCM. In addition the role of host factors such as pro-apoptotic cytokines secreted by infected macrophages in HIVCM pathogenesis is being recognised as a pivotal player leading to cardiovascular disease during long-term HIV infection (d'Amati, di Gioia, and Gallo, 2001; Lewis, 1989; Liu et al., 2001; Twu et al., 2002). Infiltrating, HIV-infected macrophages secrete a broad spectrum of pro-inflammatory and immuno-regulatory cytokines. Some of these cytokines have been reported to cause extensive apoptosis in non-infected cells located in close proximity to and by direct contact with infected cells (Heinkelein, Sopper, and Jassoy, 1995; Herbeuval and Shearer, 2006; Nardelli et al., 1995). Pro-inflammatory cytokines such as TNF, interleukin-1β (IL-1β) and IL-6 are secreted by macrophages during HIV infection and a direct link between high serum levels of these cytokines and cardiomyocyte

apoptosis has recently been suggested (Monsuez et al., 2007). Furthermore, high levels of IL-1β/6 are strongly correlated with rheumatoid arthritis (de Benedetti et al., 1991; Hermann et al., 1989; Houssiau et al., 1988; Mayet et al., 1990) and congestive heart failure (Long, 2001; Wollert and Drexler, 2001). High concentrations have also been detected in cardiac myxomas and cervical and bladder carcinomas (Scheller, Ohnesorge, and Rose-John, 2006). Recently, in a 3 year follow up study of patients with acute myocardial infarction, IL-6 levels were identified as a key predictor of mortality (Tan et al., 2009). In *ex-vivo* and murine models, IL-1β/6 have been associated with decreased cardiac contraction, extensive cardiac remodelling and increased mortality (Finkel et al., 1992; Givertz and Colucci, 1998; Li et al., 2005). Cardiac pathology caused by these cytokines is further compounded by their ability to enhance HIV replication in chronically infected $CD4^+$ T cells as well as in MDMs (Kedzierska et al., 2003), since there is a strong link between productive viral replication and cardiovascular morbidity. Therefore modulation of cytokine expression during HIV infection to normal or physiological levels could result in significant protection of cardiovascular tissue. The role of IL-1β, IL-6 and TNF produced by HIV-infected MDM in CM apoptosis was investigated in our *ex-vivo* model of HIVCM.

Cardiomyocyte (CM) apoptosis during HIV infection is a multifactorial process, involving a broad spectrum of differentially expressed cytokines. Moreover, it remains to be elucidated whether the apoptotic stimuli from cytokines act in a paracrine or autocrine fashion in CM. Finally, if the apoptotic trigger consists of cytokines secreted by HIV-infected infiltrating tissue macrophages, it would be of significant clinical importance to unravel whether regulating the host's response

would have any benefit in CM survival. This can be a potential therapeutic target, particularly if the death signal is restricted to one or two cytokines that can be modulated without causing further morbidity to the host. In this study we bridged that gap by looking at the differential expression of 27 different cytokines during HIV infection in CM, MDMs and co-cultures of HIV-infected MDMs with uninfected CM. We observed a significant deviation from homeostatic levels in co-cultures of HIV-infected MDMs and uninfected CM when compared to mock infected controls, which directly correlated with CM death. We then investigated the effects of using a a shortened synthetic derivative of anti-gp120 2′-fluoro substituted RNA aptamer with broad HIV-1 neutralizing activity called (Cohen et al., 2008; Dey et al., 2005; Khati et al., 2003) and minimal cytotoxicity (Lopes de Campos et al., 2009) on CM apoptosis and cytokine expression. The advantage of using this aptamer called UCLA1 over conventional ARVs in current clinical use is that it interferes with viral entry by binding to the CCR5/CXCR4 chemokine co-receptor binding pocket on the viral gp120 (Cohen et al., 2008), thereby inhibiting entry, as opposed to interfering with post-entry events like most ARVs such as protease and reverse transcriptase inhibitors. UCLA1 is also capable of blocking the interaction between the host cells and non-infectious virions, which surpass the number of infectious virions by several orders of magnitude (Dimitrov et al., 1993; Piatak et al., 1993). Although non-infectious, these virions still retain their ability to trigger apoptosis in HeLa cells, by interacting with the CXCR4 co-receptor (Esser et al., 2001), which has been identified as one of the key triggers in CM apoptosis (Yuan et al., 2008).

2.0 - Materials and Methods

2.1 - Cells, virus and antibody

Human cord-blood stem cell-derived cardiomyocytes were purchased from Celprogen (USA). Cardiomyocytes were cultured in Human Cardiomyocyte Expansion Media (Celprogen) and cultured as recommended.

PBMCs were and MDM were isolated from heparinized human buffy coats of normal, HIV-negative donors as previously described (Khati et al., 2003). Buffy coats were obtained from the South African National Blood Services. PBMCs were cultured in RPMI medium containing 20% foetal calf serum (Lonza, USA) and MDM in X-Vivo 10 supplemented with 2 % autologous serum.

Following 7 days of incubation, the resulting macrophages were typed via flow cytometry using an anti-CD68 antibody (R&D Systems, USA) and detected with a secondary FITC-conjugated antibody (KPL, USA). Only cultures containing over 95 % of $CD68^+$ cells were used.

The previously reported CM9 HIV-1 subtype C, CCR5/CXCR4 dual-tropic virus strain was used (Cilliers et al., 2005). In order to ensure that the virus containing supernatant was free of any viral proteins, the virions were separated from the rest of the culture medium by size-exclusion centrifugation, using a 200,000 Kda NMW (Millipore, USA) and resuspended in an equal volume of PBS.

The HIV-1 broadly-neutralizing monoclonal antibody, IgG b12, was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.2 - Culture conditions

Two types of cultures were used: a monoculture, whereby CM and MDM were seeded at a density of 50,000 cells/well in separate wells; and a co-culture system where 50,000 MDM and CM were seeded in the same well in the presence of CM growth media. For cell viability studies in co-cultures where it was required to assess both cells independently, CM were separated from MDM by trypsin treatment for 5 min at 37 ºC. Under these conditions, less than 5 % of the harvested cells were MDM as assessed by flow cytometry. These cells were then labelled as cond CM and cond MDM respectively (denoting conditioned cells). All cultures were either mock infected or infected with the HIV-1 clinical isolate, CM9.

2.3 - Virus infectivity assays

A virus titer of 100 ng/ml p24 was incubated for 1 h with 8, ½ log dilutions of UCLA1 starting at a maximum concentration of 100 nM of UCLA1. Cells were then infected in a total volume of 300 µl with treated virus, untreated virus or left uninfected. Supernatants were collected every day after day 3 for 7 days for p24 ELISA using the Vironostika® HIV-1 p24 antigen detection kit (Biomerieux, France).

2.4 - Identification and quantification of cytokine expression by multiplex flow cytometry

A total of 200 µl of culture supernatants where harvested 24 h post-infection and stored at - 80 ºC. The supernatants where then centrifuged at 14,000 rpm for 10 min at 4 ºC to remove any solid debris. A total of 50 µl from each sample was analysed for expression of a range of 27 different cytokines, via multiplexing using immuno-magnetic beads-coupled antibodies directed at the cytokines in a Bio-Plex® Suspension Array System (Bio-Rad, USA). The cytokines assayed were: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1alpha, MIP-1beta, PDGF-BB, RANTES, TNF, VEGF. Data was analysed using the Bio-Plex Manager Software Version 5.0 as well as GraphPad Prism® Version 3.02.

2.5 - Cell viability assay

Following 24 h of incubation, cell viability was determined using the ATP-based assay CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) and used according to the manufacturer's recommendations. Both the plates and the reagent were allowed to equilibrate at room temperature. Then an equal volume of reagent was added to the wells and further incubated at room temperature for 20 minutes to allow for the luminescent signal to stabilize. Following the incubation, the luminescent signal was measured in a Modulus® Microplate Reader luminometer (Promega, USA) using a count integration time of 1 second. The raw data was measured in relative light units (RLUs) and the final results were normalized (i.e. the total number of viable cells in the treated samples were respectively subtracted by the

total number of viable cells in the cell control, which was not treated with any drug or test molecule) and expressed as percentage cell death. Viability of untreated control cells was over 97%. Wells containing media alone were used as controls for background luminescence and subtracted from the test values. The general formula to calculate percentage toxicity was: % of viable cells = [(RLU of drug treated cells - RLU of medium control) ÷ (RLU of untreated cell controls - RLU of medium control)] × 100.

2.6 - Neutralization of IL-1β, IL-6 and TNF activity in HIV-infected MDM, uninfected CM co-cultures

Cytokine neutralizing antibodies were added alone or in combination, at the same time as the uninfected CM cultures were seeded with the HIV-infected MDM cultures. Antibodies against human IL-1β and IL-6 and the soluble death receptor for TNF, TNF-R1 (R&D Systems, USA) were added at a single concentration of 100 nM. Following 24 h of co-culturing, CM were harvested as described and ATP levels were measured as above as co-relates of cell viability. As controls, CM were co-cultured with uninfected MDM in the presence of 100 nM of antibody. The RLUs for control cells were used as a set as a point that represented 100 % cell viability from which the experimental percentage of cell viability was determined. The data was plotted in a bar graph showing antibody treatment against % cell survival. The results represent the mean and standard deviation of two independent experiments.

2.7 - Treatment of CM with recombinant cytokines

Log-phase CM were exposed to recombinant IL-1β, IL-6 and TNF (R&D Systems, USA), singly or in combination, at a starting concentration of 10 ng/ml of each cytokine, followed by seven ½ log serial dilutions, in a total final volume of 100 µl. Following 24 h exposure, ATP levels were measured as described. The RLUs for control cells represented 100 % cell viability from which the experimental percentage of cell viability was determined. The minimum concentration of cytokine required to kill 50 % of the cells (IC_{50}) was extrapolated from a plot relating percentage cell viability against cytokine concentration (ng/ml). The results represent the mean and standard deviation of two independent experiments.

3.0 - Results

3.1 - UCLA1 inhibits viral replication in PBMCs

Pre-treatment of the HIV-1_{CM9} clinical isolate with 100 nM of UCLA1 inhibited infection of PBMCs by 92.2 ± 3.8 %, as indicated by the near absence of p24, a surrogate marker for viral replication, p24 (Figure 1). The neutralization efficiency of UCLA1 as indicated by the IC_{50} value of 8.9 nM, was very close to that of the broad neutralizing monoclonal antibody, IgG b12 (IC_{50} = 2.9 nM), which was used as the positive control for HIV neutralization. IgG b12 decreased overall viral replication by 97.8 ± 0.3 % at 100 nM.

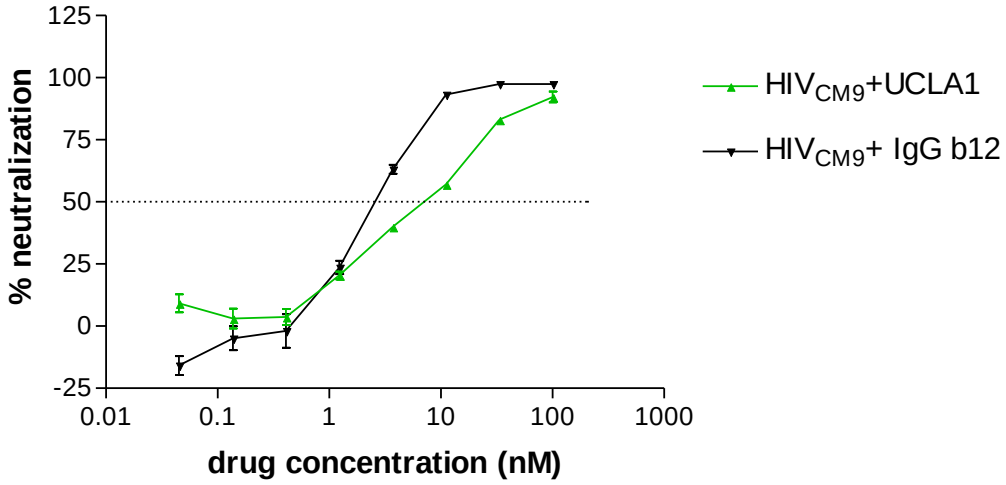


Figure 1. Neutralization of HIV-1_{CM9} infectivity by UCLA1. Virus was incubated with UCLA1, at serially diluted concentrations, for 1 h followed by infection of PBMCs. After 4 days of incubation, supernatants were analyzed for p24 production as a correlate of replication or neutralization. The broad neutralizing monoclonal antibody, IgG b12, was used as a positive control for neutralization. Strong

neutralization was observed in the presence of UCLA1, with an IC_{50} of 8.2 nM, close to that of IgG b12 (IC_{50} = 2.9 nM).

3.2 - TNF, IL-1β and IL-6 are strongly up-regulated during HIV infection and are restored to pre-infection levels by the UCLA1 aptamer

Basal levels of expression of all measured cytokines in uninfected or mock infected CMs were below 150 pg/ml (**Figure 2 A**). This contrasted with that of MDM where the basal levels of most cytokines was above 1000 pg/ml, particularly pro-inflammatory, was observed (**Figure 2 B**). Expression of all the 27 cytokines tested were significantly higher in MDMs than in CM, except for IL-2, IL-13, Eotaxin, IL-5 and GM-CSF, where levels were less than 2-fold greater than what was observed in CM supernatants (**Figures 2 A** and **B**). VEGF was the only cytokine where basal levels in CM cultures were greater than in MDM cultures by almost 6-fold (**Figures 2 A** and **B**). MDM+CM co-cultures had a cytokine profile almost identical to that of the MDM single cultures except that the levels of all 27 cytokines were lower and not cumulative as expected, since there were twice as many cells (**Figure 2 C**). HIV infection had a more pronounced effect on cytokine expression in CM than in MDMs or MDM+CM co-cultures. No cytokines were down regulated when compared to mock-infected supernatants. In infected MDM and MDM+CM co-cultures, the following cytokines increased less than two-fold when compared to uninfected controls: IL-4, IL-10, IL-12, IL-15, Eotaxin (**Figure 2.B.4**), IL-8, IP-10, MIP-1β, MCP-1 and RANTES (Figure 2.B.5). Down-regulation was observed only in MDM and the co-cultures. A 3.2-fold decrease in the pro-inflammatory cytokine MIP-1α concentration (**Figure 2.B.5**) was observed in MDM-infected cultures (from 20,655 ±

269 pg/ml to 6496 ± 173 pg/ml). The homeostatic cytokine PDGF also dropped from 5573 ± 64 pg/ml to 1688 ± 372 pg/ml, representing a 3.3-fold reduction (Figure 2.B.6). Both cytokines where also down regulated in infected co-cultures, where a 3.8-fold decrease for MIP-1α was observed (20,592 ± 161 pg/ml to 4810 ± 51 pg/ml) and a 4.3-fold decrease for PDGF (from 834 ± 53 pg/ml to 217 ± 10 pg/ml). Furthermore, basic-FGF also dropped from 65 ± 10 pg/ml to 4 ± 1 pg/ml representing a 16.3-fold decrease. There were only two cytokines that were up-regulated only in MDM and co-cultures but not in CM, namely IFN-γ and TNF. Both remained at baseline levels post-infection in CM but in infected MDM a 24-fold increase for IFN-γ (from 66±1 to 1558±22 pg/ml) and 20-fold for TNF (from 495±8 to 9824±66 pg/ml) respectively. The increase in co-cultures was less pronounced with TNF (14-fold increase, from 712±30 to 8214±8 pg/ml) but higher for IFN-γ (36-fold increase from 36±8 to 1249±39 pg/ml). Pre-treatment of the CM9 virus with 100 nM of UCLA1 for 1 h prior to infection resulted in restoration of cytokine levels to pre-infection levels in all cultures (**Figure 2**). Two key cytokines, IL-1β and IL-6 were strongly up-regulated upon HIV infection in all 3 culture models (**Figures 2.A.5**, **2.B.5**, **2.C.5**).

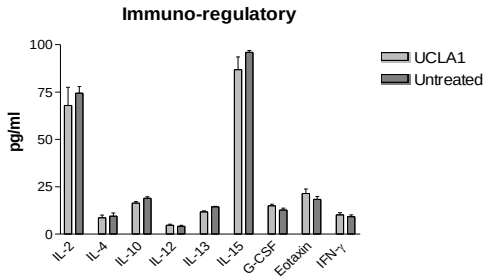
A

CM

Uninfected

Infected

1



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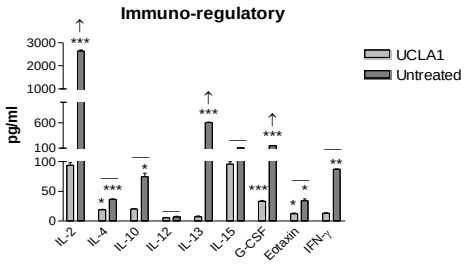

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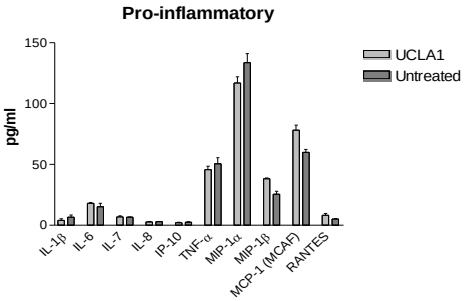

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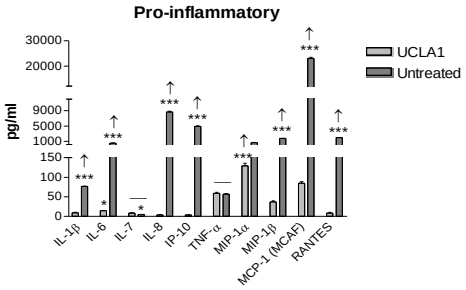

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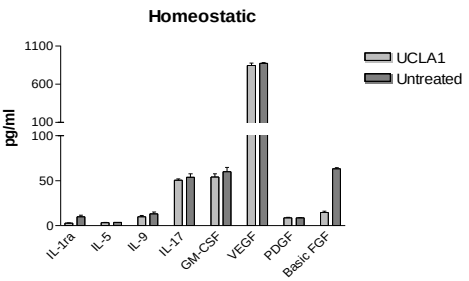

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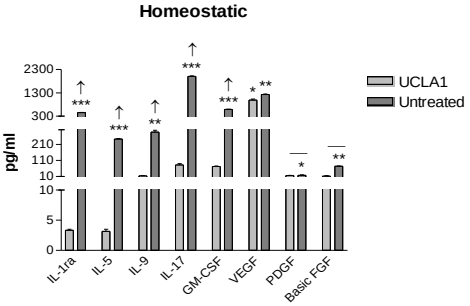


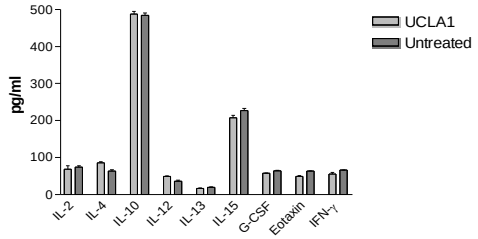
B

MDM

Uninfected

Infected

1



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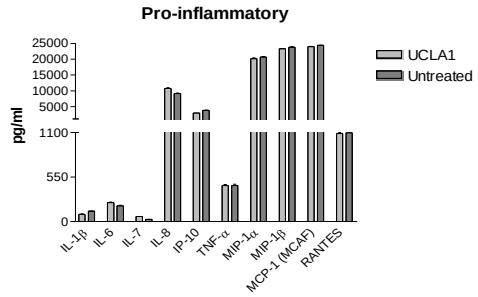

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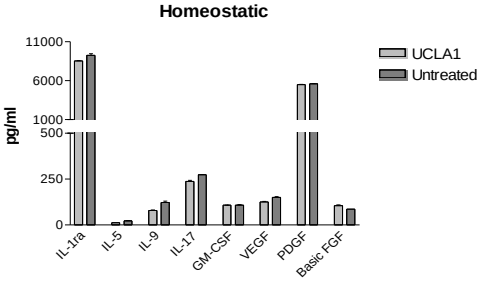

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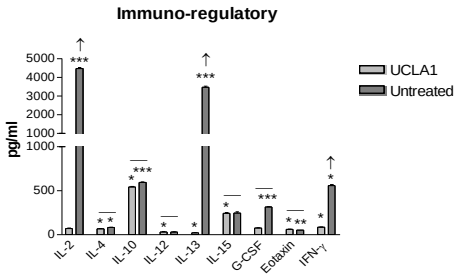

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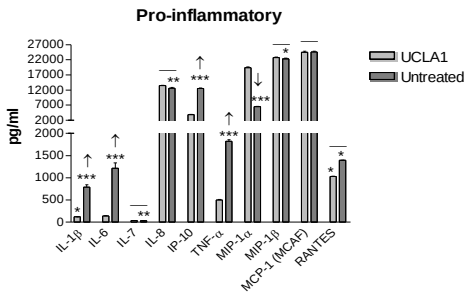

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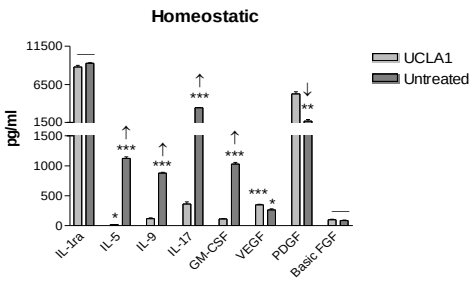


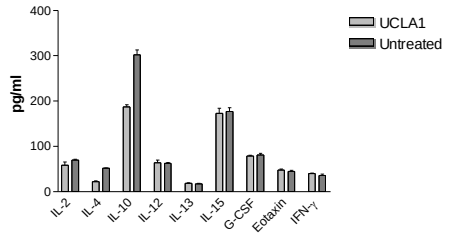
C

CM+MDM

Uninfected

Infected

1



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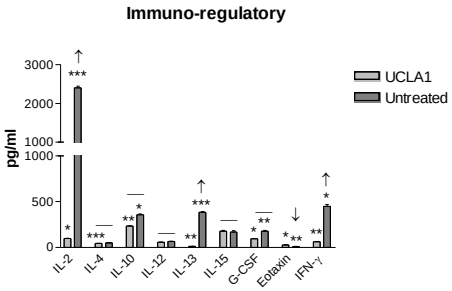

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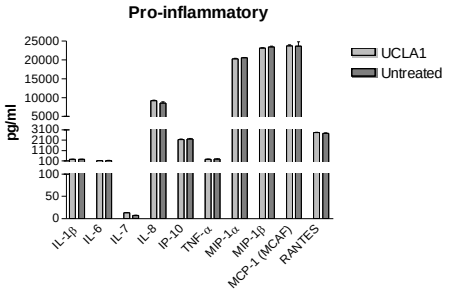

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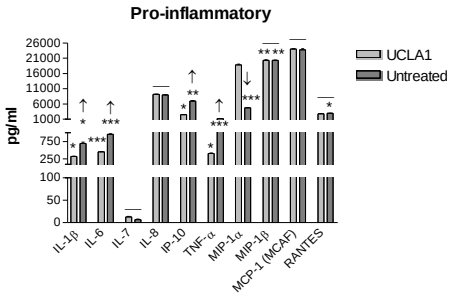

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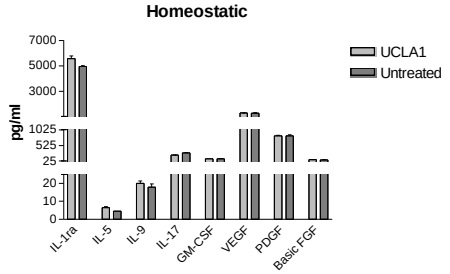

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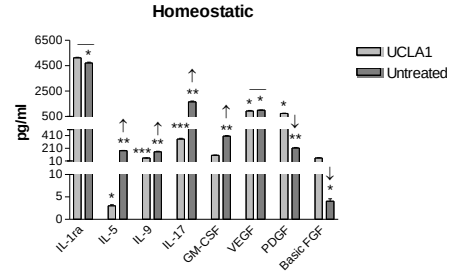

Figure 2: Cytokine profiling during HIV-1_{CM9} infection. Cytokine expression during HIV-infection was assessed in three different cultures: (A) CM, (B) MDM, (C) CM+MDM. Cultures were infected with HIV-1_{CM9} for 24 h prior to assay. In the CM+MDM co-cultures, MDM were infected for 24 h, the infected monolayer was washed and fresh growth media was added, together with uninfected CM. Following a further 24 h of incubation, the supernatants were assayed for cytokine expression. Uninfected cells were used as controls for basal cytokine expression against which infected cultures were compared. Very large deviations from baseline levels were observed in CM with all cytokines during HIV infection except for TNF and IFN-γ. MDMs had comparatively high basal levels of cytokines, particularly pro-inflammatory cytokines in uninfected cultures, when compared to MDMs. Only 10 out of 27 cytokines were significantly up-regulated. Virus-free co-cultures showed a suppression, rather than a cumulative effect in cytokine expression when compared to MDM alone, presenting a profile more closely resembling that of CM single cultures. Statistically significant deviations from uninfected control cultures, as determined by the *t*-test, are indicated by asterisks, where, *, **, *** indicate *P* values of less than 0.05, 0.005 and 0.0005 respectively. Cytokine modulation was denoted as: ↑ More than 2.5-fold up-regulation; ↓ down-regulation; − no change or minimal up-regulation, of less than 2.5-fold.

3.3 - CM apoptosis in the uninfected CM/HIV-infected MDM co-culture system was inhibited by UCLA-1 or the simultaneous neutralization of IL-1β, IL-6 and TNF

Extensive CM apoptosis was observed in infected MDM/uninfected CM co-cultures, effectively reducing cell viability to 43.7 ± 6.7 %, when compared to untreated controls (**Figure 3**). No change in cell viability was observed between infected and mock infected MDM cultures (**Figure 3**). Complete CM protection was achieved in the presence of UCLA1 (**Figure 3**) as well as by neutralizing IL-1β, IL-6 and TNF simultaneously (**Figure 4**). Treatment with TNF-R1 and neutralization of IL-1β and IL-6, conferred partial protection only (**Figure 4**). There was no statistical difference between mock infected and UCLA1-treated HIV-infected CM samples (95.8 % Vs 97.2 % cell viability, P = 0.16). An equal effect to that observed with UCLA-1 treatment, was observed with the simultaneous neutralization of IL-1β, IL-6 and TNF, resulting in complete CM protection (97.2 ± 1.6 % from treated Vs 41.8 ± 5.0 % for untreated cells, P = 0.001). This contrasted with TNF-R1-only treated, infected cultures where cell viability was maintained at 84.9 ± 3.2 % (P = 0.0012). A near identical degree of protection was conferred when cultures were treated with TNF-R1 and IL-1β or TNF-R1 and IL-6 (81.8 ± 3.5 %, P = 0.003 for TNF-R1 + IL-1β Vs 85.7 ± 2.5 %, P = 0.003 for TNF-R1 + IL-6).

The co-neutralization of IL-1β and IL-6 also improved cell survival from 40.0 ± 3.6 % to 73.2 ± 3.7 %, P = 0.0003, albeit not by as much as TNF-R1 treatment or in combination with any the other antibodies targeting IL-1β or IL-6 (**Figure 4**). This effect however, was not achieved by neutralizing each cytokine independently, since cell survival did not improve when compared to untreated controls (47.3 ± 5 % in

treated Vs 45.0 ± 4 % in untreated, $P = 0.5$ for IL-1β and; 42.9 ± 4.6 % in treated Vs 49.0 ± 4.7 % in untreated, $P = 0.2$ for IL-6). Under no conditions did MDM viability decrease.

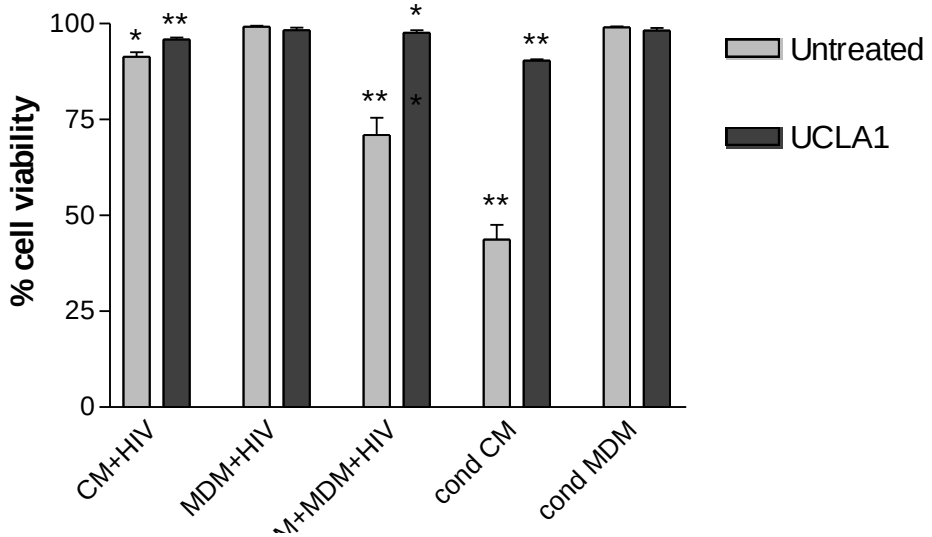

Figure 3: Cell viability during HIV-1_{CM9} infection. Cells were cultivated either as single cultures or co-cultures and mock infected or infected with 100 ng/ml, p24 of HIV-1_{CM9}. Cellular ATP was then measured as a correlate of cell viability. Cells approached 100 % viability in all infected MDM and infected cond. MDM cultures. Statistically significant variations from mock-infected cultures was observed in all infected, untreated CM cultures. Only in infected, UCLA1-treated cond CM cultures was there a significant deviation from sham infected cultures. Statistically significant deviations from sham-infected cultures were determined using the *t*-test and denoted by asterisks, where, *, **, *** indicate *P* values of less than 0.05, 0.005 and 0.0005 respectively.

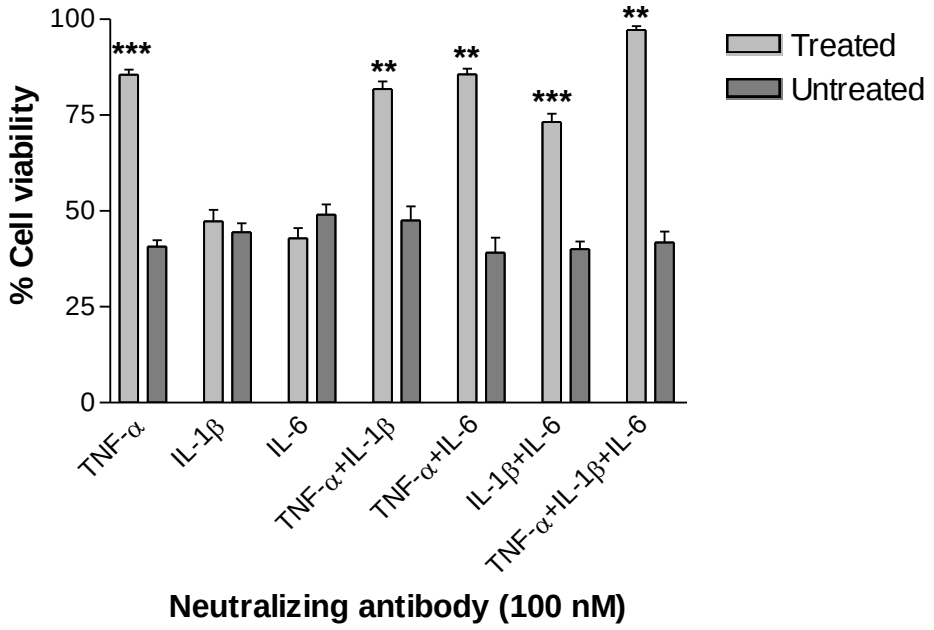

Figure 4: Protection of CM from apoptosis with neutralizing antibodies directed against TNF, IL-1β and IL-6 in the HIV-infected MDM + uninfected CM co-culture model. Day 7 MDMs were infected with HIV for 24 h. Thereafter, an equal number of CM were added to the HIV-infected MDM, together with 100 nM of neutralizing antibody targeting either one or several cytokines simultaneously. After a further 24 h, CM were removed by trypsin digestion and cellular ATP levels were measured. The data was plotted as bar graphs, showing cell viability in the presence (treated) and absence (untreated) of neutralizing antibodies.

3.4 - The cytotoxic effects on CM during co-culture with HIV-infected MDM can be mimicked in healthy, uninfected CM with recombinant TNF, IL-1β and IL-6

Neither IL-1β nor IL-6 had any detrimental effect on healthy, uninfected CM cultures at a maximum concentration of 10 ng/ml (**Table 1**). However, when used in combination with TNF, they caused a significant decrease in the IC_{50} from 650 ± 73 pg/ml to 190 ± 35 pg/ml for TNF + IL-1β and 230 ± 14 pg/ml for TNF + IL-6 ($P < 0.0001$ for all values). An even sharper decline was observed in healthy CM cultures treated with all 3 cytokines simultaneously, resulting in a decrease of the IC_{50} to 190 ± 35 pg/ml ($P < 0.0001$).

Table 1: Dose-dependent cytokine-induced cytotoxicity of CM. Cells were exposed to TNF, IL-1β and IL-6 alone or in combination at a maximum concentration of 10 ng/ml. After 24 h of incubation, cellular ATP levels were measured as a correlate of cell viability. The IC_{50} was extrapolated from a linear curve relating cytokine concentration to cell viability.

Cytokine	IC_{50} (pg/ml)*	Fold-decrease in EC_{50}#	*P* values[$]
TNF	650 ± 73	N/A	N/A
IL-1β	>10,000	-	-
IL-6	>10,000	-	-
TNF + IL-1β	190 ± 35	3.4	< 0.0001
TNF +IL-6	230 ± 14	2.82	< 0.0001
IL-1β + IL-6	>10,000	-	
TNF + IL-1β + IL-6	120 ± 46	5.4	< 0.0001

*For each individual cytokine
Compared to TNF treatment alone
[$] Measured against the IC_{50} of TNF

4.0 - Discussion

This is the first study that analysed the differential expression of a panel of 27 different cytokines in CM and MDM during HIV infection. A direct link between cytokine expression in infected MDM to CM pathogenesis was clearly established, as the data from the co-culture system reveals, shedding further light into one of the many mechanisms of the multifactorial nature of HIVCM (Barbaro, 2003b). In a similar study, adult rat cardiac myocytes presented abnormal contractile function when exposed to medium where alveolar macrophages from the same species had been activated with endotoxins (Balligand et al., 1993). The only measured anomalies were reduced CM contraction as well as increased NO levels, unlike the extensive apoptosis reported in our study. There are however, significant differences between the two studies. Firstly, we did not use conditioned media but co-cultures, which ensure a continuous and steady production of cytokines, which mimics *in vivo* scenario more closely. It also exposed the CMs to a different spectrum and concentration of cytokines than what would be observed with conditioned media, since it would be erroneous to assume that the cytokine profile as well as cytokine concentrations remains uniform over a 24 h period. Furthermore, our model allowed for a degree of direct contact between infected MDM and uninfected CM, an event that is highly cytolytic (Heinkelein, Sopper, and Jassoy, 1995). Another important difference was that we activated the MDM by HIV infection rather than endotoxins. The cytokine profile under these circumstances might be similar, but not identical. Nevertheless the study by Heinkelein and colleagues shows that in the presence of antigen, the response mounted by activated tissue macrophages can have deleterious

effects in contractile cardiac tissue, independently of direct contact between the activated MDM and CM.

We have also shown that the restoration of cytokine expression to pre-infection levels with UCLA1, strongly correlated with a significant improvement in CM survival. The potent, cross-clade HIV neutralization properties of UCLA-1 have been well documented (Cohen et al., 2008; Dey et al., 2005; Khati et al., 2003). This aptamer works by binding to the CCR5-binding pocket of the viral surface envelope glycoprotein 120 (gp120). Binding of gp120 to the CCR5 cell surface chemokine receptor is a vital step in viral entry, which is inhibited by the UCLA1 aptamer.

Although HIV did have a profound effect on the upregulation of most cytokines in all culture conditions, especially in CM, it did not seem to significantly impact on cell viability of either cell type. In fact, infected MDM have been observed to produce HIV-1 for weeks to months, with minimal cytotoxicity (Collman et al., 1989; Crowe, Mills, and McGrath, 1987). Furthermore, it has also been reported that HIV easily infects primary neonatal rat ventricular myocytes, but does not replicate (Twu et al., 2002). There was a a significant decrease in CM viability only in infected MDM and uninfected-CM cultures (**Figure 3**). The only difference between these cultures and HIV-infected CM cultures was TNF expression as well as significantly increased levels of two key pro-inflammatory cytokines in cardiovascular disease, IL-1β and IL-6 (Long, 2001; Wollert and Drexler, 2001). It was striking that only TNF increased above pre-infection levels in infected co-cultures and MDM, but not in infected CM, indicating a much more critical role in CM apoptosis than any other cytokine studies, including IL-1β and IL-6. Moreover, TNF is strongly associated with HIVCM

(Barbaro, 2005a). TNF was one of the first cytokines ever identified and is produced upon activation by the immune system, causing significant necrosis in tumors of a broad range of animal models and extensive cytotoxicity in many immortalized cell lines (Wajant, Pfizenmaier, and Scheurich, 2003). It is also well documented for its ability to significantly increase HIV-replication in HIV-infected cells of the macrophage lineage (Han et al., 1996; Kedzierska et al., 2003; Poli et al., 1990; Suresh and Wanchu, 2006), but implicated in entry inhibition in uninfected, TNF primed cells (Herbein, Montaner, and Gordon, 1996). Furthermore, its role in cardiomyocyte apoptosis, both in the clinical setting (Matsumori et al., 1994; Parrillo et al., 1985; Satoh et al., 1996) and in *ex-vivo* models (Finkel et al., 1992; Twu et al., 2002; Yokoyama et al., 1993) has been recognized. The mechanism of action through which TNF exerts its cytotoxicity is by direct interaction with the TNF type I receptor. This cell-surface receptor is ubiquitously expressed and mediates most of the death-related cellular effects of TNF (Wajant, Pfizenmaier, and Scheurich, 2003). It tightly regulates TNF activity by shedding it from the cell surface, thereby decreasing its receptor density resulting in decreased binding sites for its ligand. The resulting soluble receptor also sequesters TNF further reducing its cytotoxic effects (Garton, Gough, and Raines, 2006; Xanthoulea et al., 2004). The end result of TNF signalling is expression of inducible nitric oxide synthase (iNOS) through the activation of NF-κB (Finkel et al., 1992; Givertz and Colucci, 1998; Starling, 2005). Thus, supra physiological levels of nitric oxide (NO), the product of iNOS, a highly cardiotoxic molecule, are synthesized resulting in cardiomyocyte apoptosis. Therefore modulation of TNF, TNF-R1 and/or its downstream effector iNOS, would be essential in minimizing CM apoptosis. IL-1β and IL-6 signalling also results in NO production. However, the pleiotropy of these three cytokines and functional redundancy of IL-1β

and IL-6 have made it difficult to study their exact mechanisms of action in CM apoptosis. Nonetheless, we have shown that simultaneous neutralization of IL-1β, IL-6 and TNF protected close to 100 % of the CM from apoptosis, whereas neutralization of TNF alone or in combination with IL-1β or IL-6 only, only protected 84.9 ± 3.2 %, 81.8 ± 3.5 % and 85.7 ± 2.5 % of the CM respectively. Interestingly, there was no difference in CM survival when TNF was neutralized alone or in combination with the other two cytokines. However, when both IL-1β and IL-6 where neutralized concomitantly, there was a level of cellular protection very close to that conferred by the neutralization of TNF alone. This data is further evidence of the functional redundancy of IL-1β and IL-6, in that in the absence of one the other will induce the desired signalling cascade, but the presence of either of them is essential to augment TNF death signalling, as observed by the simultaneous neutralization of all three cytokines. The key role of these three cytokines was further demonstrated when healthy CM where treated with them in a dose-dependent manner. Although we did not observe CM apoptosis in the presence of IL-1β and IL-6 alone or in combination, we did observe a decrease of over 5-fold in the IC_{50} of TNF when cells were exposed to all three cytokines simultaneously. A significant decrease in the IC_{50} was also observed when cells were co-treated with TNF and one of the two cytokines. This further cemented the argument of the indirect role played by IL-1β and IL-6 in TNF death signalling. Indeed, IL-1β and IL-6 are secreted by several cell types stimulated with TNF (Delves and Roitt, 2000), but their overall impact in CM survival remains debatable, even though very high levels of these cytokines have been closely linked to cardiac pathology (Long, 2001; Wollert and Drexler, 2001). However, studies have focused on CM contractile dysfunction (which is reversible) in the presence of these cytokines or macrophage-activated medium rather than cell death (Balligand et al.,

1993; Finkel et al., 1992; Kan et al., 2005; Kan, Xie, and Finkel, 2006). Therefore, such intimate relationship between IL-1β, IL-6 and TNF, especially the augmentation of TNF in death signalling by these cytokines has not been previously reported.

Another intriguing aspect of the information gained with the cytokine multiplexing is whether minor, yet statistically significant changes in cytokine levels post infection are, in fact, physiologically relevant. A broad range of cytokines fall in this category (**Figure 2**), producing a wealth of data regarding the modulation of these cytokines during HIV infection. This data could be used as a framework to gain further insight into CM morbidity using end-point-measurements other than apoptosis, since some cytokines could be involved in cardiac dysfunction without measurable loss in contractile tissue.

CHAPTER FIVE

Summarizing Discussion and Conclusions

HAART has been one of the greatest triumphs to date in the ongoing fight over the HIV pandemic. Although not a cure, it has significantly decreased AIDS-related morbidity as well as minimized the spread of the disease, both vertically and horizontally (UNAIDS, 2008). However, the therapeutic burden on the patient has greatly increased as have the adverse side effects arising from chronic, life-long HAART (Barbaro et al., 2003; Chen, Hoy, and Lewin, 2007). Drug holidays whereby the patient interrupts HAART for a few months, have shown mixed, but overall, discouraging results, whereby increased blood viraemia and drug resistance emerge significantly quicker than it otherwise would (SMART study, 2009). Drug resistance in fact, remains the major concern of antiretroviral therapy, which resulted in the need for the continuous development of novel drugs, exploiting both old and novel targets within the virus life cycle. An important pre-requisite for novel ARVs is decreased toxicity. This factor is, in fact, the major hindrance to drug development. The vast majority of promising leads during drug discovery fail the toxicity trials, a fact that also holds true for ARVs. Therefore, before any drug can be considered for further testing, it has to have an acceptable, *in vitro* toxicity profile. For this reason, and before any further work was carried out with the HIV-1 neutralizing aptamer UCLA1, a comprehensive *in vitro* toxicity testing was undertaken (Lopes de Campos et al., 2009). It was encouraging to observe that B40 had no measurable effect on cell viability when compared to other ARVs and neither did it have an effect on monoamine oxidase CYP 450 3A4, amine oxidases MAO A and B and mitochondrial DNA synthesis. However, the observations made in this study are very limiting, since it encompassed the use of tissue-culture adapted cardiomyocytes in an *in vitro* system and the data may not have any correlation with *in vivo* events. In fact, the high levels of toxicity presented by PIs in this study do not directly correlate with clinical data. In

fact, PIs are better tolerated than any other class of ARVs and have a good safety profile (Walmsley, 2007). However, increasingly more frequent cardiovascular complications have been reported, particularly problems associated with cholesterol metabolism and decreased insulin sensitivity (Barbaro, 2006; Martinez, Larrousse, and Gatell, 2009). In the most severe cases, PI therapy has to be discontinued to allow for the resolution of these complications, since PIs interfere with several therapeutic agents used to treat cholesterol-associated cardiovascular complications (Hulten et al., 2009). In light of this fact, although HIV-associated cardiomyopathy has been near abolished in the presence of HAART, PIs have re-introduced cardiovascular disease from a different perspective.

HIVCM has drawn a comparatively lower degree of interest from the scientific community than the vast majority of other AIDS-associated diseases, due to the fact that it's burden during has appeared to have decreased significantly with the introduction of HAART. Evidence of this rests on very limited number of peer-reviewed articles published since the disease was first documented nearly two decades ago. One of the many possible explanations for this is that HIVCM is a condition that manifests itself very late during HIV-infection and long after the onset of AIDS, as a result of prolonged, chronic immunosuppression. The advent of ARVs, particularly of HAART, has largely eliminated this disease, but cardiac morbidity independently of HAART therapy still lingers, albeit at much lower and barely clinically relevant levels (Ntsekhe and Mayosi, 2008). However, this has only happened in the Western world, where all HIV-positive patients have ready access to HAART. In resource-limited countries, more specifically in sub-Saharan Africa, where over 70 % of HIV-infected patients live, significantly less HIV-positive patients have access to HAART

(Sendagire et al., 2009). One other facet of HIVCM that has possibly discouraged more intense research is its multifactorial nature from etiology to clinical outcome. Infection of cardiac tissue by opportunistic pathogens, especially TB and cardiotropic viruses has been recognized as the major cause of HIVCM (Barbaro, Fisher, and Lipshultz, 2001; Bowles et al., 1999; Currie et al., 1998; Hadigan et al., 2001; Herskowitz et al., 1994; Lewis, 2001; Twagirumukiza et al., 2007). However, a growing number of cases have been reported where no known pathogens could be identified *in situ* in patients presenting with early stages of the disease. A direct role of HIV in cardiac pathology has been suspected in these cases, provoked either by direct infection of HIV or by cardiotoxic viral proteins, but evidence is still scant (Carr and Ory, 2006; Grody, Cheng, and Lewis, 1990; Lipshultz et al., 1990; Twu et al., 2002). Data collected i*n vitro* has been more conclusive but, at times, controversial with most of the efforts focused on viral proteins, more specifically gp120 (Fiala et al., 2004; Finkel et al., 1992; Li et al., 2005; Raidel et al., 2002; Kan et al., 2000; Rebolledo et al., 1998; Roggero et al., 2001). Direct effects arising from the interaction between virions and cardiomyocytes has been poorly investigated, and research groups reporting on this issue have never pursued it as indicated by the absence of any follow up studies. A key question of HIV pathogenesis in cardiomyocytes was their ability to infect and replicate in these cells. I've shown that HIV does indeed infect CM, but viral replication is blocked after reverse transcription. This observation has been made in the past, but in neonatal rat ventricular myocytes (Twu et al., 2002). The question as to why the virus life cycle is interrupted following reverse transcription remains to be answered, but observations made in a different study using foetal human cardiomyocytes allows for some postulation (Rebolledo et al., 1998). In this study it was observed that HIV could in fact replicate relatively

efficiently in this early, embryonic cardiomyocytes. The CM employed in my study was an immortalized cell line obtained from mature cord-blood cell derived CM. Adult CM are terminally differentiated and this is one of the several reasons as to why CM are unable to complete their life cycle, even though this CM line is mitotically active. Embryonic CM, have some proliferative capability and are phenotypically distinct. Since there is no pro-viral DNA integration as observed in my study where a time-dependent decrease in pro-viral DNA was observed coupled with an inhibition in proviral DNA synthesis in the presence of AZT, a viral reverse transcriptase inhibitor, it was possible to identify the stage at which the viral life cycle was interrupted. Following proviral DNA synthesis, the proviral DNA is transported to the nucleus for integration, a process that is aided by viral and host factors (Campbell and Hope, 2008; Gomez and Hope, 2005). It is likely that in adult cardiomyocytes, some of the proteins involved in the nuclear translocation process are absent, thereby arresting the viral life cycle at this stage. Future studies should focus on elucidating which host factors are responsible from stopping HIV from actively replicating in cardiomyocytes since these are some of the most metabolically active cells in the body. Usually what restrains productive infection is the entry step, but HIV successfully invaded these cells. Understanding the underlining cellular mechanisms stopping post-transcription/pre-integration events, which was were the life cycle of the virus was arrested, could provide novel targets for HIV therapy.

I then looked into the relationship between cardiomyocyte infection and co-receptor utilization. HIV infection is almost exclusively receptor mediated, requiring an initial interaction between the viral surface envelope glycoprotein gp120 and the cell surface receptor CD4 (McClure, Marsh, and Weiss, 1988; Pauza and Price, 1988). This event

is followed by interaction with a second co-receptor, either the CCR5 or CXCR4 (Jiang, 1997; Wu et al., 1996), although others have been identified but play minor roles during HIV infection *in vivo* (Cilliers et al., 2005; He et al., 1997). Co-receptor utilization defines virus tropism, which could be either R5, X4 or dual, R5X4. By making use of clinical isolates with known tropism (Cilliers et al., 2005) as well as inhibitors that blocked virus invasion by targeting either X4 or R5, I've shown that HIV-1 infection of this CM cell line was receptor independent. Receptor independent HIV infection has been reported in neuronal cells, believed to be mediated by macropinocytosis and lipid rafts (Liu et al., 2002), although the exact mechanism is yet to be fully elucidated. All clinical isolates with different tropisms successfully invaded CM, an observation made by the successful amplification of proviral DNA in CM following virus incubation. Furthermore, infection could not be blocked in the presence of either AMD3100 (CXCR4 inhibitor) and RANTES (CCR5 inhibitor). In PHA-treated, IL-2 stimulated PBMC control cells, all viruses were neutralized in a tropism-dependent manner.

Further studies were conducted aimed at dissecting the downstream events following virus the interaction of HIV-1 with cardiomyocytes, even though the virus was unable to complete its life cycle. Extensive cell death was observed following three days of exposure of CM to HIV. CXCR4 and CCR5/CXCR4 utilizing viruses were, on average, three-fold more toxic than CCR5 tropic strains. Inhibition of cell death by AMD3100 and UCLA1 but not RANTES, indicated that the CXCR4 chemokine receptor may be the receptor that transduces the death signal. Earlier studies in MDM and more recent studies in neonatal rat ventricular myocytes have mapped this receptor as the death signaling receptor in the presence of soluble gp120 (Berndt et

al., 1998; Herbein et al., 1998; Yuan et al., 2008). Furthermore, inhibition of cell death by the highly selective caspase-9 inhibitor Z-LEHD-FMK, pointed to apoptosis as the death mechanism and the mitochondria or intrinsic pathway as the preferred pathway. Further evidence of this was the minimal protective effect against apoptosis by the selective caspase-8 inhibitor Z-IETD-FMK, since caspase-8 is the canonical caspase in the death-receptor or extrinsic apoptotic pathway (Wallach, Kang, and Kovalenko, 2008). The selective caspase-9 inhibitor also decreased the amount of Cytchrome *c* released from HIV-infected CM as well as nuclear DNA fragmentation in the same cells. Cytochrome *c* release into the cell cytosol is intimately associated with intrinsic apoptosis and, in fact, is required to activate the caspase-9 from its pro-caspase precursor (Riedl and Salvesen, 2007). The caspase-8 inhibitor on the other hand, had virtually no protective effect. All this data argues for a mitochondria-initiated pathway, triggered by the interaction between cell-free HIV-1 and the cell surface chemokine receptor CXCR4 as the key events in HIV-1 initiated CM apoptosis.

Interestingly, a shift from caspase-9 to caspase-8 mediated apoptosis was observed when CM were co-cultured with HIV-infected MDM. In this scenario, neither the selective caspase-9 inhibitor Z-LEHD-FMK nor UCLA1 had a protective effect of CM viability. Protection was only achieved with the selective caspase-8 inhibitor Z-IETD-FMK and the soluble TNF death receptor TNF-R1. Furthermore, there was no change in the apoptotic potential of HIV based on tropism, since both X4 and dual-tropic viruses had a near identical effect on CM survival. This indicated a shift in the apoptotic pathway, from CXCR4-triggered, mitochondrial-mediated to Fas-ligand/death receptor triggered, caspase-8 mediated. Multiplexing for 27 different

cytokines under these culture conditions revealed that TNF was only produced in HIV-infected MDM but not in HIV-infected CM. This was further indication that the change in the apoptotic pathway occurred as a result of a shift in the apoptotic stimuli.

The evidence for the switch in apoptotic pathway was further strengthened when neutralizing antibodies against IL-1β and IL-6 were added to HIV-infected MDM, CM co-cultures, in addition to TNF-R1. I observed that single neutralization of IL-1β or IL-6 had no effect on cell survival, but when both were neutralized at the same time, the effect on cell survival was very similar to that conferred in the presence of TNF-R1 alone. This indicated that both IL-1β and IL-6 had an additive effect on the death signal generated by TNF, and either one of them was capable of triggering this effect, indicating a degree of functional redundancy of IL-1β and IL-6, although the protective effect brought about by the concomitant neutralization of all 3 cytokines was even greater than that obtained with TNF-R1 and either IL-1β or IL-6 neutralization. This data further indicated that the up-regulation of these pro-inflammatory cytokines were directly linked to CM apoptosis, rather than HIV itself. Moreover, when recombinant TNF was added to healthy CM cultures, a dose-dependent decrease in cell viability was observed during the course of 24 h. It was further observed that when the pro-inflammatory cytokines IL-1β and IL-6 were added to the same culture medium there was a significant reduction in the EC_{50} of TNF, thereby augmenting its death-signal by between 2.82 and 5.4-fold.

In the co-culture model of HIV-initiated cardiomyocyte apoptosis, UCLA1 had no protective effect. This was somewhat expected since UCLA acts directly on the virus, preventing infection. Once infection has established in MDM, the death signal shifts

from the virus to the up-regulation of TNF, IL-1 and IL-6, upon which UCLA1 has no effect. However, the role of UCLA1 in CM survival during HIV infection cannot be denied, even in the co-culture system, since if HIV is pre-incubated with UCLA1 prior to infection of MDM, there is no infection, resulting in no loss of MDM. UCLA1 would also prevent infection of bystander cells by neutralizing infectious virions produced by infected cells, thereby containing the infection. However, this is also achieved by conventional ARVs in the clinical setting. What is unique to UCLA1 is that, by binding to cell-free virus directly and interfering with gp120-CXCR4 signaling, the mitochondrial initiated apoptotic pathway triggered by non-infectious virions is blocked.

The evidence gathered from this body of work suggests that both viral and host factors are key contributors for HIVCM. It however points to a more direct role of HIV in cardiomyocyte pathogenesis than previously acknowledged. All research efforts and evidenced obtained pointed to viral proteins, more specifically gp120 to the almost complete exclusion of virions, as the viral mediators of apoptosis (Biard-Piechaczyk et al., 2000; Fiala et al., 2004; Otis et al., 2008; Roggero et al., 2001; Twu et al., 2002; Yuan et al., 2008). Furthermore, the role of IL-1β and IL-6 in increasing CM sensitivity to TNF had yet to be reported. It has been widely described that they are key inflammatory cytokines and are involved in a wide array of cardiac inflammatory diseases (Long, 2001; Wollert and Drexler, 2001), but the manner in which they may bring about the loss of contractile cardiac tissue is still poorly understood. The observations made in this *n vitro* model of cardiomyocyte apoptosis during HIV infection may provide a framework for future therapeutic interventions targeting the host cytokines IL-1β and IL-6 concomitantly. The importance of

neutralizing both cytokines simultaneously for maximum therapeutic effects was evident in this model. However, this model remains a crude approximation of *in vivo* events. A mouse model needs to be established, using the parameters set in this study whereby IL-1β, IL-6 and TNF are administered to live mice and its effects not only in cardiomyocyte apoptosis but also in the heart as a whole, can be observed.

This study shows once again the multifactorial nature of HIVCM, whereby viral, host and therapeutic factors are intimately involved in the progression to cardiac failure. However, HIV infection is the trigger and, ultimately, this is where our efforts must be focused. The clinical use of UCLA1 is not yet feasible, but its efficacy and unique mode of action judging by the mounting body of evidence gathered over the last 6 years, coupled with its toxicity profile, suggests that it may prove to be an effective antiretroviral. However, as discussed in the *Introduction* chapter of this dissertation, HIV neutralizing aptamers need to be made orally available, inexpensive and have a favourable pharmacokinetic profile.

There is a general perception that HIVCM has been eradicated in first world countries with the introduction of HAART, which has decreased the interest of the medical and scientific community in further understanding the underlying mechanisms of this complex disease. This apparent neglect may have profound consequences in the near future as the HAART-treated population surpasses its first decade. There has been a recent surge in idiopathic cardiovascular complications in HIV positive patients, independently of HAART, even though viral loads are almost always maintained at levels below the threshold of detection. However, there are still basal levels of viral replication and an increase in blood viraemia is observed on a regular basis as escape

mutants. This issue is further compounded by increasing number of patients that are kept on a virologically failing regime for short-term immunological or virological benefit (Cozzi-Lepri et al., 2007; Deeks et al., 2001). Although there are no clinical manifestations of disease, both from a serological as well as physiological perspective, the presence of virions in the blood may eventually lead to cardiac complications, if there is any correlate between the *in vitro* data gathered during the course of this study and clinical cardiac morbidity. In future, we are very likely to observe an even greater increase in idiopathic cardiovascular morbidity which may be directly related to chronic HIV infection.

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Cytotoxicological Analysis of a gp120 Binding Aptamer with Cross-Clade Human Immunodeficiency Virus Type 1 Entry Inhibition Properties: Comparison to Conventional Antiretrovirals▽

Walter Rangel Lopes de Campos,[1] Dayaneethie Coopusamy,[1] Lynn Morris,[2] Bongani M. Mayosi,[3] and Makobetsa Khati[1]*

CSIR Biosciences, P.O. Box 395, Pretoria 0001, South Africa[1]; National Institute for Communicable Diseases, Private Bag X4, Sandringham 2131, South Africa[2]; and Department of Medicine, Groote Schuur Hospital and University of Cape Town, Observatory 7925, South Africa[3]

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The long-term cumulative cytotoxicity of antiretrovirals (ARVs) is among the major causes of treatment failure in patients infected with human immunodeficiency virus (HIV) and patients with AIDS. This calls for the development of novel ARVs with less or no cytotoxicity. In the present study, we compared the cytotoxic effects of a cross-clade HIV type 1-neutralizing aptamer called B40 with those of a panel of nonnucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), and the entry inhibitor (EI) T20 in human cardiomyocytes and peripheral blood mononuclear cells. An initial screen in which cell death was used as the end-point measurement revealed that the B40 aptamer and T20 were the only test molecules that had insignificant ($0.61 < P < 0.92$) effects on the viability of both cell types at the maximum concentration used. PIs were the most toxic class ($0.001 < P < 0.00001$), followed by NNRTIs and NRTIs ($0.1 < P < 0.00001$). Further studies revealed that B40 and T20 did not interfere with the cellular activity of the cytochrome P450 3A4 enzyme ($0.78 < P < 0.24$) or monoamine oxidases A and B ($0.83 < P < 0.56$) when the activities of the enzymes were compared to those in untreated controls of both cell types. Mitochondrion-initiated cellular toxicity is closely associated with the use of ARVs. Therefore, we used real-time PCR to quantify the relative ratio of mitochondrial DNA to nuclear DNA as a marker of toxicity. The levels of mitochondrial DNA remained unchanged in cells exposed to the B40 aptamer compared to the levels in untreated control cells ($0.5 > P > 0.06$). These data support the development of B40 and related EI aptamers as new ARVs with no cytotoxicity at the estimated potential therapeutic dose.

The introduction of highly active antiretroviral therapy (HAART) has significantly reduced the rates of morbidity and mortality among patients infected with human immunodeficiency virus (HIV) and patients with AIDS. HAART, however, requires life-long treatment and results in toxicity in up to 50% of patients following 6 to 12 months of therapy (4, 53). Treatment failure arising from toxicity has highlighted the need for close medical supervision and, ultimately, the development of novel, less toxic antiretrovirals (ARVs). This is of even greater concern on the resource-poor African continent, where, unlike in developed countries, monitoring for and diagnosis and management of ARV-associated toxicity are not routinely conducted (54). This problem is likely to increase as the use of ARVs becomes more widespread in resource-poor settings (3).

Mitochondrial toxicity is one of the major complications associated with the long-term use of HAART (6, 7, 37, 38, 67, 68). The nucleoside reverse transcriptase inhibitors (NRTIs) used in HAART inhibit DNA polymerase γ, which is solely responsible for mitochondrial DNA (mtDNA) replication (47). Through this mechanism, NRTIs induce the depletion of mtDNA as well as that of mtDNA-encoded enzymes (8). This results in mitochondrial dysfunction and eventually leads to a range of complications, such as bone marrow suppression and cardiomyopathy (22).

In addition to mitochondrial toxicity, other markers of cytotoxicity caused by ARVs are cell death, modulation of cytochrome P450 (CYP450) and the monoamine oxidase (MAO) A and B enzymes. CYP450 enzymes are mostly amine oxidases and key metabolizers with regard to their catalytic versatility and broad spectrum of oxidative transformation of both exogenous and endogenous molecules (33). This enzyme superfamily plays a vital role in tissue and cardiovascular health (25). On the other hand, MAO enzymes are flavoenzymes that catalyze the oxidative deamination of a large number of biogenic and xenobiotic amines (24, 39). Therefore, any drug that interacts with MAOs, regardless of its function, can lead to a decrease in normal MAO cellular activity. This, in turn, can result in the intracellular accumulation of its natural substrates, such as serotonin, to potentially lethal levels (18).

Because of the toxicity associated with the use of ARVs, we evaluated in the present study the cytotoxic effects of one of the potent and cross-clade HIV type 1 (HIV-1)-neutralizing RNA aptamers, called B40, which was recently isolated and described (17, 21, 42). The B40 aptamer blocks viral entry by binding to core conserved residues on gp120 at the heart of the CCR5 binding site (17, 20). While this is the first study that describes the in vitro toxicity of the experimental HIV-1 gp120 binding RNA aptamer, previous studies have shown that the vascular endothelial growth factor (VEGF) binding aptamer

* Corresponding author. Mailing address: CSIR Biosciences, P.O. Box 395, Pretoria 0001, South Africa. Phone: 27(12)8414770. Fax: 27(12)8313080. E-mail: Mkhati@csir.co.za.
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did not have any intrinsic toxicity in preclinical assessments (23, 29). Single-dose and repeated-dosing toxicity studies with the anti-VEGF aptamer conducted in rats, rabbits, and rhesus monkeys showed that there was no observable adverse effect level or dose-limiting toxicity (23, 28, 29).

In the present study, we evaluated the toxicity of the B40 anti-gp120 aptamer using cell viability; caspase 3/7 activity; and CYP450 3A4 (CYP3A4), MAO A and B, and mtDNA levels as markers of cytotoxicity in human cardiomyocytes and peripheral blood mononuclear cells (PBMCs). Toxicity in human cardiomyocytes was evaluated due to the association of cardiomyopathy with the use of ARVs (34, 49), and toxicity in PBMCs was evaluated because the $CD4^+$ T cells and macrophages found in PBMCs are the backbone of the immune system. HIV-1 predominantly infects and replicates in these cells.

MATERIALS AND METHODS

ARVs. All ARVs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Four classes of ARVs were tested in this study: the nonnucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine (NVP) and efavirenz (EFV); the NRTIs abacavir (ABC), dideoxycytidine (ddC), zidovudine (AZT), stavudine (d4T), tenofovir (TDF), and lamivudine (3TC); the protease inhibitors (PIs) saquinavir (SQV), ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV), tipranavir (TPV), indinavir (IND), amprenavir (AMP), and darunavir (DRV); and the entry inhibitor (EI) enfuvirtide (T20).

In vitro transcription and validation of monoclonal aptamers. Plasmid DNA containing the B40 aptamer was amplified by PCR, as described previously (42). The PCR product was purified with Wizard SV gel and a PCR cleanup system (Promega) and was transcribed to RNA in vitro by using 2′-fluoro-pyrimidines (TriLink), as described previously (42). The resulting 2′-F-RNA aptamer was quantified with a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific). Prior to use, the RNA aptamer was refolded by heating in water to 95°C for 5 min and then cooling to room temperature for 5 min. This was followed by the addition of 1/20 volume of 20× refolding buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 2.7 mM KCl [final concentrations]) and incubated at room temperature for an additional 10 min. The refolded aptamer was validated by binding to recombinant gp120 derived from HIV-1_{BaL} by using a BIAcore 3000 surface plasmon resonance (GE Healthcare), as described previously (42).

Recombinant HIV-1_{BaL} gp120. HIV-1_{BaL} gp120 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Culture and phenotyping of human cord blood stem cell-derived cardiomyocytes. The human cord blood stem cell-derived cardiomyocytes were purchased from Celprogen. The cardiomyocytes were cultured in human cardiomyocyte expansion medium (a proprietary medium containing, serum, growth factors, and antibiotics) and in tissue culture flasks containing a proprietary growth matrix at 37°C with 5% CO_2. The medium and the flasks were also purchased from Celprogen.

The cardiomyocytes were phenotyped by immunocytochemistry with a cardiac protein-specific monoclonal antibody that targets the first 32 amino acids of the C terminus of the human cardiac protein troponin I (R&D Biosystems) and were detected by using an anti-goat rhodamine-labeled antibody produced in chickens (Santa Cruz Biotechnology). Briefly, a cell monolayer growing on a chamber slide (Lab-Tek; Nunc) were fixed and permeabilized in 2% paraformaldehyde (PFA) containing 0.1% Triton X-100 (Sigma-Aldrich, Germany) for 30 min. Following this, primary antibody was added at a concentration of 10 μg/ml in the presence of 1% normal goat serum (Invitrogen), and the mixture was incubated for 1 h on ice. The cells were then washed four times with ice-cold phosphate-buffered saline (PBS), prior to the addition of the anti-goat rhodamine-conjugated secondary antibody at a concentration of 5 μg/ml. Following incubation for 1 h, the cardiomyocyte monolayer was covered with a glass coverslip in the presence of mounting medium (UltraCruz; Santa Cruz Biotechnology) for immunofluorescence analysis.

Isolation and cultivation of human PBMCs. PBMCs were isolated from heparinized human buffy coats from healthy, HIV-negative donors, as described previously (42). The buffy coats were obtained from the South African National Blood Services. Briefly, the buffy coats were layered into Ficoll-Paque Plus (GE Healthcare), and the PBMCs were isolated by density gradient centrifugation. Following centrifugation at 2,000 rpm for 30 min at 20°C, the PBMC layer was harvested and washed once with ice-cold PBS (Lonza). The cell pellet was then suspended in 30 ml of a hypotonic solution of ice-cold ammonium chloride, and the mixture was incubated at room temperature for 20 min to lyse the remaining red blood corpuscles. The cells were harvested by centrifugation and seeded on tissue culture flasks (Corning) at a density of 2×10^6 cells/ml in RPMI medium containing 20% fetal bovine serum (Sigma-Aldrich), followed by stimulation with phytohemagglutinin (PHA) and treatment with interleukin-2 (IL-2), as described previously (42). For comparison, we also cultured PBMCs without mitogen (PHA) and without IL-2 in X-Vivo-10 medium (BioWhittaker) supplemented with 2% autologous serum because we previously showed that this system produces a slowly proliferating mixed culture of $CD4^+$ T lymphocytes and macrophages that supports a higher level of replication of HIV-1 primary isolates (42).

Cell viability assay. PHA-stimulated, IL-2-treated PBMCs and cardiomyocytes in log-phase growth were seeded at a density of 20,000 cells/well in 96-well, white-opaque-wall tissue culture plates (Nunc, Thermo Fisher Scientific) in 100 μl growth medium. Unstimulated PBMCs cultured in X-Vivo-10 medium (BioWhittaker) supplemented with 2% autologous serum were also seeded, as described above. The overlay medium in either system was replaced with the relevant culture medium containing the drug at a concentration equivalent to the maximum concentration in plasma (C_{max}) in patients at a predetermined therapeutic dose or an equivalent amount of the aptamer (Table 1). Following 24 h of incubation, the overlay medium was removed and replaced with fresh medium. The overlay medium was replaced every 2 days for the duration of the experiment. The assay was done in triplicate and was independently repeated twice. Following 7 days of incubation, the cell viability was determined by using an ATP-based assay, the CellTiter-Glo luminescent cell viability assay (Promega). Both the plates and the reagent were allowed to equilibrate at room temperature. An equal volume of reagent was then added to the wells, and the plates were further incubated at room temperature for 20 min to allow the luminescent signal to stabilize. Following the incubation, the luminescent signal was measured in a Modulus microplate reader luminometer (Promega) by use of a count integration time of 1 s. The raw data were measured in relative light units (RLU), and the final results were normalized (i.e., the total number of viable cells in each of the treated samples was divided by the total number of viable cells in the cell control, which was not treated with any drug or test molecule) and expressed as percent cell death. The cell controls, which were not treated, were, on average, more than 95% viable. Wells containing medium alone were used as controls for background luminescence, and the values for those wells were subtracted from the values for the test wells. Dimethyl sulfoxide (DMSO) at a concentration of 0.1% (vol/vol) was used as a solvent for most ARVs; hence, a control for DMSO-initiated toxicity was included whereby cells were treated with 0.1% DMSO for the duration of the study. The general formula for the calculation of percent toxicity was percent viable cells = [(RLU of drug-treated cells − RLU of medium control)/(RLU of untreated cell controls − RLU of medium control)] × 100. The percentage of dead cells was equal to 100% minus the percentage of viable cells.

Caspase 3/7 activity. Representatives from each class of ARVs were selected and tested to determine if they upregulated the activity of the apoptosis executioner caspase 3/7 in human cardiomyocytes and PBMCs. The ARVs selected were EFV (an NNRTI), ABC and TDF (NRTIs), RTV and IND (PIs), and T20 and the B40 aptamer (EIs). The cells were seeded at a density of 50,000 cells/well in 24-well tissue culture plates (Corning) and were incubated with the ARVs at the C_{max} for 1, 4, and 7 days. After each time point, the cells were immediately frozen at −80°C. On day 7, the cells were frozen for 24 h prior to the assay. The cells were then equilibrated to room temperature and an equal volume of Caspase-Glo 3/7 detection reagent (Promega) was added. Following 2 h of incubation, the resulting luminescent signal was measured in a Modulus microplate reader luminometer (Promega) by use of a count integration time of 1 s. The results were expressed as RLU from which the value for the blank was subtracted (RLU, blank subtracted).

MAO A and B enzyme activity assay. The MAO A and B enzyme activity assay was carried out with an MAO-Glo assay kit (Promega). A 96-well, white-opaque-wall tissue culture plate was seeded with either cardiomyocytes or PBMCs and incubated in triplicate with the test ARV or aptamer at a maximum starting concentration of 2 μM, followed by incubation with half-log serial dilutions. The overlay medium was removed, and the cell monolayer was washed with PBS (Lonza) and lysed with 50 μl proprietary lysis buffer (Promega). The cell debris was removed by centrifugation at 2,000 rpm, and the supernatant was recovered and incubated with MAO A or B enzyme substrate for 3 h at room temperature. This was followed by the addition of the detection reagent. After 1 h of incuba-

TABLE 1. C_{max}s in patients and in vitro data for ARVs

Class	ARV	C_{max} (μM)[a]	In vitro data				
			Cell[b]	Strain[c]	IC_{50} or IC_{95} (μM)[d]	TI[e]	Reference
NNRTIs	NVP	16.9	C8166	IIIB	0.04	8,000	51
	EFV	15.7	PBMC	Primary isolates	0.0015–0.003*	>50,000	71
NRTIs	ABC	14.7	PBL	IIIB	4	40	50
	ddC	0.12	PBMC	LAV	0.011	>9,000	15
	AZT	48.8	PBMC	IIIB	0.05	1,000	52
	d4T	5.3	PBMC	LAV	0.009	7,778	15
	TDF	1.0	MT-2	IIIB	0.003	16,667	58
	3TC	8.8	CEM	RF	0.18	>2,000	16
PIs	SQV	3.7	C8166	RF	0.0002	>50,000	59
	RTV	21.0	PBL	Primary isolates	0.045	1,089	41
	NFV	7.0	MT-2	RF	0.043	651	57
	LPV	15.7	MT-4	NL4-3	0.005	>20,000	13
	TPV	0.2	MT-4	NL4-3	0.016	>2,000	19
	IND	15.4	MT-4	IIB	0.1*	>4,000	66
	AMP	15.1	MT-4	IIIB	0.054	>1,600	1
	DRV	13.6	MT-2	LAI	0.003	24,667	44
EIs	T20	1.0	PBMC	LAI			69
	B40[f]		PBMC	Primary isolates	<0.01, <0.1*	>5,000	17, 21, 42

[a] The C_{max}s of all registered ARVs were taken from package inserts of the respective drugs.
[b] Cell type used to obtain the respective in vitro data.
[c] Strain of HIV-1 used to obtain the respective in vitro data.
[d] IC_{95}s are indicated by asterisks.
[e] TI, therapeutic index. The therapeutic index is the relation between the inhibitory concentration that reduces cellular growth or the viability of uninfected cells by 50% (data not shown) and the IC_{50}, and it is obtained by the general formula 50% cytotoxic concentration/IC_{50}.
[f] The B40 aptamer is an experimental anti-HIV-1 molecule and is not yet registered for clinical use. For this reason, its C_{max} in blood plasma of patients has not been established. It was used at 2 μM, which is twice the C_{max} of T20 and more than 200-fold its IC_{50}. The 50% cytotoxic concentration of B40 could not be obtained even after the use of the aptamer at 50 μM, which had an effect similar to that of the aptamer at 2 μM.

tion, the luminescent signal was measured in a Modulus microplate reader luminometer (Promega) by use of a count integration time of 1 s. The raw data (in RLU) were measured, and the final results were normalized as described above for the cell viability assay and expressed as the percentage of enzyme activity. A sample with no substrate was included as the control for background luminescence, and the values for that sample were subtracted from the values for the test samples. Clorgyline (Sigma-Aldrich) was used as a positive control for MAO A inhibition, and deprenyl (Sigma-Aldrich) was used as a positive control for MAO B inhibition. The general formula used to calculate percent enzyme activity was [(RLU of drug-treated cells − RLU of medium control)/(RLU of untreated cell controls − RLU of medium control)] × 100.

CYP3A4 activity. The P450-Glo assay kit (Promega) was used for the determination of CYP3A4 activity. Cells were seeded as described above in the presence of the aptamer or ARVs and were incubated for 2 days. The overlay medium was then removed, and 60 μl of fresh medium which contained 50 μM of the luciferin substrate was added. After 4 h of incubation, 50 μl of the culture supernatant from each well was transferred to 96-well, white-opaque-wall tissue culture plates (Nunc, Thermo Fisher Scientific) and an equal volume of detection reagent was added. The luminescent signal was measured after 1 min of incubation in a Modulus microplate reader luminometer (Promega), as described above. A sample with no substrate was included as a control for background luminescence, and the value for that sample was subtracted from the values for the test samples. The PI RTV was used as a positive control for CYP3A4 inhibition. The CYP3A4 activity in the cell culture supernatant from untreated cells was used as the baseline for CYP3A4 activity.

Real-time PCR for quantification of mtDNA toxicity. Cells were exposed to the B40 aptamer T20 or ddC at 2 μM for 7 days. Following exposure to the test molecule for 7 days, the cell monolayer was washed with warm PBS and the cells were lysed in 50 μl of a lysis buffer (Promega) that was compatible with the PCR mixture. A volume of 2 μl lysate was subjected to PCR for both genomic DNA and mtDNA in a LightCycler instrument (Roche, Switzerland). The target nuclear gene was the human polymerase γ accessory subunit (*ASPOLG*), and the target mitochondrial gene was cytochrome *c* oxidase I (*CCOI*). For *ASPOLG* the forward primer 5′-GAC CTG TTG ACG GAA AGG AG-3′ and the reverse primer 3′-CAG AAG AGA ATC CCG GCT AAG-5′ were used for PCR amplification. The *CCOI*-specific primer pair consisted of forward primer 5′-TTC GCC GAC CGT TGA CTA TT-3′ and reverse primer 3′-AAG ATT ATT ACA AAT GCA TGG GC 5′. PCRs were carried out with the master mixture Power SYBR green 5 (Applied Biosystems) in the presence of 5 mM $MgCl_2$ (Promega). The cycling conditions consisted of a single enzyme activation step of 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 0 s, annealing at 60°C for 10 s, and elongation at 72°C for 5 s, with a temperature transition rate of 20°C/s being used. The relative amounts of mtDNA and nuclear DNA (nDNA) were extrapolated from a standard curve, and the results were calculated as the mean ratio of the amount of mtDNA to the amount of nDNA. Each experiment was carried out in triplicate, and the results represent the averages between two independent experiments. The mtDNA/nDNA ratio for the untreated controls was arbitrarily set at 1.0 and was used as the baseline measurement.

RESULTS

Effect of B40 aptamer in comparison to effects of ARVs on viability of human PBMCs and cardiomyocytes. We evaluated the toxicity of a novel gp120 binding and HIV-1-neutralizing RNA aptamer called B40 in comparison to the toxicities of conventional ARVs in human cord blood stem cell-derived cardiomyocytes (Fig. 1) and human PBMCs. An initial screen by an ATP cell-based assay to measure cell death as the endpoint measurement of cell toxicity showed that the B40 aptamer, which is a novel EI, did not cause the death of either human cardiomyocytes (1% ± 0%; $P = 0.74$) or PBMCs (0% ± 5%; $P = 0.92$) at the maximum concentration used, 2 μM (Fig. 2). The maximum concentration of B40 used in this study (2 μM) was more than 200-fold the B40 concentration (<10 nM) required to inhibit 50% of the HIV-1 isolates in PBMCs (50% inhibitory concentration [IC_{50}]) (17, 21, 42). The ability of the B40 aptamer to cause no cell death at the max-

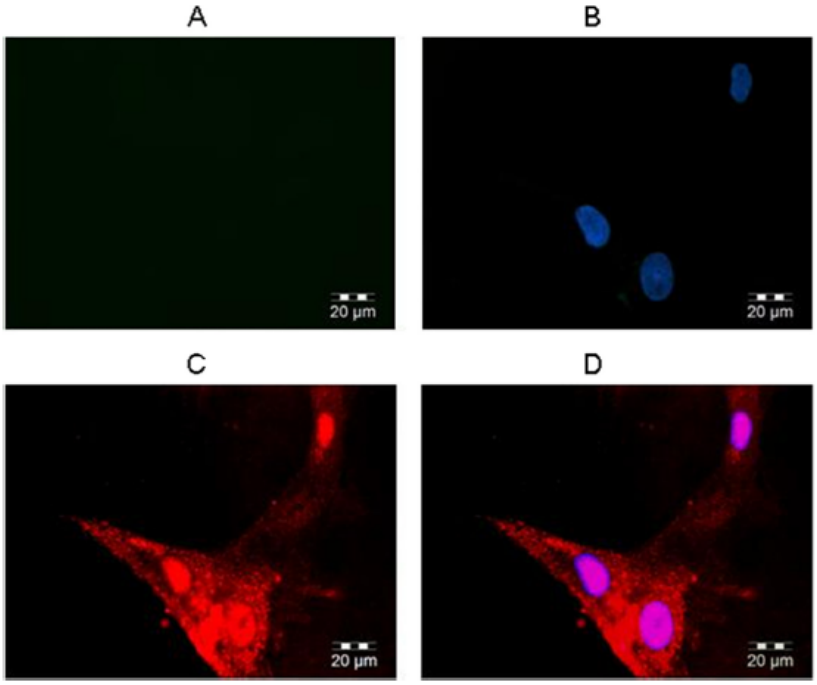

FIG. 1. Phenotyping of human cord blood stem cell-derived cardiomyocytes. (A) Cardiomyocytes were cultured in chamber slides, permeabilized, and treated with rhodamine-labeled secondary antibody. (B) 4′,6-Diamidino-2-phenylindole nuclear stain (B). (C) cTnI primary antibody and rhodamine-labeled secondary antibody. (D) A composite image created by superimposing the images in panels B and C.

imum concentration used compared favorably to the ability of the registered EI, called T20, which also did not cause the death of cardiomyocytes (1% ± 3%; $P = 0.74$) or PBMCs (0% ± 6%; $P = 0.61$). All other classes of ARVs tested caused significant cell death of either or both cardiomyocytes and PBMCs (Fig. 2). PIs were generally the most toxic class of ARVs for both cardiomyocytes and PBMCs, causing up to almost 100% cell death ($0.0002 < P < 0.00001$) at the maximum concentration used (Fig. 2). The two NNRTIs tested, NVP and EFV, caused the death of 80% ($0.1 < P < 0.002$) and 60% ($0.1 < P < 0.002$) of PBMCs, respectively, while they each caused the death of about 20% ($0.004 < P < 0.002$) of the cardiomyocytes (Fig. 2). NRTIs were generally the second least toxic class of ARVs after the EIs, causing the death of less than 20% of the cardiomyocytes and having between 20% and 72% toxicity for PBMCs (Fig. 2).

Cell death was caspase 3/7 independent. To complete the cytotoxicity profile, we further probed the mechanism through which ARVs were inducing cell death by measuring the activity of the executioner caspase 3/7. Despite the significant cell death induced by most ARVs in this study (Fig. 2), no significant increase in caspase 3/7 activity in either cardiomyocytes or PBMCs was observed after treatment with any of the ARVs compared to the activity in untreated control cells (Fig. 3A and B). However, a sharp and statistically significant decrease ($P < 0.002$) in caspase 3/7 activity was observed at days 4 and 7 for EFV-, ABC-, RTV-, and IND-treated cardiomyocytes (Fig. 3A). There was also a significant decrease in caspase 3/7 activity in PBMCs treated with EFV, ABC, TDF, and RTV for at least 4 and 7 days (Fig. 3B). The marked decrease in caspase 3/7 activity in cardiomyocytes and PBMCs treated with the ARVs directly correlated with the observed decrease in cell viability (Fig. 2). T20- and B40-treated cells were the only cells that had no significant changes in caspase 3/7 activity levels at any of the time points at which activity was measured, again correlating with the cell viability data (Fig. 2). Taken together, these data suggest that cell death was independent of the upregulation of caspase 3/7 activity. The significant decrease in the caspase 3/7 activity of cells treated with the ARVs compared to the activity of untreated control cells simply confirmed that most cells were dead and therefore unable to produce physiological levels of the caspase.

The B40 aptamer did not affect MAO A and B enzyme levels. Since the B40 aptamer and T20 (both EIs) were the only test molecules that had no effect on cell viability and caused insignificant cell death, an end-point marker of cytotoxicity, we selected them for more in-depth cytotoxic analysis at the enzyme and cell metabolism levels. We measured the interaction of the B40 aptamer with the MAO A and B enzymes, because any drug that interacts with MAO A or B, regardless of its function, can lead to a decrease in normal MAO cellular activity. This, in turn, can result in the intracellular accumulation of its natural substrates, such as serotonin, to potentially lethal levels. It is therefore important to screen potential drugs for a

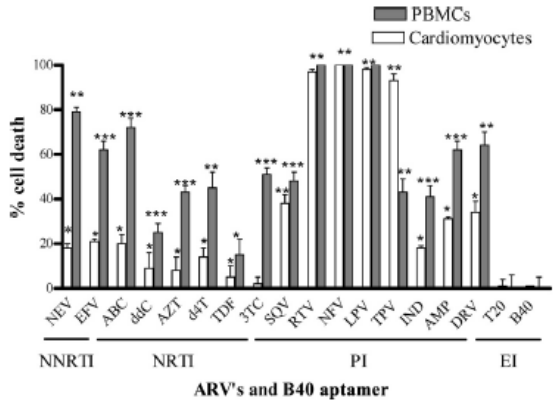

FIG. 2. Effect of the B40 aptamer in comparison to the effects of a panel of 17 ARVs on the viability of human cardiomyocytes and PBMCs. Human cord blood stem cell-derived cardiomyocytes or freshly isolated PBMCs were seeded at a density of 20,000 cells/well in 96-well plates and exposed to the respective ARVs at a concentration equivalent to the C_{max} shown in Table 1 or the B40 aptamer at 2 μM. All experiments were done in triplicate and were independently repeated twice. ATP levels were measured as a correlate of cell viability after 7 days of exposure by relating the RLU of treated cells to the RLU of untreated cells. The results were normalized and expressed as the percent cell death for the treated cell samples by comparison to the number of untreated cells. The data on the graph show the means, and the error bars represent the standard deviations for three replicates in two sets of independent experiments. ARVs whose toxicity was statistically different from that of the untreated controls, as determined by the *t* test, are indicated as follows: *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.

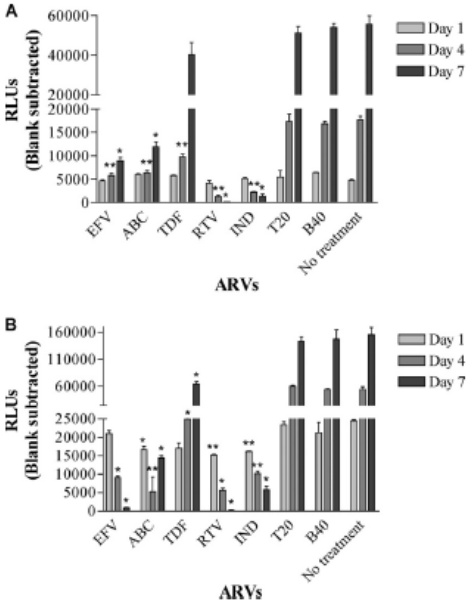

FIG. 3. Caspase 3/7 activity in cardiomyocytes (A) and PBMCs (B) upon exposure to ARVs. Cells seeded at a density of 50,000 cells/well were exposed to EFZ, ABC, TDF, RTV, IND, T20, or the B40 aptamer at the C_{max} for 1, 4, and 7 days. No statistically significant increases in caspase 3/7 activity were observed for any ARV, including the B40 aptamer, in cardiomyocytes or PBMCs compared to the activity in untreated control cells. A statistically significant decrease in caspase 3/7 activity was observed at least at day 4 and 7 in cardiomyocytes (A) and PBMCs (B) treated with EFV, ABC, TDF, and RTV compared to the activity in untreated control cells, as determined by the *t* test. *, $P < 0.002$; **, $P < 0.0002$. The data on the graphs represent the means, and the error bars represent the standard deviations for three replicates in two sets of independent experiments.

possible interaction with MAO enzymes. In this study, we measured the levels of both the MAO A and the MAO B enzymes in the presence of the B40 aptamer and, for comparison, in the presence of T20. The results clearly indicated that neither of the MAO isozymes was inhibited or induced by the B40 aptamer or T20 in either cardiomyocytes or PBMCs when the effects on treated cells were compared to those on untreated control cells (Fig. 4A to D). However, MAO A was inhibited by clorgyline in a concentration-dependent manner (Fig. 4A and B) and MAO B was inhibited by deprenyl in a similar manner (Fig. 4B and D). Clorgyline and deprenyl, which were used as inhibitors of MAO A and MAO B, respectively, served as good controls, confirmed the presence and the activities of both isozymes in cardiomyocytes and PBMCs, and confirmed that these isozymes are amenable to interference by certain drugs or specific inhibitors.

The B40 aptamer did not affect the level of the CYP3A4 enzyme. CYP3A4 is another key enzyme involved in cell metabolism and commonly affected by the PI class of ARVs. For this reason, we tested if the B40 aptamer modulates the activity of this enzyme in human cardiomyocytes and PBMCs. The effect of the B40 aptamer compared favorably to that of T20 and did not significantly affect the level of CYP3A4 when the effect was compared to that on untreated negative control cardiomyocytes ($P = 0.78$ for T20 and $P = 0.37$ for the B40 aptamer) and PBMCs ($P = 0.62$ for T20 and $P = 0.24$ for B40 aptamer), even at the highest concentration used (Fig. 5A and B). However, as a positive control, CYP3A4 was significantly inhibited in a concentration-dependent manner by the PI RTV in human cardiomyocytes ($P = 0.0001$) and PBMCs ($P = 0.0008$).

The B40 aptamer did not cause mtDNA toxicity. Mitochondrial toxicity is one of the major complications associated with the long-term use of ARVs, specifically NRTIs. To complete the circle, we further tested if the B40 aptamer causes the depletion of mtDNA in human cardiomyocytes or PBMCs. Similar to T20, the B40 aptamer did not cause a significant decrease in the mtDNA levels in either cardiomyocytes or PBMCs compared to the levels in untreated control cells (Fig. 6). The ratio of mtDNA/nDNA in cardiomyocytes treated with the B40 aptamer was 1.17 ± 0.19 ($P = 0.3$), and that in PBMCs was 1.16 ± 0.11 ($P = 0.06$), while the ratio of mtDNA/nDNA in cardiomyocytes treated with T20 was 1.01 ± 0.17 ($P = 0.5$) and that in PBMCs treated with T20 was 1.08 ± 0.10 ($P = 0.3$). The NRTI ddC was used as a positive control for mtDNA depletion, and treatment with ddC resulted in mtDNA/nDNA ratios of 0.72 ± 0.12 ($P = 0.006$) in cardiomyocytes and 0.41 ± 0.18 ($P = 0.0004$) in PBMCs (Fig. 6).

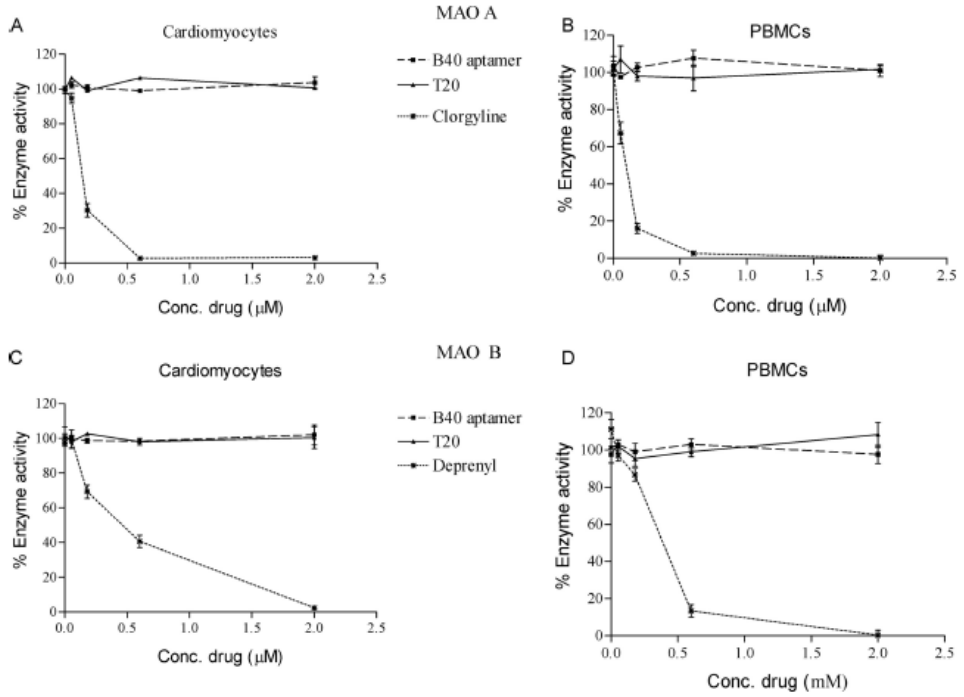

FIG. 4. MAO A and B enzyme modulation by the B40 aptamer and T20 in cardiomyocytes (A and C) and PBMCs (B and D). Cells were incubated in triplicate with the serially diluted test molecule at a maximum concentration of 2 μM for 2 h. A cell lysate was incubated with MAO A or B substrate for 1 h, followed by the addition of detection reagent, and the resulting luminescence signal was quantified in a luminometer. The no-treatment control was used as the baseline for maximum enzyme activity. No change in enzyme activity was detected for either MAO A or MAO B in the presence of the B40 aptamer and T20 for both cardiomyocytes and PBMCs. However, there was a dose-dependent inhibition of MAO A by clorgyline and MAO B by deprenyl, which are well-documented inhibitors of MAO A and MAO B, respectively, indicating the presence and activity of both enzymes in human cardiomyocytes and PBMCs. Enzyme activity was calculated by relating the RLUs of untreated control cells to that of the treated cells as a percentage. The data on the graphs represent the means, and the error bars represent the standard deviations for three replicates in two sets of independent experiments.

DISCUSSION

This study describes the cytotoxic effects of a novel, cross-clade HIV-1-neutralizing RNA aptamer called B40 and compares the cytotoxic effects of B40 with those of conventional ARVs. We used cell death; caspase 3/7 activation; and MAO A and B, CYP3A4, and mtDNA levels as markers of toxicity in human cardiomyocytes and PBMCs. First, in contrast to most registered ARVs tested, the B40 aptamer did not cause the death of cardiomyocytes and PBMCs even at the highest concentration tested (2 μM). This is encouraging, particularly because the IC_{50} for HIV-1_{BaL} and several HIV-1 primary isolates of the B40 aptamer in PBMCs is less than 10 nM (17, 21, 42). We have also observed that cell death is independent of caspase 3/7 activation. These are executioner caspases that are involved in all caspase-mediated apoptotic pathways (14, 64). Our data clearly demonstrate that NRTI- and NNRTI-induced apoptosis is caspase independent. These observations are in general agreement with findings described in the literature, in which ARV-induced cell death is frequently reported to be the result of mitochondrial injury (45). Mitochondrial toxicity is one of the major side effects and causes of treatment failures associated with the mid- to long-term use of ARVs, especially NRTIs (45). The severity of mitochondrial toxicity can range from being clinically silent to causing life-threatening conditions such as lactic acidosis (12). One of the most accepted hypotheses of mitochondrial toxicity is that ARVs cause a depletion of mtDNA due to inhibition of the mtDNA polymerase γ (47, 48). This results in the impaired production of mitochondrial enzymes, which participate in oxidative phosphorylation, ultimately leading to diverse organ-specific and/or systemic pathological changes. It was for this reason that we examined the B40 aptamer for its mitochondrial toxicity, and we have unequivocally shown that B40 does not interfere with mtDNA synthesis, ruling out the possibility that potentially deleterious mitochondrial interactions via the known pathways of ARV-induced mitochondrial toxicity are associated with the therapeutic use of this compound. Due to the high incidence of these cases, the NRTI ddC is used with extreme caution in the clinical setting. Furthermore, PI-related toxicity was also caspase independent, as cell death was not preceded by an increase in the level of caspase 3/7 activity (Fig. 3). The sharp decrease in the level of caspase activity induced by most ARVs

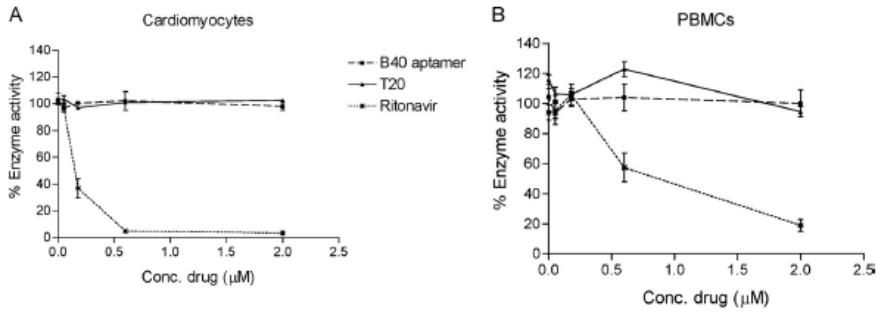

FIG. 5. Effects of the B40 aptamer and T20 on CYP3A4 enzyme activity in cardiomyocytes (A) and PBMCs (B). Cells were seeded at a density of 20,000 cells/well and exposed to the respective test molecule in triplicate for 2 days at serially diluted concentrations. The overlay medium was removed, and fresh medium containing a luciferin luminogenic substrate for the CYP3A4 enzyme was added. After 4 h of incubation, the luciferin detection reagent was added and the resulting luminescence signal was quantified in a luminometer. The no-treatment control was used as the baseline for maximum enzyme activity, and RTV, a known inhibitor of CYP3A4 (36, 56), was used as a positive control. The data on the graphs represent the means, and the error bars represent the standard deviations for three replicates in two sets of independent experiments.

(Fig. 3) was the result of a decrease in the total number of viable cells (Fig. 2). The exact mechanism by which PIs induce cell death has not been fully dissected (2), but cell cultures treated with RTV showed an accumulation of growth-arrested cells in the G_1 phase and high levels of expression of the universal cyclin-Cdk kinase inhibitor p21 (30). It has been speculated that the accumulation of p21 could be due to the inhibition of the cellular proteosome, resulting in cell death (62). Therefore, necrosis due to chemical insult is the most likely cause of cell death, although caspase-independent apoptosis cannot be ruled out (2).

Another favorable property of the B40 aptamer shown in this study is that it did not interact with the MAO A and B enzymes. The two MAO isoforms are present in most tissues, including peripheral tissues and the myocardium (32, 55, 60, 61, 63, 65). Drugs interacting with MAO A or B can cause either the upregulation or the downregulation of the enzymes, leading to the concomitant accumulation or depletion of neurotransmitters, respectively, thus resulting in serious clinical effects. Clorgyline and deprenyl, which were used as positive controls in this study, are well-documented inhibitors of MAO A (35, 43) and MAO B (5, 26), respectively. No ARV currently in clinical use has been shown to interact with either MAO A or MAO B. Notwithstanding this lack of a proven interaction, given the metabolic importance of MAO A and MAO B and their putative interactions with other drugs, it was imperative and prudent to look at the potential interaction of MAOs A and B with the B40 aptamer.

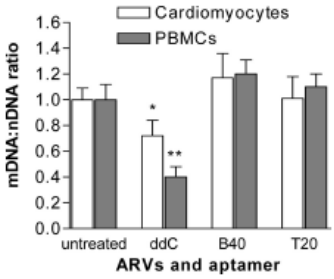

FIG. 6. Effects of the B40 aptamer and T20 on mitochondrial toxicity, as measured by determination of the mtDNA/nDNA ratio in cardiomyocytes and PBMCs. Cardiomyocytes and PBMCs were exposed to 2 μM B40 aptamer, T20, or ddC in triplicate for 7 days. The relative amounts of nDNA and mtDNA were quantified by real-time quantitative PCR amplification of the *CCOI* nuclear gene and the *ASPOLG* mitochondrial gene, respectively. The data were extrapolated from a standard curve relating the cycle numbers to the relative DNA concentration. The mtDNA/nDNA ratio for the untreated sample was arbitrarily set equal to 1.0. Only the ddC-treated cells showed a statistically significant decrease in the mtDNA/nDNA ratio compared to that for the untreated control, as determined by a *t* test and as indicated as follows: *, $P < 0.001$; **, $P < 0.0001$. The data on the graph represent the means, and the error bars represent the standard deviations for three replicates in two independent experiments.

The B40 aptamer also did not affect CYP3A4 activity in either PBMCs and cardiomyocytes, which compared favorably with the findings for T20. Most ARVs, notably, PIs, are substrates and potent competitive inhibitors of CYP3A4 (27). The CYP450 superfamily of heme-containing enzymes is the major catalyst for the oxidative transformation of most therapeutic drugs and xenobiotics in general, as well as a vast array of endogenous substances (40). The interaction of ARVs with members of the CYP450 enzyme superfamily often causes inactivation of the enzyme, leading to decreased plasma PI levels (40). This effect is often counteracted in the clinical setting by the coadministration of RTV, which is a potent inhibitor of CYP3A4 (36, 56), with other PIs (70). In this case, RTV plays a dual role both because of its antiretroviral activity and because of its inhibition of the CYP3A4 enzyme to boost the plasma levels of other PIs. However, this is closely associated with an increased incidence of both metabolic and somatic cardiac changes, which are often silent diseases because they are not readily diagnosed. Lipodystrophy, hyperlipidemia, and insulin resistance are some of the most common such disorders (9–12). Furthermore, the potent inhibition of CYP3A4 by most PIs, especially RTV (36, 56), seriously restricts the use of other auxiliary drugs that are also metabolized by the same enzyme, as this could lead to life-threatening cardiac events such as arrhythmias. The property of the B40 aptamer to po-

tently and broadly neutralize HIV-1 (21, 42) without inhibiting CYP3A4 therefore makes it an ideal candidate drug as a complement to existing ARVs.

Taken together, these data show that the B40 aptamer does not interfere with any of the major pathways of ARV-related cytotoxicity. These data agree with toxicological observations made with pegaptanib sodium (Macugen), which is the first and only aptamer-based therapeutic agent in clinical use (46). Pegaptanib sodium was approved for clinical use in 2005 and did not exhibit any intrinsic toxicity when it was evaluated in preclinical studies (23, 28, 29). The only side effect reported arises almost exclusively from the injection procedure rather than the drug itself (31).

The cytotoxicological data observed in this study further suggest that B40 in particular and aptamers in general may be safer than most ARVs. This argues for the development of the B40 aptamer and related anti-gp120, HIV-1-neutralizing aptamers (42) as new ARVs (entry inhibitors) with no cytotoxicity. While entry inhibitors such as the B40 aptamer are expected to be less toxic or not toxic because they act extracellularly, the results of this study should be treated with caution because there may be in vitro artifacts. A key challenge now is to evaluate the efficacy, pharmacokinetics, and toxicity of B40 and related aptamers in preclinical studies with a more dynamic in vivo animal model.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Lopes de Campos

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M071031**
PROJECT	Analysis of HIV-Induced Cardiomyopathy Using Anti-gp 120 Aptamers
INVESTIGATORS	Mrs WR Lopes de Campos
DEPARTMENT	School of Pathology/Virology
DATE CONSIDERED	07.10.26
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.10.31 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof L Morris

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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