

**APOPTOSIS IN PERIPHERAL BLOOD MONONUCLEAR  
CELLS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)  
INFECTED PATIENTS UNDERGOING  
HIGHLY ACTIVE ANTIRETROVIRAL THERAPY**

**BY**

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## ABSTRACT

Highly active antiretroviral therapy (HAART) is currently the only treatment that effectively reduces the morbidity and mortality of individuals infected with Human Immunodeficiency Virus-1 (HIV-1). Standard HAART regimens typically comprise 2 nucleoside reverse transcriptase inhibitors and either one non-nucleoside reverse transcriptase inhibitor or a protease inhibitor. These drugs bind to and inhibit the HIV-1 Reverse Transcriptase and Protease enzymes respectively, thereby suppressing viral replication.

The nucleoside reverse transcriptase inhibitors promote mitochondrial (mt) dysfunction by strongly inhibiting mt polymerase gamma (Pol- $\gamma$ ) and subsequently, mtDNA replication. In contrast, the non-nucleoside reverse transcriptase inhibitors, efavirenz (EFV) and nevirapine (NVP) do not inhibit Pol- $\gamma$  although EFV has been shown to induce mt depolarisation ( $\Delta\psi m^{\text{low}}$ ) *in vitro* at supra-therapeutic concentrations. However, the capacity of non-nucleoside reverse transcriptase inhibitor drugs to induce mt toxicity *in vivo* previously remained undetermined. The objective of this study was to determine the influence of EFV and NVP on peripheral lymphocyte mt transmembrane potential ( $\Delta\psi m$ ) and apoptosis in HIV-1–infected patients treated with these non-nucleoside reverse transcriptase inhibitors.

Thirty-two HIV-1–infected patients on HAART between 4 and 24 months (12 on EFV, 20 on NVP) and 16 HAART-naive HIV-1–infected patients were enrolled into this study. All participants were black South African patients. Spontaneous peripheral lymphocyte apoptosis and  $\Delta\psi m^{\text{low}}$  were measured *ex vivo* by flow cytometry for all patients.

CD4 T-helper apoptosis for the EFV and NVP cohorts was  $19.38\% \pm 2.62\%$  and  $23.35\% \pm 1.51\%$  (mean  $\pm$  SEM), respectively, whereas total lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  was  $27.25\% \pm 5.05\%$  and  $17.04\% \pm 2.98\%$ , respectively. Both parameters for each cohort were significantly lower ( $P < 0.05$ ) than that of the HAART-naive patients. The NVP cohort exhibited both a significant time dependent increase in peripheral lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  ( $P = 0.038$ ) and correlation between T-helper apoptosis and  $\Delta\psi\text{m}^{\text{low}}$  ( $P = 0.0005$ ). These trends were not observed in the EFV cohort.

This study provides evidence that both EFV and NVP induce peripheral lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  in HIV-1-infected patients on non-nucleoside reverse transcriptase inhibitor-based HAART, which in the case of NVP is sufficient to induce the apoptosis cascade.

## DECLARATION

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

The research described in this study was carried out in the Discipline of Medical Biochemistry, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Prof. A.A. Chuturgoon and Dr. H. Dawood.

A handwritten signature in black ink, appearing to read 'L. Karamchand', written over a horizontal line.

L. Karamchand

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## ABBREVIATIONS

$\psi$	HIV-1 RNA Packaging Signal
$\Delta\psi_m$	mitochondrial inner transmembrane potential
$\Delta\psi_m^{\text{low}}$	mitochondrial depolarisation
3TC	Lamivudine
ABC	Abacavir
ADP	Adenosine Diphosphate
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis Inducing Factor
Annexin-V-FITC	Annexin-V-Fluorescein Isothiocyanate
ANT	Adenine Nucleotide Transporter
Apaf-1	Apoptotic Protease-Inducing Factor-1
APC	Allophycocyanin
ARV	Antiretroviral
Asp	Aspartate
ATP	Adenosine Triphosphate
AZT	Zidovudine
BCL-2	B-cell CLL/Lymphoma 2
CA	Capsid (p24)
CAD	Caspase-activated DNase
CAP-3	Cytotoxicity-dependant APO-1 associated protein
CARD	Caspase Recruitment Domain
Caspase	Cysteine-dependant aspartate-specific protease
CCR5	CC-chemokine Receptor 5
CD4, CD8, CD95	Cluster of Differentiation 4/ 8/ 95
CD4 <sup>+</sup> / CD8 <sup>+</sup>	Cluster of Differentiation 4/ 8 positive
CRF	Circulating Recombinant Form
CTL	Cytotoxic T Lymphocyte
CXCR4	CXC-chemokine Receptor 4

<b>CYP<sub>450</sub></b>	Cytochrome P450
<b>CyP-D</b>	Cyclophilin D
<b>Cys</b>	Cysteine
<b>d4T</b>	Stavudine
<b>dATP</b>	deoxy-Adenosine Triphosphate
<b>ddC</b>	Zalcitabine
<b>ddI</b>	Didanosine
<b>DD</b>	Death Domain
<b>DED</b>	Death Effector Domain
<b>DIS</b>	Dimer Initiation Site
<b>DISC</b>	Death-Inducing Signalling Complex
<b>dsDNA</b>	Double-stranded Deoxyribonucleic Acid
<b>EFV</b>	Efavirenz
<b>Env</b>	Envelope
<b>FADD</b>	Fas-associated death domain
<b>FasL</b>	Fas Ligand
<b>FL1-H, FL2-H, FL3-H</b>	Fluorescence 1/ 2/ 3-Height
<b>FTC</b>	Emtricitabine
<b>gp41, gp120, gp160</b>	Glycoprotein 41/ Glycoprotein 120/ Glycoprotein 160
<b>gag</b>	Group Antigen
<b>GagPol</b>	Group Antigen-Polymerase polypeptide
<b>gRNA</b>	Genomic Ribonucleic Acid
<b>HAART</b>	Highly Active Antiretroviral Therapy
<b>HIV-1/ -2</b>	Human Immunodeficiency Virus-1/ -2
<b>HLA</b>	Human Leukocyte Antigen
<b>IAP</b>	Inhibitor of Apoptosis
<b>ICAD</b>	Inhibitor of Caspase-activated DNase
<b>IDV</b>	Indinavir
<b>IN</b>	Integrase (p31)
<b>IMM</b>	Inner Mitochondrial Membrane
<b>JNK</b>	Jun N-terminal kinase

<b>kb</b>	Kilobase
<b>kDa</b>	KiloDalton
<b>LDS</b>	Lipodystrophy Syndrome
<b>LTR</b>	Long Terminal Repeat
<b>LPV</b>	Lopinavir
<b>MA</b>	Matrix (p17)
<b>MOMP</b>	Mitochondrial Outer Membrane Permeabilisation
<b>mRNA</b>	Messenger Ribonucleic Acid
<b>mtDNA</b>	Mitochondrial Deoxyribonucleic Acid
<b>NC</b>	Nucleocapsid (p7)
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor-kappa B
<b>NFV</b>	Nelfinavir
<b>NNRTI</b>	Non-Nucleoside Reverse Transcriptase Inhibitor
<b>NRTI</b>	Nucleoside/ Nucleotide Reverse Transcriptase Inhibitor
<b>NRTI-TP</b>	Nucleoside Reverse Transcriptase Inhibitor Triphosphate
<b>NVP</b>	Nevirapine
<b>OMM</b>	Outer Mitochondrial Membrane
<b>PARP</b>	Poly (ADP-ribose) Polymerase
<b>PBMC</b>	Peripheral Blood Mononuclear Cell
<b>PBS</b>	Phosphate Buffered Saline
<b>PE</b>	Phycoerythrin
<b>PerCP</b>	Peridinin Chlorophyll Protein
<b>PI</b>	Protease Inhibitor
<b>PIC</b>	Preintegration complex
<b>PKC<math>\delta</math></b>	Protein Kinase C delta
<b>Pol</b>	HIV-1 Polymerase
<b>Pol-<math>\gamma</math></b>	Mitochondrial Polymerase gamma
<b>PpI</b>	Propidium Iodide
<b>PR</b>	HIV-1 Protease
<b>PrBS</b>	Primer Binding Site
<b>PS</b>	Phosphatidylserine

<b>PT</b>	Permeability Transition
<b>PTPC</b>	Permeability Transition Pore Complex
<b>R5X4</b>	CCR5-CXCR4 dual-tropic
<b>RIP</b>	Receptor Interacting Protein
<b>RNase H</b>	Ribonuclease H
<b>ROS</b>	Reactive Oxygen Species
<b>RT</b>	HIV-1 Reverse Transcriptase
<b>RTV</b>	Ritonavir
<b>Smac/DIABLO</b>	Second mitochondria-derived activator of caspase/ direct IAP-binding protein with low pI
<b>SQV</b>	Saquinavir
<b>ssRNA</b>	Single-stranded Ribonucleic Acid
<b>TAR</b>	Trans-activation Response element
<b>tBID</b>	Truncated BID
<b>T<sub>H</sub>L</b>	T-helper Lymphocyte
<b>TK 1/2</b>	Thymidine Kinase 1/2
<b>TNF<math>\alpha</math></b>	Tumour Necrosis Factor alpha
<b>TNFR1/2</b>	Tumour Necrosis Factor Receptor 1/ 2
<b>TRADD</b>	TNFR-associated Death Domain
<b>TRAF2</b>	TNFR-associated factor 2
<b>TRAIL</b>	TNF-related apoptosis-inducing ligand
<b>TRAIL-R1/2</b>	TRAIL Receptor 1/2
<b>tRNA<sup>Lys3</sup></b>	transfer Ribonucleic Acid Lysine 3
<b>UCP</b>	Uncoupling Protein
<b>VDAC</b>	Voltage-Dependant Anion Channel

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## ***INTRODUCTION***

The Human Immunodeficiency Virus (HIV) is the aetiological agent of the acquired immunodeficiency syndrome (AIDS). Infection with either HIV-1 or HIV-2 leads to immune compromise with the onset of opportunistic infections and development of AIDS-related diseases. However HIV-1 is the primary cause of the present global AIDS pandemic. As at December 2007, approximately 33.2 million people worldwide were living with HIV-1. During 2007 alone, there were approximately 2.5 million new infections and 2.1 million AIDS-related deaths worldwide (UNAIDS-WHO 2007).

At present, 68% and 90% of the world's HIV-1 infected adults and children respectively live in Sub-Saharan Africa, with 76% of the total AIDS-related deaths in 2007 occurring in this region. AIDS is the single largest cause of mortality in Sub-Saharan Africa, making this region the most seriously affected by HIV. The majority of adults in Sub-Saharan Africa that are infected with HIV-1 are women (prevalence of 61%), a characteristic unique to this region (UNAIDS-WHO 2007). South Africa currently has the largest number of HIV-1 infections in the world, the HIV-1 prevalence being highest in the KwaZulu-Natal province (39%) (Department of Health South Africa, 2007).

To date numerous multi-national attempts at producing an effective vaccine against HIV-1 have been unsuccessful. The treatment of HIV-infected patients with anti-retroviral (ARV) drugs currently remains the only effective method of retarding the progression of HIV-1 infection to AIDS. ARV drugs are typically administered as a "cocktail" of three drugs which constitutes highly active anti-retroviral therapy (HAART). When managed correctly, HAART is highly

effective in reducing the morbidity and mortality of individuals infected with HIV-1. However, despite dramatically improving the prognosis of patients infected with HIV-1, HAART remains ineffective in eradicating the virus from an individual once infected. Of the estimated 5.5 million South Africans that are already infected with HIV-1, approximately 1 million individuals presently require ARV therapy, of which only about 300000 are receiving it. In light of this statistic, the South African National AIDS Council adopted a decisive AIDS management strategy only in April 2007 to tackle the South African epidemic. This strategy set two main objectives to be achieved by the end of 2011, these being, firstly to halve the national infection rate and secondly, to increase the accessibility of ARV therapy to 80% of the infected individuals that require it.

The high cost of ARV drugs, poor health care infrastructure and lack of trained doctors and nurses presents major impediments to the successful implementation of HAART in South Africa's rural regions. Furthermore, the need for HIV-1 infected patients to be on life-long HAART together with the narrow therapeutic range of ARV drugs predisposes these patients to the risk of developing short- and long-term adverse toxic side-effects. Of even greater concern is the emergence of drug-resistant viral mutants that arise during patient-initiated treatment interruptions. These are the potential dire consequences of HAART when it is not monitored stringently. This emphasises the need for ARV drug rollout programmes to be coupled with efficient mechanisms to ensure patient adherence to HAART regimens and to closely monitor their development of adverse drug side-effects.

# CHAPTER 1

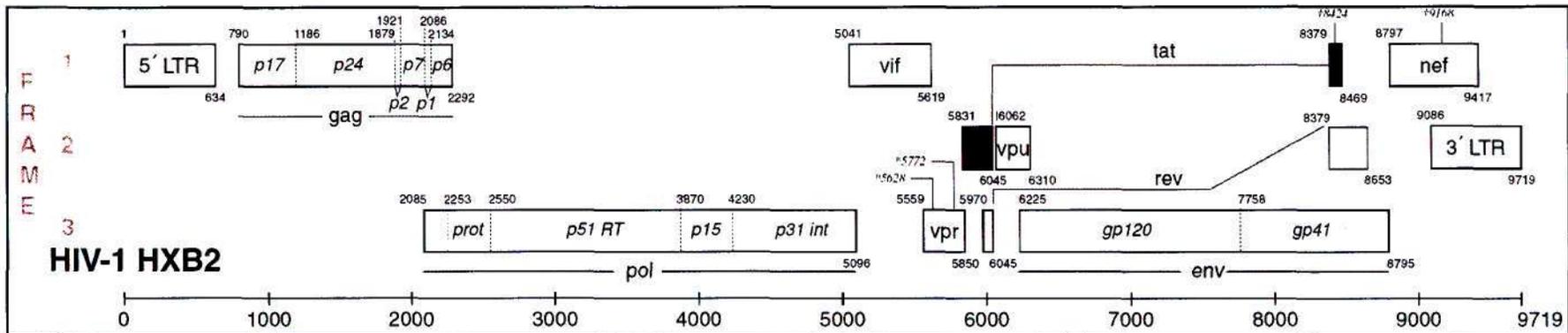
## *LITERATURE REVIEW*

### 1.1 THE HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1)

#### 1.1.1 THE HIV-1 GENOME

HIV-1 and its subtypes are retroviruses and belong to the family of lentiviruses. The HIV-1 genome is a 9.2 kilobase (kb) single-stranded (ss) positive (+) RNA molecule. HIV-1 virions contain two copies (dimers) of genomic (g) ssRNA, which upon infection are integrated as double-stranded (ds) DNA proviruses into the host cell genome. The organisation of the HIV-1 genome is typical of retroviruses: **5' LTR-gag-pol-env-LTR 3'** (LTR: Long Terminal Repeat; gag: Group Antigen; pol: Polymerase; env: Envelope) (Fig. 1.1).

The LTRs are transcription regulatory sequences that flank either end of the protein-coding sequence and are each subdivided into three regions designated U3, R and U5 [1]. The U3 region contains binding sites for cellular transcription factors such as Nuclear Factor-kappa B (NF-κB) [2, 3]. The R region contains the trans-activation response element (TAR) to which Tat protein binds and maintains high transcription levels of the proviral dsDNA [4, 5]. The U5 region contains a binding site for transfer RNA lysine 3 (tRNA<sup>Lys3</sup>) [1], which acts as a primer for the reverse transcription of viral ssRNA into dsDNA [6].



**Figure 1.1.** Organisation of the HIV-1 genome (HXB2 strain). The position of the start and stop codons of each gene are designated by numbers at the top left and bottom right corners respectively of the gene boxes. The reading frame of each gene is indicated to the left side of the diagram. The spliced exons of the *tat* and *rev* genes are indicated by shaded boxes. The position of the *pol* start codon (2085) is shifted -1 relative to the start codon of *gag-p1* (2086) [7].

The sequence between the U5 region and the *gag* AUG start codon contains the 5' leader domain, consisting of the primer binding site (PrBS), dimer initiation site (DIS) and packaging signal ( $\psi$ ) [8, 9]. These regions adopt secondary and tertiary structures that divert the viral mRNA strand from translation and into packaging as gRNA dimers in assembling virions [10, 11].

The *gag* region encodes the major structural proteins of the virus, p17 Matrix (MA), p24 Capsid (CA), p7 Nucleocapsid (NC) and p6, which are initially translated as a single p55 Gag polyprotein precursor [12]. The *pol* gene is located immediately downstream from the *gag* gene, with its reading frame shifted -1 relative to that of *gag* (Fig. 1.1, frame 3). The Protease (PR), p51 Reverse Transcriptase (RT), p15 RNase H and p31 Integrase (IN) enzymes are encoded by the *pol* gene. The *env* region encodes the gp120 and gp41 proteins which are involved in recognising and attaching to the host cell. The HIV-1 genome also encodes the genes of the regulatory (*tat* and *rev*) and accessory (*vif*, *vpr*, *vpu* and *nef*) proteins, whose reading frames are indicated relative to the *gag*, *pol* and *env* genes (Fig. 1.1).

### **1.1.2 GENETIC VARIANTS OF HIV-1**

HIV-1 is comprised of three major groups, designated M (main), O (outlier) and N (non-M non-O). Group M is further sub-divided into 9 clades or subtypes (A-D, F-H, J and K). The subtypes are classified and distinguished from each other on the basis of phylogenetic analysis of full length viral genomes, with each subtype represented by its own consensus sequence [13]. Subtype C accounts for 50% of the HIV-1 infections worldwide and is the most prevalent subtype in China, India and Central, Eastern and South Africa [14]. Subtypes A, B, G and D

represent 12, 10, 6 and 3% respectively of the total worldwide prevalence of HIV-1 group M, and subtypes F, H, J and K account for approximately 1% [14].

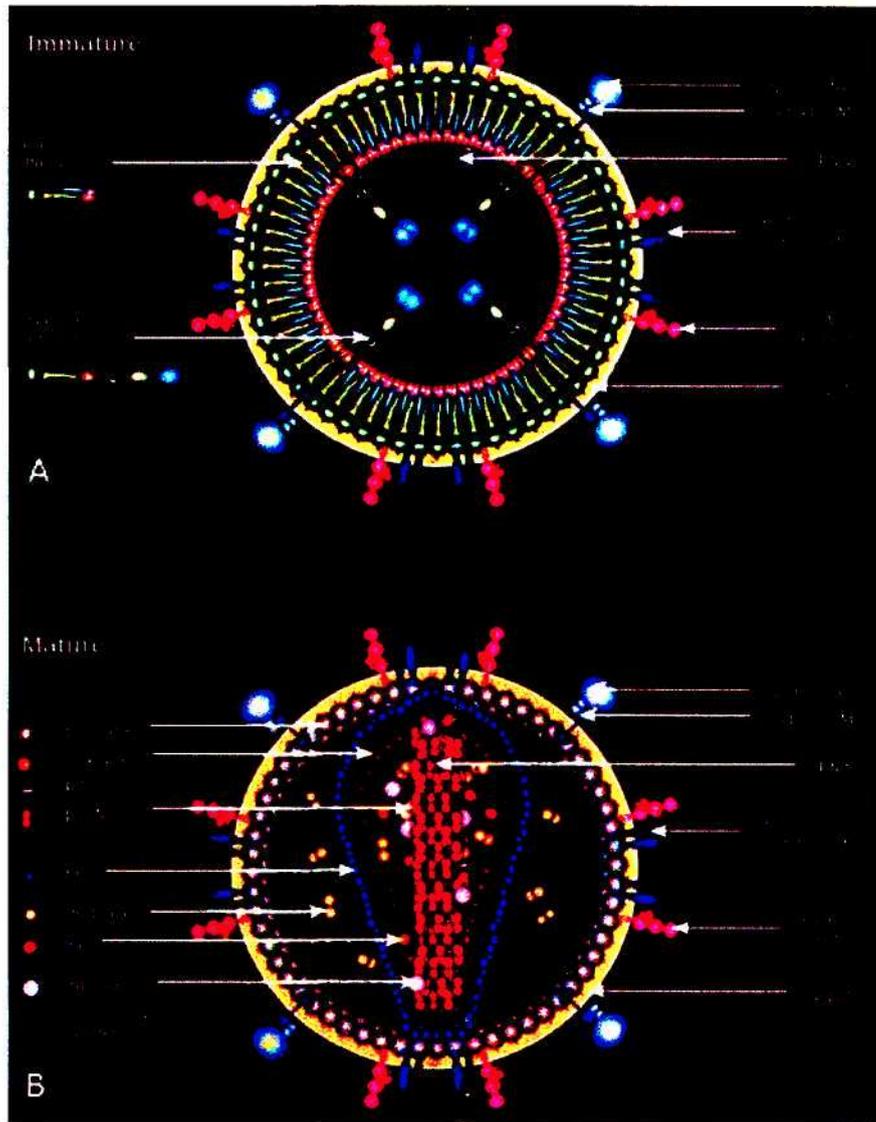
The remaining 18% of group M is represented by circulating recombinant form (CRF) viruses [14]. The CRFs are viruses whose genomic sequences are recombinants (mosaics) of more than one phylogenetic subtype and are common in geographic regions with a high prevalence of more than one viral subtype. To date 43 group M CRFs have been identified [15]. Inter-subtype recombination occurs when a host cell is productively super-infected with two distinct viral subtypes, with the gRNA strands of each subtype being compatible to form heterodimers during viral packaging [16]. Following infection, the viral genomic ssRNA is reverse transcribed into dsDNA, during which HIV-1 RT frequently switches between the two packaged copies of viral gRNA templates [17], a process known as copy choice [18]. The switching of RT between heterozygous RNA strands during multiple-subtype super-infection [19], results in the synthesis of recombinant proviral DNA [20]. If the new recombinant proviral genome confers enhanced immune evasion [21, 22] and/or anti-retroviral (ARV) drug resistance [23], the virus is then transmitted as a CRF.

### **1.1.3 VIRAL ULTRASTRUCTURE**

Mature HIV-1 virions have a spherical morphology and range between 100 – 120nm in diameter. A lipid bilayer envelope, of host cell origin, encapsulates the virion into which approximately 72 Env-glycoprotein complexes are integrated. Each complex is a trimer of the non-covalently linked external-gp120 and transmembrane-gp41 heterodimer [24, 25] (Fig. 1.2B). The gp120 moiety contains binding sites for the CD4-ligand and chemokine co-receptor (CCR5 or CXCR4),

the latter formed by the V1/V2 and C4 regions and V3 loop [26]. GP41 contains the HR1 and HR2 helices and hydrophobic fusion peptide that penetrates and anchors the virion into the host cell membrane [27, 28]. Host cell receptors such as Human Leukocyte Antigen (HLA) class I and II are also incorporated into the viral envelope during budding [29].

MA (p17) proteins form a shell lining the inner surface of the viral envelope through the interaction of the N-myristylated terminal of MA with the host membrane cytoplasmic leaflet [30]. The hollow viral conical core is produced from the assembly of CA (p24) proteins into hexameric rings [31]. The capsid core contains two copies of viral ssRNA in complex with NC protein and RT and IN enzymes [32] (Fig. 1.2B). The aspartyl protease (PR), tRNA<sup>Lys3</sup> [6, 33] and p6 [34], vpr [35], vif [36] and nef [37] proteins are also packaged within the capsid core (Fig. 1.2B).



**Figure 1.2.** Schematic diagrams of the immature (A) and mature (B) HIV-1 virion [38]. The immature virion notably lacks a capsid core, with the structural and enzymatic proteins still contained as part of the Gag and GagPol polyproteins. The virion attains maturity and is rendered infectious upon PR-mediated auto-catalytic cleavage of Gag and GagPol polyproteins.

### 1.1.4 HOST CELLS

The CD4 ligand, a 58kDa monomeric glycoprotein, is expressed on the surface of T-helper lymphocytes, bone marrow and thymic T-cell progenitors, monocytes and macrophages, dendritic and microglial cells [39]. CD4 is the primary receptor for HIV-1 [40, 41], whilst the chemokine receptors CCR5 [42-44] and CXCR4 [45] (G-protein-coupled 7-transmembrane receptors) are the major co-receptors utilised by HIV-1 *in vivo*. CXCR4 is mainly expressed on naïve CD4<sup>+</sup> T-helper lymphocytes (T<sub>H</sub>L), whereas activated memory CD4<sup>+</sup> T<sub>H</sub>Ls, monocytes and macrophages express CCR5 [46, 47]. Viruses that utilise either only CCR5 or only CXCR4 are designated R5- and X4-tropic respectively, whilst viruses capable of utilising both co-receptors are designated R5X4 [48].

The most significant long-term cellular reservoirs of HIV-1 are memory CD4<sup>+</sup> T<sub>H</sub>L [49, 50] and macrophages [51], due to the long life span of these cells. Immature dendritic cells, such as Langerhans cells, present in the genital epithelium, also support the replication of HIV-1 and play an important role during the early stages of sexual transmission of the virus [52]. Furthermore, circulating and follicular dendritic cells are capable of trapping extracellular virions on their membrane processes [53], and upon interaction with CD4<sup>+</sup> T<sub>H</sub>Ls in lymph nodes facilitate infection of these cells [54]. Epithelial dendritic cells express 10-fold more surface CCR5 than CXCR4 [55], and thus selectively propagate R5 strains [56]. HIV-1 R5 strains are preferentially transmitted via the sexual route [47] and predominate during the early stages of infection. The phenotypic switch from the R5 to the X4 or R5X4 strains *in vivo* generally occurs only several years after infection, which is accompanied by accelerated CD4<sup>+</sup> T<sub>H</sub>L loss and progression to AIDS [57]. In contrast to this observation, X4 viruses are far less frequent than R5 viruses in

individuals who are infected with subtype C HIV-1 [58]. The switch in viral tropism is determined by amino acid changes primarily in the V3 loop of gp120 [59-61].

### **1.1.5 VIRAL LIFE CYCLE**

The viral life cycle is a series of 6 sequential stages: 1) viral attachment and entry, 2) reverse transcription, 3) integration, 4) transcription, 5) translation, 6) viral assembly, budding and maturation. The initial binding event between the viral particle and host cell is the interaction of gp120 with the host cell CD4 receptor. GP120-CD4 binding induces a conformational change in gp120 that exposes its chemokine co-receptor binding site [62], with subsequent binding to either CCR5 or CXCR4. Co-receptor recruitment in turn triggers the insertion of the gp41 hydrophobic fusion peptide [63] into the host cell membrane thereby inducing the HR1 and HR2 domains of gp41 to adopt a stable six-helix bundle structure [64]. The viral envelope and host cell membrane then fuse delivering the p24 capsid core into the host cell cytoplasm where it is subsequently disassembled.

Capsid disassembly (viral uncoating) releases the dimeric gRNA which is reverse transcribed into dsDNA by the viral RT enzyme [65]. The preintegration complex (PIC), a complex of linear dsDNA with IN, MA, NC, PR, RT, and Vpr proteins [32, 66], is transported along microtubules [67] towards the nucleus. Active transport of the PIC across the nuclear pore complex [68] into the nucleus is mediated by MA and Vpr [69-71], a feature that allows HIV-1 to replicate in interphasic or quiescent cells [72]. Productive infection is achieved when the viral linear dsDNA is integrated, as the provirus, into the host cell genome by viral IN [73]. Viral transcription is activated by the binding of cellular factors, such as NFκB, to the proviral LTR [74]. Multiple-

spliced mRNAs are initially produced from which the regulatory proteins, Tat, Nef and Rev, are expressed. Rev promotes the accumulation of non-spliced or singly-spliced mRNAs [75] which are directed to cytoplasmic polysomes [76] for translation or packaging as gRNA dimers.

P55 Gag, GagPol and gp160 are the predominant viral proteins synthesised during the late stages of the viral replication cycle. Following glycosylation in the endoplasmic reticulum, gp160 is cleaved in the Golgi apparatus by cellular proteases into the gp120 and gp41 subunits [77], which are subsequently inserted into the plasma membrane. The PR, RT, RNase H and IN enzymes are derived from the GagPol polyprotein. Gag and GagPol are synthesised in an approximately 20:1 ratio, the latter produced following a -1 ribosomal frameshift during translation [78].

Viral particles are assembled from the oligomerisation of Gag and GagPol polyproteins under the host cell membrane [31, 79] (Fig. 1.2A). Viral assembly and budding is mediated via three specific domains of p55 Gag polyprotein precursor, designated the M (membrane targeting), I (interaction) and L (late assembly) domains. The N-myristylated terminal in the MA (M) domain of p55 Gag facilitates the insertion of Gag into the host plasma membrane [80, 81]. The NC (I) domain of p55 Gag chaperones the dimerisation of viral ssRNA [82, 83] via its two zinc-finger motifs [84, 85] and also facilitates the switch of newly synthesised viral mRNA to gRNA [11, 86]. This directs the packaging of viral RNA into progeny virions. The I-domain also mediates Gag-Gag interactions at the plasma membrane [79, 87]. The p6 (L) domain mediates viral budding [88] and promotes the incorporation of Vpr into the assembling virions [89]. The budding viral particle composed of Gag and GagPol precursors (Fig. 1.2A) is immature and non-infectious. During budding, viral PR cleaves the Gag and GagPol polyproteins into their

individual structural protein and enzymatic components (Fig. 1.2B), which is essential for viral maturation and viral budding to occur with maximum efficiency [90].

## **1.2 THE PATHOGENESIS OF HIV-1**

The hallmark feature of HIV-1 – infected patients is a progressive decline in their CD4<sup>+</sup> T<sub>H</sub>L and to a lesser degree, CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) levels, resulting in immunodeficiency which renders the patient susceptible to opportunistic infections and malignancies. The primary mechanism of T-cell depletion during HIV-1 infection is elevated CD4<sup>+</sup> T<sub>H</sub>L and CD8<sup>+</sup> CTL apoptosis [91-95]. Rapid disease progression has been shown to correlate with both elevated patient viral load [96] and CD4<sup>+</sup> T<sub>H</sub>L apoptosis [97] levels, whilst the latter correlates inversely with CD4<sup>+</sup> T<sub>H</sub>L counts [98]. Paradoxically, the majority of CD4<sup>+</sup> T<sub>H</sub>L cells undergoing apoptosis are uninfected by HIV-1 [99].

### **1.2.1 APOPTOSIS**

Apoptosis is a controlled energy-dependant, physiological death process that maintains cellular homeostasis in multicellular animal organisms by removing individual unwanted as well as aberrant and virus-infected cells, whilst maintaining the integrity of adjoining cells. Apoptotic cells are morphologically distinguishable from normal cells by the appearance of chromatin condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage that ultimately results in cellular fragmentation into smaller membrane-bound vesicles (apoptotic bodies) [100, 101]. The regulated biochemical and morphological disintegration of an apoptotic cell is executed by the class of proteolytic enzymes known as caspases.

### 1.2.1.1 CASPASES

Caspases (cysteine-dependant **aspartate-specific proteases**) [102] are highly specific proteases which catalyse a cysteine (Cys)-mediated hydrolysis of peptide bonds that immediately follow aspartate (Asp) residues [103]. Initially synthesised as inactive single chain proenzymes (zymogens), procaspases are activated by proteolytic cleavage at two Asp sites flanking the linker segment that separates the large (17-20kDa) and small (9-12kDa) subunits [103, 104]. The active caspase is a heterotetramer of two large and two small subunits [105-107]. This mechanism of activation facilitates the recruitment of latent procaspases in an amplifying proteolytic cascade [108-110]. Caspases involved in apoptosis belong to two classes based on their point of action in the caspase cascade: (a) the initiators, subdivided into the intrinsic (caspase-9) and extrinsic (caspases-2, -8 and -10) apoptosis pathway activators, and (b) effectors (caspases -3, -6 and -7) [111, 112].

Initiator caspases possess autocatalytic activity i.e. the ability to auto-activate [113]. However, autocatalytic activation of the initiator caspases occurs only upon recruitment and oligomerisation [114, 115] of several procaspase-2/8/10 and procaspase-9 molecules to either the cell surface death receptors (extrinsic pathway) or apoptosome (intrinsic pathway) respectively. The death effector domains (DED) [116, 117] and caspase recruitment domains (CARD) [118] are located in the long N-terminal peptides of procaspase-8/-10 and procaspases-2/-9 respectively. These domains mediate the targeting and association of the intrinsic and extrinsic initiator caspases with the complementary DEDs of the death receptor or CARD of the apoptosome respectively [108, 119]. Once activated, the initiator caspases cleave and activate the downstream effector procaspases [120-122]. Both procaspases-3 and -7 can also be cleaved and activated directly by

granzyme-B [123] [proteases delivered into a cell via perforin (pore-forming proteins) channels inserted by natural killer cells and CTLs].

Effector caspases usually mediate only a single cleavage of a protein substrate which either activates or inactivates that protein [104]. A broad range of cellular substrates are cleaved by effector-caspases including apoptotic and survival signalling kinases (MEK kinase-1 [124], protein kinase C delta (PKC $\delta$ ) [125], phosphatidylinositol-3 kinase/Akt-1 and Raf-1 [126]), pro- and anti-apoptotic BCL-2 family proteins (Bid [127], BCL-2 [128], BCL-XL [129]), structural proteins (nuclear lamins [123, 130], actin [131],  $\alpha$ -fodrin [132, 133] and gelsolin [134]), the DNA repair protein poly (ADP-ribose) polymerase (PARP) [135] and the endonuclease CAD/ICAD [136, 137]. The translocation of phosphatidylserine (PS) from the inner plasma membrane leaflet to the outer leaflet early in apoptosis is also caspase dependant [138], which triggers the removal of the apoptotic cell by macrophages [139]. The inhibitor of apoptosis proteins (IAPs) XIAP, c-IAP1 and c-AIP2, expressed in mammalian cells, bind directly to and inhibit active caspase-3 and -7 [140, 141].

During HIV-1 infection, lymphocyte apoptosis is mediated primarily via the death receptor (extrinsic) and/or mitochondrial (intrinsic) pathways.

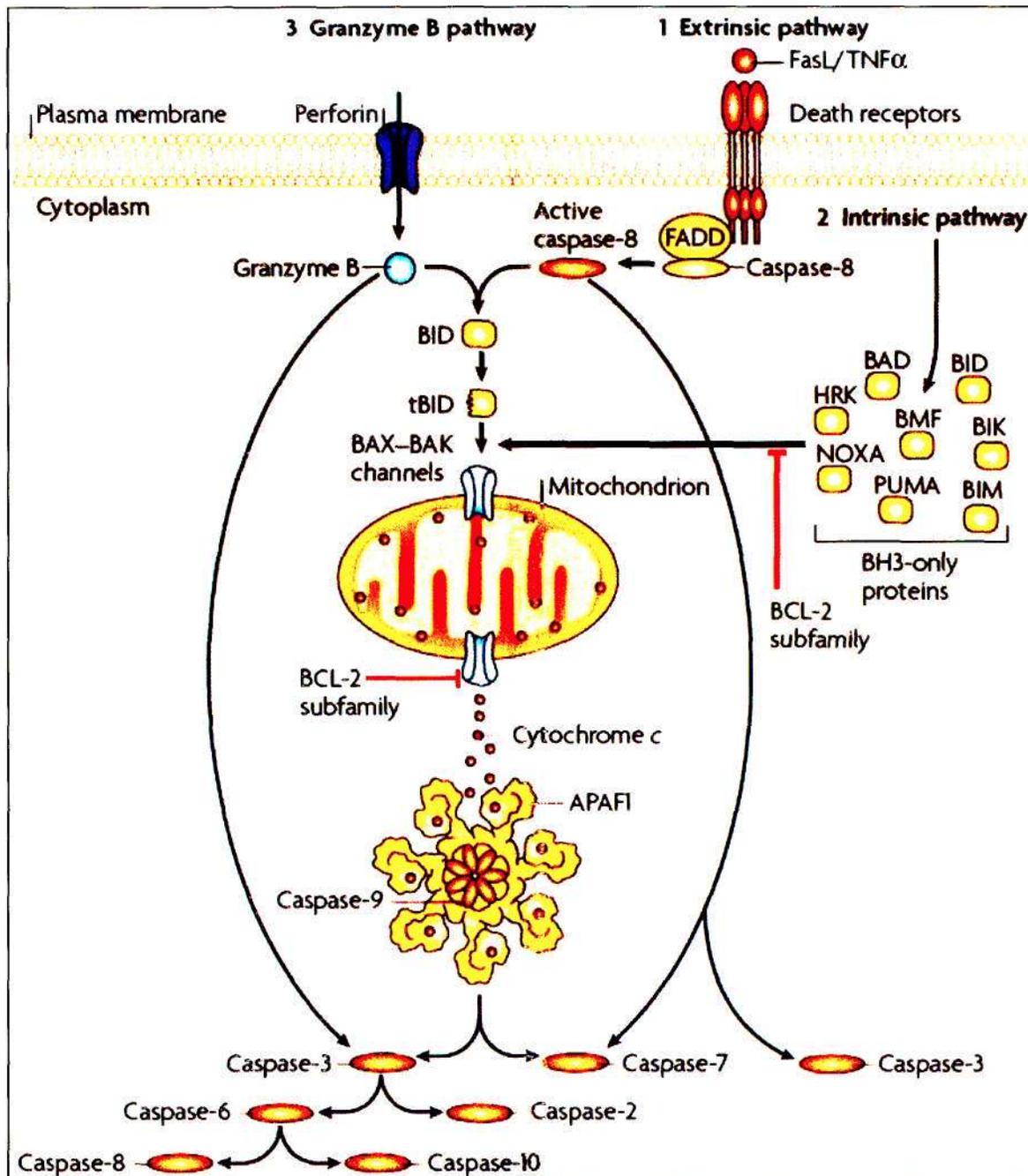
### **1.2.1.2 THE EXTRINSIC PATHWAY**

The cell surface transmembrane death receptors belong to the tumour necrosis factor (TNF) superfamily, and are characterised by cysteine-rich extracellular domains [142] and an intracellular cytoplasmic “death domain” (DD) sequence [143]. Death receptor-induced T-cell apoptosis in HIV-1 infected patients is mediated primarily by the ligation of Fas (CD95/APO1)

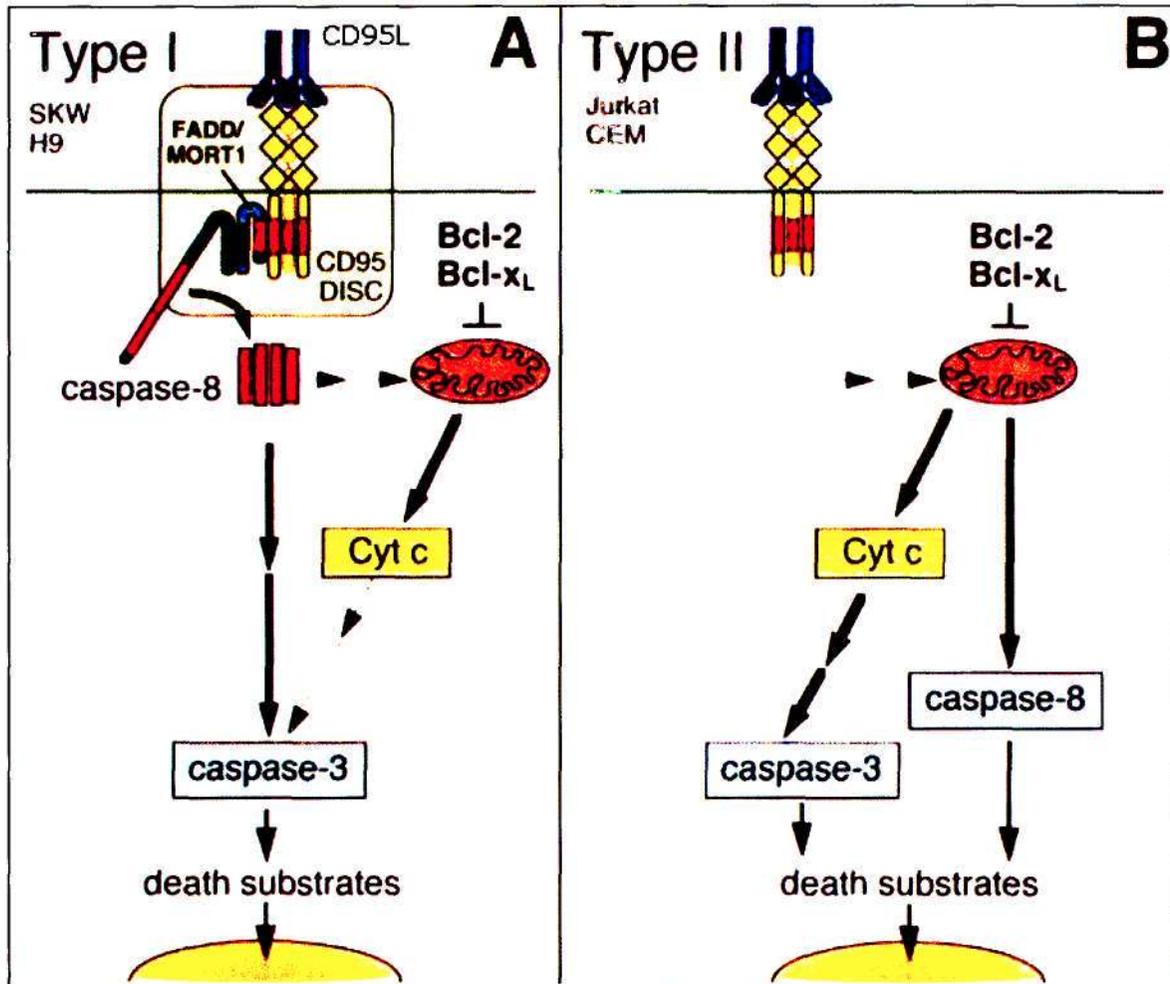
[144-147], TNFR1/2 [148-150] and TNF-related apoptosis-inducing ligand (TRAIL-R1/2) [151-153] receptors by their respective cognate ligands (FasL, TNF and TRAIL/APO2L) (Fig. 1.3). T-cells (T<sub>H</sub>L and CTL) of HIV-1 infected patients exhibit increased expression of particularly Fas [154-157] and TNFR1 receptors [158-160] (including their cognate ligands), thus enhancing their susceptibility to Fas- and TNF-mediated apoptosis respectively. Both TNFR1 and TRAIL-R1/2 receptors share similar mechanisms of caspase activation with the Fas receptor (Fig. 1.3).

Upon binding of homotrimeric FasL, Fas receptors undergo trimerisation [161] resulting in the recruitment and aggregation of Fas-associated death domain (FADD) adapters [162] to the receptors' cytoplasmic domains. Fas/FADD interactions are mediated by the DD motifs located in both proteins [162-163]. FADD additionally contains an N-terminal DED motif that facilitates the recruitment of multiple procaspase-8 zymogens [117, 164] (via their DED motifs) to Fas. CAP-3 (cytotoxicity-dependant APO-1 associated protein), which contains a DED motif, is also recruited to FADD and functions in procaspase-8 recruitment [117]. This multi-protein complex is known as the death-inducing signalling complex (DISC) [165]. Oligomerisation at the membrane induces procaspase-8 auto-activation which in turn activates procaspase-3 zymogens [113-115]. The above pathway is distinctive of cells, such as the H9 cell line, that undergo apoptosis via the type-I Fas signaling pathway (Fig. 1.4). The type-I Fas pathway typically bypasses mitochondria leading directly to caspase activation. In type-II Fas cells, such as the Jurkat cell line, DISC formation is restricted which leads to mitochondrial permeabilisation and subsequent activation of caspases-8 and -3 downstream of mitochondria [166] (Fig. 1.4). Consequently, only type-II Fas-mediated apoptosis can be blocked by the over-expression of anti-apoptotic Bcl-2 and Bcl-X<sub>L</sub> proteins, which inhibit mitochondrial permeabilisation [166].

In TNF-mediated apoptosis the adapter molecule, TNFR-associated death domain (TRADD), facilitates the binding between the DDs of TNFR1 and FADD [167]. Upon receptor ligation, TRADD additionally recruits the secondary adapters RIP (receptor interacting protein) and TRAF2 (TNFR-associated factor 2) which both activate the NF- $\kappa$ B and JNK survival signalling pathways [168-170], thereby counteracting the apoptotic signal. Furthermore, HIV-1 induces the production and secretion of TNF $\alpha$  by lymphocytes and peripheral blood mononuclear cells (PBMCs) [171]. TNF $\alpha$  induces the autocrine activation of NF $\kappa$ B [172-173] which in turn binds to the HIV-1 LTR [3] thereby inducing transcription of the HIV-1 provirus [174] and further stimulating TNF $\alpha$  production.



**Figure 1.3.** Schematic diagram of the intrinsic and extrinsic apoptosis induction pathways [175]. Signals from all pathways notably converge at the mitochondrion, culminating in the activation of the proteolytic caspase cascade.



**Figure 1.4.** Schematic overview of the Fas type I (A) and type II (B) pathways [166]. In type I cells, DISC formation predominates following Fas-FasL ligation leading directly to the activation of procaspase-8 and procaspase-3, independently of the mitochondria. In type II cells, DISC formation is limited and is characterised by mitochondrial PT followed by the release of cytochrome *c* into the cytoplasm and caspase activation.

### **1.2.1.3 THE INTRINSIC (MITOCHONDRIAL) PATHWAY**

The mitochondrion is the central organelle at which signals from numerous intracellular stresses converge, including calcium accumulation, reactive oxygen species (ROS), sphingolipids and DNA damage, resulting in mitochondrial outer membrane permeabilisation (MOMP) [176-178]. MOMP is induced by the opening of the permeability transition pore complex (PTPC). The PTPC, a transmembrane channel between the inner and outer mitochondrial membranes (IMM, OMM), is formed primarily by the interaction of VDAC (voltage-dependant anion channel) in the OMM with ANT (adenine nucleotide transporter) and cyclophilin D (Cyp-D) in the IMM [179].

Opening of the PTPC is regulated by the pro- and anti-apoptotic BCL-2 (B-cell CLL/Lymphoma 2) family of proteins (Fig. 1.3). The anti-apoptotic proteins include BCL-2, BCL-XL, BCL-W and MCL1, whilst the pro-apoptotic subfamily is subdivided into the pore-forming proteins (Bak and Bax) and BH3 domain-only proteins (BID, BAD, BIM, BIK, BMF, NOXA, PUMA and HRK) (Fig. 1.3). BCL-XL inhibits the opening of the PTPC by integrating into the OMM and binding directly to the VDAC [180], whilst Bax, Bak and BIM promote PTPC opening by binding to VDAC [181, 182]. Furthermore, MOMP is also induced upon homo-oligomerisation of Bax and Bak in the OMM [183, 184]. Bax can be directly activated by p53 tumour suppressor protein to induce MOMP [185], whilst both Bax and Bak homo-oligomerisation is induced by truncated BID (tBID) [184, 186, 187] (Fig. 1.3). Death receptor ligation results in caspase-8 cleavage of cytosolic BID to tBID [127, 188, 189]. tBID also directly induces MOMP by forming homo-oligomer pores in the OMM [190] and thus relays apoptotic signals from the cell surface to the mitochondria (Fig. 1.3).

Following MOMP, small pro-apoptotic proteins, including cytochrome *c* [191], second mitochondria-derived activator of caspase/ direct IAP-binding protein with low pI (Smac/Diablo) and Omi/HtrA2 (IAP inhibitors)[192, 193], apoptosis-inducing factor (AIF) and endonuclease G (nucleases) [194, 195], are released from the mitochondrial intermembrane space into the cytosol. The release of cytochrome *c* is accompanied by the collapse of the mitochondrial inner transmembrane potential ( $\Delta\psi_m$ ) [196], known as permeability transition (PT) or depolarisation ( $\Delta\psi_m^{\text{low}}$ ), which irreversibly commits the cell to apoptosis [197, 198]. In the cytosol, cytochrome *c* assembles with dATP, apoptotic protease-inducing factor-1 (Apaf-1) and procaspase-9 (the latter two interacting via their CARD motifs) to generate the apoptosome [199-201]. Formation of the apoptosome induces auto-catalytic activation of procaspase-9, which subsequently recruits and activates procaspase-3 [202] (Fig. 1.3).

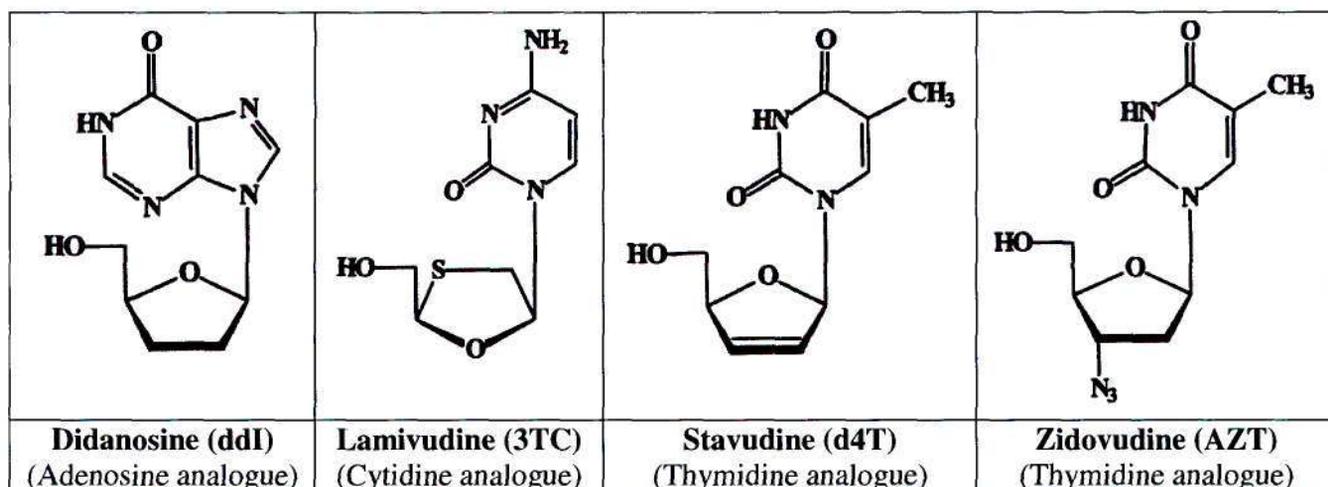
Peripheral circulating T-cells of HIV-1 infected patients are characterised by an increase in  $\Delta\psi_m^{\text{low}}$  [203], an event crucial to T-cell apoptotic death during AIDS [204]. The collapse of  $\Delta\psi_m$  and PS-externalisation are early apoptotic events both of which can be measured flow cytometrically on whole cells stained with the JC-1 and Annexin-V-FITC fluorophores respectively [205, 206].

### **1.3 ANTI-RETROVIRAL DRUGS**

The anti-retroviral (ARV) drugs currently licensed in South Africa (SA) for the treatment of HIV-1 infection target and inhibit the viral RT and PR enzymes, thereby retarding viral progeny synthesis. The ARV drug classes comprise the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitor (NNRTIs) and protease

inhibitor (PIs). The co-administration of at least two NRTIs with either one NNRTI or PI constitutes highly active anti-retroviral therapy (HAART) and is now standard of care of HIV-1 infected individuals [207]. HAART dramatically reduces plasma viral load with a concomitant increase in circulating CD4<sup>+</sup> T<sub>H</sub>Ls [208-210]. However, HAART targets only actively replicating virus and is ineffective in eliminating dormant virus in reservoir and sanctuary sites [211]. This necessitates an HIV-1 infected individual to be on life-long HAART to maintain suppression of viral replication. *Ex vivo* studies have reported the persistence of T-lymphocyte apoptosis in patients undergoing HAART, particularly with NRTI-NNRTI based regimens [212, 213]. In addition to their anti-viral properties, NRTIs, NNRTIs and PIs modulate lymphocyte apoptosis at the level of the mitochondrion, which will be discussed further.

### 1.3.1 THE NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS



**Figure 1.5.** Chemical structures of the NRTIs (nucleoside derivatives) [214] commonly prescribed in South Africa for the treatment of HIV-1 infection.

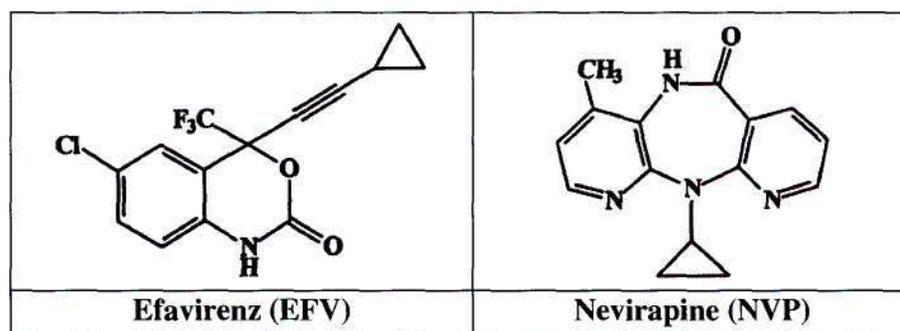
The NRTIs are dideoxyribonucleoside derivatives that lack a 3'-OH group on the deoxyribose sugar moiety. Within the cell, NRTIs are phosphorylated in a step-wise manner by various cellular kinases to their 5'-triphosphate derivatives (NRTI-TPs) [215-217]. Thymidine analogues are most efficiently phosphorylated in activated PBMCs, whereas ddi, 3TC and zalcitabine (ddC) are preferentially phosphorylated in resting PBMCs [218, 219]. The NRTI-TPs compete with endogenous deoxyribonucleotides for the catalytic site of RT. Incorporation of an NRTI-TP at the 3'-end of the elongating viral DNA strand results in premature termination of reverse transcription [216, 220].

### **1.3.1.1 MITOCHONDRIAL TOXICITY OF THE NRTIs**

In light of their mechanism of action, NRTI-TPs also potentially compete as substrates for cellular DNA polymerases  $\alpha$ ,  $\beta$ ,  $\epsilon$  and  $\gamma$  [221]. However, mitochondrial DNA polymerase-gamma (Pol- $\gamma$ ), the enzyme required for replication of mitochondrial DNA (mtDNA), is most potently inhibited by NRTI-TPs [221, 222]. The *in vitro* hierarchy of Pol- $\gamma$  inhibition by NRTI-TPs, in descending order, is as follows: ddC  $\geq$  ddi  $\geq$  d4T > 3TC > emtricitabine (FTC) > AZT > abacavir (ABC) [221]. Pol- $\gamma$  inhibition results in mtDNA depletion, which has been observed *in vitro* in NRTI-treated cell lines [223-225] and *in vivo* in fat [226, 227], skeletal muscle [228, 229] and liver tissue [230] of patients presenting with lipodystrophy syndrome (LDS). Whilst mtDNA depletion was reported in human peripheral lymphocytes exposed both *in vivo* [231, 232] and *in vitro* [233, 234] to NRTIs, individuals asymptomatic for LDS whilst undergoing HAART did not exhibit lymphocyte mtDNA depletion [235].

mtDNA depletion results in decreased synthesis of mtDNA-encoded respiratory chain complex subunits [232, 233], which manifests predominantly during long-term HAART [236, 237]. This ultimately compromises oxidative phosphorylation resulting in increased anaerobic respiration and lactic acid production, culminating in elevated serum lactate levels (hyperlactatemia) [233, 238-240]. AZT-induced respiratory chain dysfunction also increases the generation of ROS within the mitochondrion [241-243], resulting in apoptosis [244]. Cells lacking mtDNA undergo apoptosis via induction of PT [245] possibly as a result of ROS-mediated attack of the ANT and/or VDAC components of the PTPC, independent of pro-apoptotic Bcl-2 proteins [246, 247].

### 1.3.2 THE NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

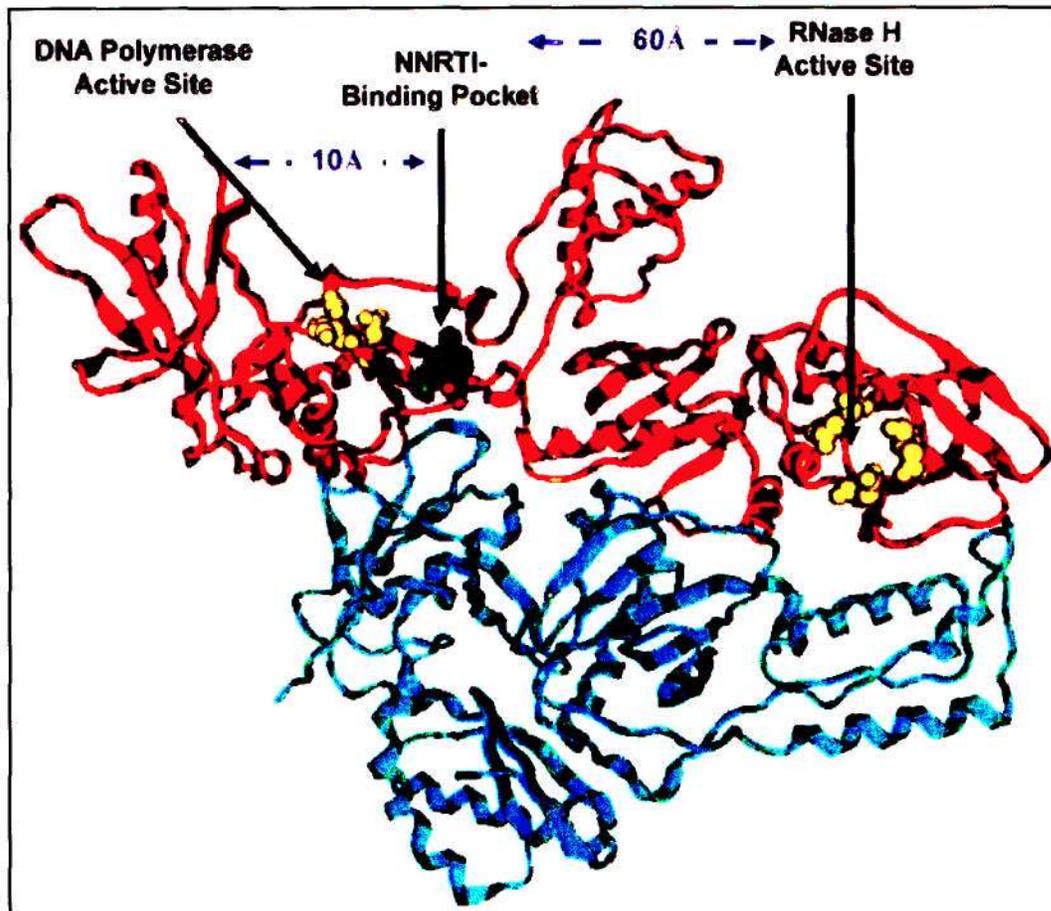


**Figure 1.6.** Chemical structures of the NNRTIs [214] commonly prescribed in South Africa for the treatment of HIV-1 infection.

NNRTIs are a heterogeneous class of small (<600Da) hydrophobic non-nucleoside analogue compounds that specifically inhibit HIV-1 RT, but not HIV-2 RT [248]. In contrast to NRTIs, NNRTIs do not require intracellular metabolism for activation. NNRTIs non-competitively inhibit reverse transcription by binding to the “NNRTI-binding pocket”, located allosterically to

the substrate binding (DNA polymerase) site in the p66 subunit of HIV-1 RT [249, 250] (Fig. 1.7). The NNRTI-binding pocket is spatially and functionally associated with the DNA polymerase site [251, 252] (Fig. 1.7), which upon binding a NNRTI drug locks the DNA polymerase site in an inactive conformation [253].

In addition, EFV (but neither NVP nor Delavirdine) enhances the intracellular proteolytic processing of Gag and GagPol polyproteins with a concomitant decrease in viral particle production [254]. This is mediated by the binding of EFV to RT embedded in the GagPol polyprotein thereby promoting the oligomerisation of GagPol polyproteins. This induces premature activation of the PR enzyme embedded within GagPol, and subsequent cleavage of the polyproteins into their constituent structural and enzyme subunits. As a result, this decreases the amount of full-length Gag and GagPol polyproteins that are necessary for viral assembly and budding from the host cell membrane [254].



**Figure 1.7.** Ribbon representation of HIV-1 RT in complex with EFV (grey). The p66 and p51 subunits of HIV-1 RT are coloured red and green respectively. Residues located in the DNA polymerase and RNase H active sites are indicated with yellow spheres [255].

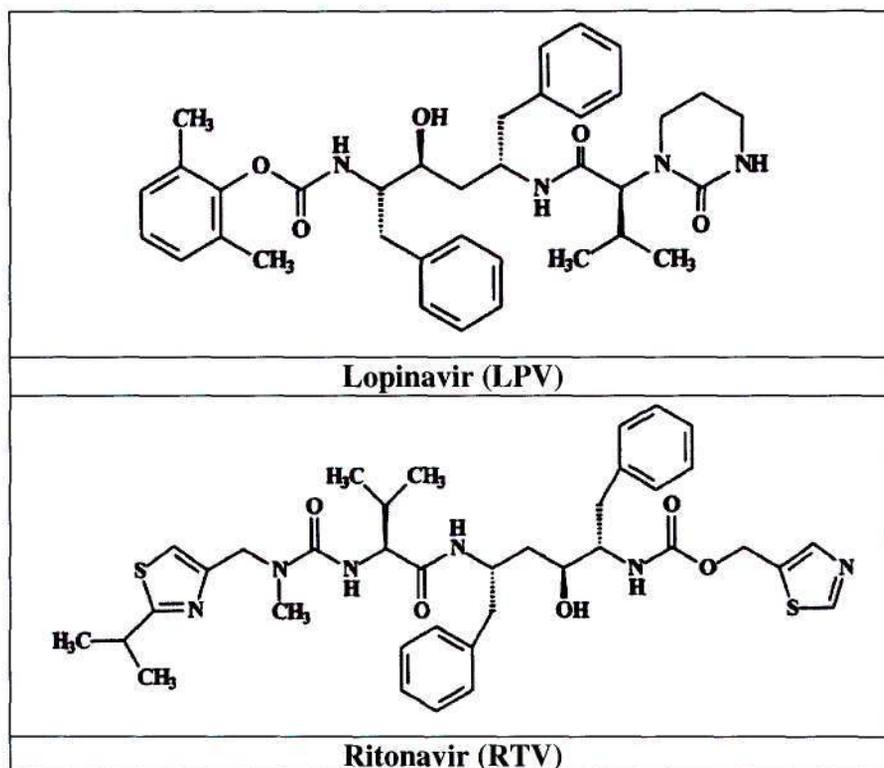
### 1.3.2.1 MITOCHONDRIAL TOXICITY OF THE NNRTIs

In contrast to the NRTIs, much less is known about the apoptosis-inducing potential of the NNRTIs and the mechanism/s thereof. EFV has been shown to induce apoptosis *in vitro* in both the Jurkat cell line and PBMCs, which were characterised by a dose-dependant increase in  $\Delta\psi_m^{\text{low}}$ , cytochrome *c* release, procaspase-9 and -3 activation and PS externalisation [256]. This finding suggests a mitochondrial mechanism of NNRTI-induced lymphocyte cytotoxicity,

however the precise mechanism of NNRTI-induced mitochondrial PT in lymphocytes remains unknown.

NNRTIs appear to influence the expression of uncoupling proteins (UCPs) in brown adipocytes. UCPs are proton transporters, present in the IMM, that catalyse a regulated discharge of the proton gradient across the IMM i.e. reduction of the  $\Delta\psi_m$ . UCP1 is expressed exclusively in brown adipose tissue, UCP3 in brown adipose and muscle tissue, UCP 4 and BMCP1 in the brain, whilst UCP2 expression is widespread [257]. In an *in vitro* study, both d4T and NVP significantly induced the UCP1 mRNA expression in treated brown adipocytes, with the level of induction by NVP being significantly higher than by d4T [258]. Neither of these drugs influenced the expression of UCP2 mRNA, whilst EFV had no effect on the expression of UCP1 and UCP2 mRNA in brown adipocytes with respect to the control [258].

### 1.3.3 THE PROTEASE INHIBITORS



**Figure 1.8.** Chemical structures of the PIs [214] commonly prescribed in South Africa for the treatment of HIV-1 infection. Other drugs in this class include Indinavir (IDV), Nelfinavir (NFV) and Saquinavir (SQV).

The importance of HIV-1 PR in viral maturation lends itself as a potent target for the inhibition of viral replication. PIs are transition state peptidomimetic analogues that bind more tightly than the natural substrates to aspartate residues in the PR catalytic active site, thus competitively inhibiting the PR enzyme [214, 259]. Inhibition of HIV-1 PR results in the production of immature and non-infectious viral particles.

### 1.3.3.1 MITOCHONDRIAL PROTECTIVE EFFECT OF PIs

Numerous studies reported that after initiation of PI-containing HAART, patients exhibited marked increases in their circulating CD4<sup>+</sup> T<sub>H</sub>L counts before significant decreases in plasma viral RNA load [209, 260-262]. This has been attributed to the independent inhibition of lymphocyte apoptosis by PI drugs both *ex vivo* and *in vitro* [263, 264]. Phenix *et al* [265] reported that NFV inhibited Jurkat cell apoptosis within 1 hour of treatment, at clinically achievable concentrations, in the presence of apoptotic stimuli. NFV blocked PTPC opening at the level of the ANT and thus the collapse of  $\Delta\psi_m$  and cytochrome *c* release in the presence of apoptotic stimuli, but did not inhibit active caspase-3, -6, -7, or -8 proteolytic activity [265]. NFV however did not inhibit Fas-mediated apoptosis in the type-I Fas H9 cell line [265]. The inhibition of ANT function in PTPC formation by NFV was also confirmed in another independent study [266].

Several studies subsequent to Phenix *et al* [265] reported the inhibition of  $\Delta\psi_m^{\text{low}}$  and mitochondrial-driven apoptosis by protease inhibitors [267-270]. In addition, Matarrese *et al* [269] reported the novel finding that LPV, IDV and SQV reduced UCP2 protein expression, in a dose-dependant manner, in T-lymphocytes isolated from human PBMCs, with a concomitant reduction in mitochondrial PT and ROS production. Thus PIs maintain the integrity of the lymphocyte mitochondrial  $\Delta\psi_m$  by preventing opening of the PTPC and UCP-mediated discharge of the  $\Delta\psi_m$ .

## **CHAPTER 2**

### **SCIENTIFIC PAPER PUBLICATION**

#### ***PREFACE***

The following paper published in the Journal of Acquired Immune Deficiency Syndromes (48(4); August 2008, 381-388), documents the persistence of peripheral lymphocyte mitochondrial depolarisation and apoptosis in Black South African HIV-1 infected patients undergoing HAART that contained either one of the NNRTIs, EFV or NVP.

This study was undertaken in light of the lack of literature regarding the potential of the above NNRTIs to induce lymphocyte mitochondrial dysfunction and apoptosis *in vivo*.

# Lymphocyte Mitochondrial Depolarization and Apoptosis in HIV-1-Infected HAART Patients

Leshern Karamchand, MSc,\* Halima Dawood, MD, FCP,† and Anil A. Chuturgoon, PhD\*

**Background:** Efavirenz (EFV) and nevirapine (NVP), unlike nucleoside reverse transcriptase inhibitor drugs, do not inhibit mitochondrial (mt) polymerase gamma (Pol- $\gamma$ ), although EFV has been shown to induce mt depolarization ( $\Delta\psi_{m}^{low}$ ) in vitro at supratherapeutic concentrations. However, the capacity of nonnucleoside reverse transcriptase inhibitor drugs to induce mt toxicity in vivo remains undetermined.

**Objective:** To determine the influence of EFV and NVP on peripheral lymphocyte mt transmembrane potential ( $\Delta\psi_{m}$ ) and apoptosis in HIV-1-infected patients treated with these nonnucleoside reverse transcriptase inhibitors.

**Methods:** Thirty-two HIV-1-infected patients on highly active antiretroviral therapy (HAART) between 4 and 24 months (12 on EFV, 20 on NVP) and 16 HAART-naive HIV-1-infected patients were enrolled into this study. All participants were black South African patients. Spontaneous peripheral lymphocyte apoptosis and  $\Delta\psi_{m}^{low}$  were measured ex vivo by flow cytometry for all patients.

**Results:** CD4 T-helper apoptosis for the EFV and NVP cohorts was  $19.38\% \pm 2.62\%$  and  $23.35\% \pm 1.51\%$  (mean  $\pm$  SEM), respectively, whereas total lymphocyte  $\Delta\psi_{m}^{low}$  was  $27.25\% \pm 5.05\%$  and  $17.04\% \pm 2.98\%$ , respectively. Both parameters for each cohort were significantly lower ( $P < 0.05$ ) than that of the HAART-naive patients. The NVP cohort exhibited both a significant time-dependent increase in peripheral lymphocyte  $\Delta\psi_{m}^{low}$  ( $P = 0.038$ ) and correlation between T-helper apoptosis and  $\Delta\psi_{m}^{low}$  ( $P = 0.0005$ ). These trends were not observed in the EFV cohort.

**Conclusions:** This study provides evidence that both EFV and NVP induce peripheral lymphocyte  $\Delta\psi_{m}^{low}$  in HIV-1-infected patients on nonnucleoside reverse transcriptase inhibitor-based HAART, which in the case of NVP is sufficient to induce the apoptosis cascade.

**Key Words:** efavirenz, nevirapine, peripheral lymphocytes, mitochondrial depolarization, apoptosis

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## INTRODUCTION

Highly active antiretroviral therapy (HAART) is currently the only treatment that effectively reduces the morbidity and mortality of individuals infected with HIV-1.<sup>1</sup> HAART results in a reduction in plasma viral load with a subsequent increase in circulating CD4 T-helper ( $T_H$ ) lymphocytes<sup>2–4</sup> and decreased lymphocyte apoptosis.<sup>5–7</sup> Standard HAART regimens typically comprise 2 nucleoside reverse transcriptase inhibitors (NRTIs) and either one nonnucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). Continuous suppression of viral replication requires long-term therapy during which patients develop severe side effects. Lipodystrophy syndrome (LDS), the result of long-term NRTI therapy, is characterized by dyslipidemia, body fat redistribution, and metabolic abnormalities.<sup>8,9</sup>

NRTI-associated LDS is attributed to the mitochondrial (mt) toxicity of this drug class,<sup>9,10</sup> which is mediated by the inhibition of the mt enzymes DNA polymerase gamma (Pol- $\gamma$ ),<sup>11,12</sup> adenylate kinase,<sup>13</sup> thymidine kinase (TK) type-2,<sup>14</sup> and ADP/ATP translocator.<sup>15</sup> The inhibition of Pol- $\gamma$ , the enzyme responsible for mitochondrial DNA (mtDNA) replication, is however the most significant contributor to mt toxicity. The in vitro hierarchy of Pol- $\gamma$  inhibition by NRTIs (triphosphorylated) in descending order is as follows: zalcitabine (ddC)  $\geq$  didanosine (ddI)  $\geq$  stavudine (d4T)  $>$  lamivudine (3TC)  $>$  emtricitabine (FTC)  $>$  zidovudine (AZT)  $>$  abacavir (ABC).<sup>12</sup> mtDNA depletion and deletion have been observed in vitro in NRTI-treated cell lines<sup>16–18</sup> and in vivo in fat,<sup>19,20</sup> skeletal muscle,<sup>21</sup> and liver tissue<sup>22</sup> of LDS patients.

In recent studies, human peripheral lymphocytes exposed in vivo<sup>23,24</sup> and in vitro<sup>25,26</sup> to NRTI-containing HAART also exhibited significant mtDNA depletion. Furthermore, reduced expression and activity of mtDNA-encoded complexes of the mt respiratory chain were observed in NRTI-treated T lymphocytes<sup>25</sup> and peripheral blood mononuclear cells (PBMCs) of LDS patients, respectively.<sup>24</sup> Impairment of respiratory chain enzyme expression inhibits oxidative phosphorylation,<sup>27</sup> with possible induction of apoptosis via the mt pathway.<sup>28</sup> Conversely, PIs have been shown to prevent apoptosis, at the mt level by preventing collapse of the mt transmembrane potential ( $\Delta\psi_{m}$ ).<sup>29</sup> The effect of NNRTIs on mitochondria is less well documented, although efavirenz (EFV) has been reported to induce apoptosis in vitro via the mt pathway in the Jurkat cell line and primary T cells of uninfected donors.<sup>30</sup> However, the in vivo effect of the commonly prescribed NNRTIs, EFV, and nevirapine (NVP) on patient lymphocyte mitochondria remains unknown. We therefore assessed ex vivo mt depolarization ( $\Delta\psi_{m}^{low}$ ) and apoptosis in lymphocytes of

HIV-1-infected patients who were treated with either EFV or NVP as a component of triple-drug HAART, for 4–24 months.

## METHODS

### Study Design and Patient Recruitment

The study was approved by the University of KwaZulu-Natal, Biomedical Research Ethics Administration (H129/04). Patients were recruited from an antiretroviral rollout clinic at a tertiary-level hospital after obtaining informed consent. All patients were black South Africans with HIV-1 infection. Thirty-two patients on NNRTI-based HAART were enrolled (8 males:24 females). Sixteen HIV-1-infected patients (5 males:11 females) who were HAART naive were recruited as control subjects.

### Drug Regimens

Three HAART regimens are currently prescribed to HIV-1-infected patients in South Africa: regimen 1a: stavudine (d4T), lamivudine (3TC), and EFV; regimen 1b: d4T, 3TC, and NVP; and regimen 2: zidovudine (AZT), didanosine (ddI), and lopinavir/ritonavir. Only patients on regimen 1a or 1b (males:females; 6:6 and 2:18, respectively) were recruited. Patients on PI-based regimens were excluded so as to preclude the antiapoptotic effects of the PIs (lopinavir/ritonavir) on PBMCs. HAART drugs were dosed as follows: regimen 1a/1b: d4T [30 mg/12 h if body weight (body weight < 60 kg, 40 mg/12 h if body weight ≥ 60 kg)]; 3TC (150 mg/12 h); and EFV (600 mg/24 h)/NVP (200 mg daily for initial 2 weeks, followed by 200 mg/12 h). Four of the 32 patients on NNRTI-based HAART (2 from each regimen) were treated with AZT (300 mg/12 h) instead of d4T. All HIV-infected patients were 18 years and older and 50 years and younger. Patients in the NNRTI-based HAART group were on therapy for a minimum of 4 months and did not have signs and symptoms of LDS. Patients with an abnormal liver function profile were excluded. Patients with a current diagnosis or undergoing treatment for opportunistic infections or malignancy were excluded. All patients were on trimethoprim and sulfamethoxazole (160/800 mg daily) as prophylaxis against *Pneumocystis carinii* pneumonia and *Toxoplasma gondii*.

### Flow Cytometry Reagents

Monoclonal anti-human CD4-APC antibody, BD TriTEST CD4-FITC/CD8-PE/CD3-PerCP antibody kit, and JC-1 MitoScreen kit were from Becton Dickinson (BD Biosciences, San Jose, CA). The Annexin-V-FLUOS kit was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Histopaque-1077 and 0.4% trypan blue solution were from Sigma-Aldrich (Durban, South Africa).

### Isolation of PBMCs

All blood specimens were processed for apoptosis assays within 6 hours of being drawn. PBMCs were isolated from heparinized whole blood by centrifugation on a Histopaque-1077 density gradient at 400g for 30 minutes at room temperature (RT). Isolated PBMCs were washed twice in phosphate buffered saline (PBS) (pH 7.4) (350g for 10 minutes

at RT) before cell counting and viability determination via trypan blue dye exclusion.

### Flow Cytometric Analysis of Lymphocyte Apoptosis and $\Delta\psi_m$

Separate aliquots of approximately  $1 \times 10^6$  PBMCs were stained and analyzed for each assay.

### Annexin-V-FLUOS Assay

Lymphocyte apoptosis was determined by measuring the translocation of phosphatidylserine to the outer cell membrane surface using Annexin-V-FLUOS. Necrotic cells were distinguished from apoptotic cells by staining with propidium iodide.<sup>31</sup> PBMCs were incubated with 100  $\mu$ L Annexin-V-FLUOS reagent and 5  $\mu$ L CD4-APC in the dark at RT for 15 minutes.

### JC-1 MitoScreen Assay

Lymphocyte  $\Delta\psi_m$  was measured flow cytometrically with the JC-1 dye as previously reported.<sup>32</sup> PBMCs were incubated in 500  $\mu$ L JC-1 solution at 37°C (5% CO<sub>2</sub>-humidified atmosphere) for 15 minutes. Stained PBMCs were washed twice in JC-1 MitoScreen wash buffer (350g for 10 minutes at RT). As CD4-APC-positive lymphocyte populations were not distinguishable when costained with the JC-1 dye,  $\Delta\psi_m$  data were acquired for total lymphocyte populations only. All flow cytometric data were acquired immediately after staining on an FACSCalibur flow cytometer with CellQuest PRO v4.0.2 software (BD Biosciences). A total of 50,000 PBMC events were acquired for each assayed sample. Lymphocytes were gated on forward scatter and side scatter parameters to exclude debris and nonlymphoid cells using FlowJo v7.1 software (Tree Star, Inc). Lymphocytes were gated on their fluorescent probes for the respective assays (Fig. 1).

### CD4 Count and Viral Load Determination

HIV-1 RNA viral load levels were determined using the Nuclisens Easy Q HIV-1 assay (bioMérieux) (assay detection limit: 25 copies/mL). CD4 T<sub>H</sub> absolute counts were evaluated flow cytometrically with the BD TriTEST kit on whole blood samples (MultiSET v.1.1.1, BD Biosciences).

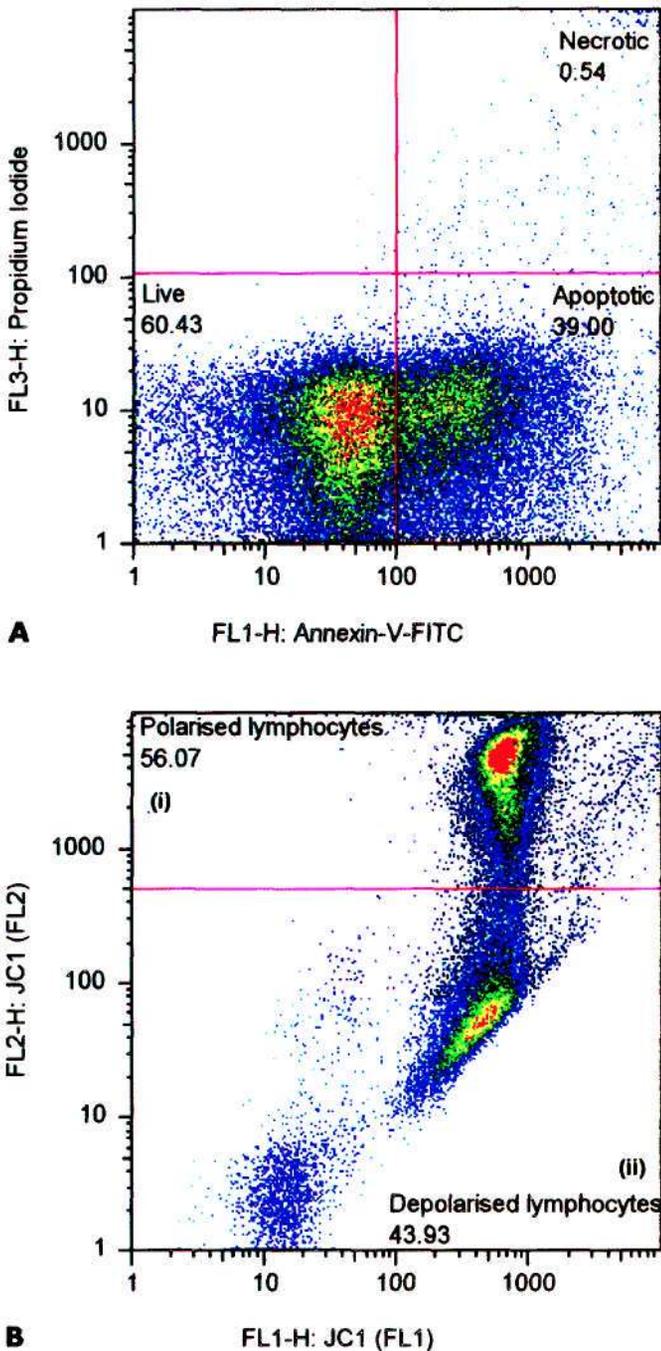
### Statistical Analysis

All statistical analyses were performed using GraphPad InStat v3.06 (GraphPad Software, San Diego, CA). Differences between the control and HAART regimens for lymphocyte apoptosis and  $\Delta\psi_m^{\text{low}}$  were compared by parametric unpaired *t* tests (2-tailed *P* value). Viral loads and CD4 T<sub>H</sub> counts were also compared by parametric unpaired *t* tests (1-tailed *P* value). Differences between total lymphocyte and T<sub>H</sub> apoptosis within each regimen were compared by paired *t* tests. Correlations between lymphocyte apoptosis and  $\Delta\psi_m^{\text{low}}$  and with the duration of HAART were analyzed with Spearman tests. A *P* value < 0.05 was considered significant in all statistical comparisons.

## RESULTS

### Clinical Parameters

HIV-1 plasma viral loads for all HAART-treated patients were below the lower detection limit of the assay and



**FIGURE 1.** Flow cytometric scatter plots of HIV-1-infected patient lymphocytes stained with (A) Annexin-V-FLUOS and (B) JC-1 dye. A, Live, apoptotic, and necrotic lymphocytes were gated as live cells: Annexin-V-FITC log FL1-H < 10<sup>2</sup>, Ppl log FL3-H < 10<sup>2</sup>; apoptotic cells: Annexin-V-FITC log FL1-H ≥ 10<sup>2</sup>, Ppl log FL3-H < 10<sup>2</sup>; necrotic cells: Annexin-V-FITC log FL1-H ≥ 10<sup>2</sup>, Ppl log FL3-H ≥ 10<sup>2</sup>. B, JC-1 dimerizes in lymphocytes with an intact Δψm resulting in a higher red fluorescence emission detected in the FL2-H channel in addition to green fluorescence detected in the FL1-H channel (i). JC-1 remains in monomeric state in lymphocytes with a collapsed Δψm and emits only green fluorescence (FL1-H) (ii). Ppl, propidium iodide; FL, fluorescence.

significantly lower than that of the HAART-naive cohort ( $P < 0.0001$ ). Both regimen 1a and 1b cohorts had significantly higher mean CD4 T<sub>H</sub> counts than the HAART-naive cohort ( $P = 0.014$  and  $P = 0.0008$ , respectively) (Table 1).

**Apoptosis and Δψm<sup>low</sup>**

Mean total lymphocyte apoptosis of the HAART-naive cohort was higher than in both regimen 1a and 1b cohorts, with only the latter comparison being significant ( $P = 0.037$ ). Mean T<sub>H</sub> apoptosis of the HAART-naive cohort was significantly higher than that of both regimen 1a and 1b cohorts ( $P = 0.0006$  and  $P = 0.0003$ , respectively). In both HAART cohorts, mean total lymphocyte apoptosis was significantly higher than mean T<sub>H</sub> apoptosis ( $P = 0.026$  and  $P = 0.001$ , respectively), whereas these parameters correlated significantly to each other in both regimens 1a ( $P = 0.035$ ) and 1b ( $P = 0.0004$ ). This correlation however was not observed in the HAART-naive cohort ( $P = 0.141$ ). Conversely, mean T<sub>H</sub> apoptosis of the HAART-naive cohort was higher than mean total lymphocyte apoptosis, although this difference was not significant either ( $P = 0.330$ ) (Table 2). In addition, differences between the 2 HAART cohorts with respect to total lymphocyte and T<sub>H</sub> apoptosis were also tested, with neither of these comparisons being significant.

Mean total lymphocyte Δψm<sup>low</sup> of the HAART-naive cohort was significantly higher than that of both cohorts 1a and 1b ( $P = 0.017$  and  $P < 0.0001$ , respectively). Moreover, mean lymphocyte Δψm<sup>low</sup> in the regimen 1a cohort was approximately 1.5 times higher than in the regimen 1b cohort, although this difference was not significant ( $P = 0.072$ ). In comparison to the HAART-naive cohort, both cohorts 1a and 1b notably exhibited reductions in mean total lymphocyte Δψm<sup>low</sup> in relation to their respective mean total lymphocyte apoptosis (Table 2). The significance of these changes was determined by testing the degree of correlation between total lymphocyte Δψm<sup>low</sup> and apoptosis for all 3 groups. The only statistically significant correlation was observed between T<sub>H</sub> apoptosis and lymphocyte Δψm<sup>low</sup> for the regimen 1b cohort ( $P = 0.039$ ;  $r = 0.464$ , Fig. 2A). Interestingly, the correlation between T<sub>H</sub> apoptosis and lymphocyte Δψm<sup>low</sup> in the HAART-naive cohort was not significant ( $P = 0.105$ ).

**Correlation of Apoptosis and Δψm<sup>low</sup> With Duration of HAART**

Regimen 1b (NVP) patients demonstrated a significant time-dependent increase in lymphocyte Δψm<sup>low</sup> (Fig. 2B,  $P = 0.0005$ ;  $r = 0.704$ ). Such a correlation was not observed for regimen 1a (EFV) patients. Furthermore, total and T<sub>H</sub> lymphocyte apoptosis correlations against treatment duration were not significant in either regimen 1a or 1b cohorts.

Patients in each cohort were stratified into 1 of 4 subcategories, according to their respective durations on HAART up to the point of recruitment (Table 3). Statistical differences between each cohort for the corresponding subcategories with regard to lymphocyte apoptosis and Δψm<sup>low</sup> were tested. Between the initial 4-6 months of therapy, both mean total lymphocyte and T<sub>H</sub> apoptosis in the regimen 1a (EFV) cohort were significantly lower than that of the regimen 1b (NVP) cohort ( $P = 0.004$  and  $P = 0.027$ , respectively).

**TABLE 1.** Clinical Parameters of HAART-Naive and HAART-Treated HIV-1-Infected Patients

	HAART-Naive HIV-1-Infected Patients (n = 16)	HAART-Treated HIV-1-Infected Patients	
		Regimen 1a (n = 12)	Regimen 1b (n = 20)
CD4 <sup>+</sup> T <sub>H</sub> count (cells/ $\mu$ L)	144 $\pm$ 23.25	254 $\pm$ 45.32*	315 $\pm$ 40.70†
Plasma viral load (log <sub>10</sub> copies/mL)	4.40 $\pm$ 0.24	1.06 $\pm$ 0.02‡	1.22 $\pm$ 0.10‡
Duration on HAART (months)	—	12 $\pm$ 1.73	9 $\pm$ 1.11

All values are reported as mean  $\pm$  SEM.  
\**P* = 0.014; †*P* = 0.0008; ‡*P* < 0.0001 (Difference from HAART-naive cohort).

However, during this period, mean total lymphocyte  $\Delta\psi$ <sup>low</sup> in the regimen 1a (EFV) cohort was nearly 4 times greater than that of the regimen 1b (NVP) cohort (*P* = 0.006). Furthermore, mean T<sub>H</sub> apoptosis of the regimen 1a (EFV) cohort, during months 13–18, was approximately 1.8 times lower than that of the regimen 1b (NVP) cohort for the same period (*P* = 0.019). Comparisons between the other subcategories were not statistically significant.

In addition, the subcategories within each regimen were compared against each other for the respective assays. The regimen 1a (EFV) cohort exhibited significant time-dependent increases in both total lymphocyte and T<sub>H</sub> apoptosis parameters up to only the 12th month of treatment (*P* = 0.026 and *P* = 0.029, respectively). This was the only significant trend noted for regimen 1a, whereas no significant trends in the total lymphocyte and T<sub>H</sub> apoptosis parameters were observed for regimen 1b. In regimen 1b, however, the only significant trend observed was a time-dependent increase in lymphocyte  $\Delta\psi$ <sup>low</sup> between 4 and 18 months (4–6 months < 7–12 months and 4–6 months < 13–18 months) of treatment (Table 3 and Fig. 2B). Conversely, regimen 1a patients exhibited a non-significant time-dependent decrease in lymphocyte  $\Delta\psi$ <sup>low</sup> up to 18 months of treatment. Notably, total lymphocyte apoptosis was consistently higher than T<sub>H</sub> apoptosis in all subgroups of both regimens, a finding similar to the comparison of the means of these parameters (Table 1).

**DISCUSSION**

Elevated T<sub>H</sub> and cytotoxic T-lymphocyte apoptosis is the primary mechanism of HIV-1-induced T-lymphocyte depletion<sup>33–35</sup>; however, the majority of T<sub>H</sub> that are committed to apoptosis are uninfected bystander cells.<sup>36</sup> Bystander T<sub>H</sub>

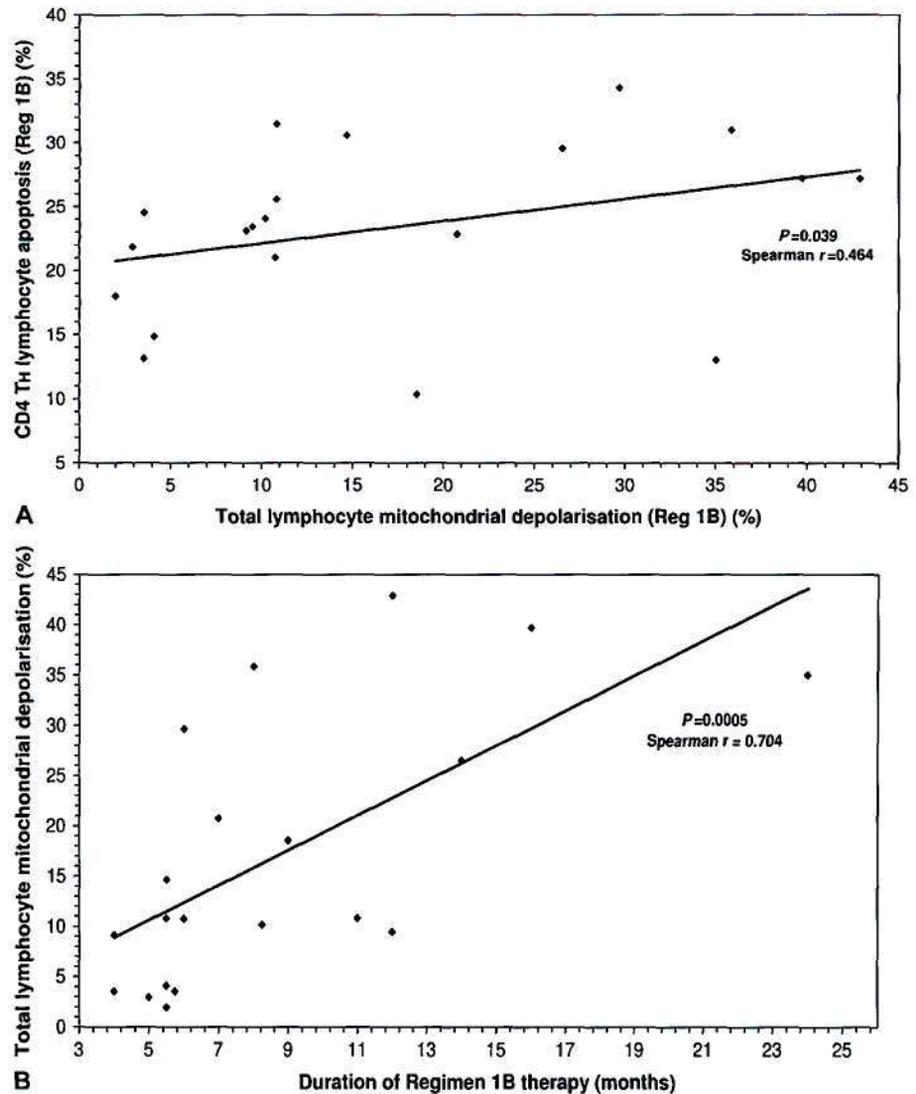
apoptosis is primarily mediated by Fas ligand<sup>37–40</sup> and/or tumor necrosis factor-related apoptosis-inducing ligand-dependent<sup>41</sup> activation-induced cell death, whereas infected T<sub>H</sub> cells are spared from autonomous Fas- or TNF-related apoptosis-inducing ligand-mediated apoptosis via the inhibition of apoptosis signal-regulating kinase-1 by Nef protein.<sup>42</sup> In the HAART-naive cohort, mean T<sub>H</sub> apoptosis was higher than, although not significantly different from total lymphocyte apoptosis. Significant reductions in T<sub>H</sub> apoptosis below total lymphocyte apoptosis and that of HAART-naive T<sub>H</sub> apoptosis in both treatment cohorts suggest the reduction of apoptosis in bystander and directly infected T<sub>H</sub> cells by HAART (Table 2). This is further supported by the absence of detectable plasma virus and significantly higher peripheral T<sub>H</sub> counts in patients of both treatment cohorts.

Furthermore, the loss of  $\Delta\psi$  is a crucial event in T<sub>H</sub> apoptosis during HIV-1 infection.<sup>43</sup> A mean lymphocyte  $\Delta\psi$ <sup>low</sup> of ~45% in our HAART-naive cohort that was significantly higher than in both HAART cohorts corresponds with this finding. However, the lack of a significant correlation between lymphocyte apoptosis and lymphocyte  $\Delta\psi$ <sup>low</sup> in the HAART-naive cohort seemed paradoxical. This could be explained by the effect of the soluble HIV-1 viral protein R on mitochondria, whereby it promotes the loss of  $\Delta\psi$  in both infected and uninfected cells by inducing the opening of the mt permeability transition pore complex.<sup>44</sup> Viral protein R, however, does not induce the release of apoptosis-inducing factor from the permeabilized mitochondrion into the cytoplasm.<sup>45</sup> The subsequent translocation of phosphatidylserine from the cytoplasmic to the extracellular plasma membrane surface, as catalyzed by apoptosis-inducing factor, would be reduced and thus also the binding of Annexin-V to the depolarized lymphocyte.

**TABLE 2.** Lymphocyte Apoptosis and  $\Delta\psi$ <sup>low</sup> Data for HAART-Naive and HAART-Treated HIV-1-Infected Patients

	HAART-Naive HIV-1-Infected Patients (n = 16)	HAART-Treated HIV-1-Infected Patients	
		Regimen 1a (n = 12)	Regimen 1b (n = 20)
Total lymphocyte apoptosis (%)	35.67 $\pm$ 2.42 $\phi$ ▼	27.45 $\pm$ 4.04 $\delta$ Δ	28.77 $\pm$ 2.08 $\#$ ¶⊖
CD4 <sup>+</sup> T <sub>H</sub> apoptosis (%)	39.53 $\pm$ 4.04 $\phi$ ▼	19.38 $\pm$ 2.62* $\delta$ Δ	23.35 $\pm$ 1.51** $\#$ ¶⊖●
Total lymphocyte $\Delta\psi$ <sup>low</sup> (%)	44.22 $\pm$ 4.36	27.25 $\pm$ 5.05‡	17.04 $\pm$ 2.98‡‡●

All values are reported as mean  $\pm$  SEM.  
#*P* = 0.037; \**P* = 0.0006; \*\**P* = 0.0003; ‡*P* = 0.017; ‡‡*P* < 0.0001 (Difference from HAART-naive cohort).  
●*P* = 0.039 (Correlation between control T<sub>H</sub> apoptosis and  $\Delta\psi$ <sup>low</sup>).  
 $\phi$ *P* = 0.330;  $\delta$ *P* = 0.026; ¶*P* = 0.001 (Difference between total and T<sub>H</sub> lymphocyte apoptosis).  
▼*P* = 0.141; Δ*P* = 0.035; ⊖*P* = 0.0004 (Correlation between total and T<sub>H</sub> lymphocyte apoptosis).



**FIGURE 2.** A, Spearman correlation between total lymphocyte  $\Delta\psi_m^{low}$  and  $T_H$  apoptosis in regimen 1b-treated patients; (B) Spearman correlation between duration of regimen 1b treatment and total lymphocyte  $\Delta\psi_m^{low}$ .

We report reduced but persistent spontaneous peripheral lymphocyte apoptosis and  $\Delta\psi_m^{low}$  during HAART in our study, with similar levels of lymphocyte apoptosis reported in previous *in vivo* studies.<sup>46,47</sup>  $T_H$  lymphocyte apoptosis was consistently lower than total lymphocyte apoptosis in both HAART cohorts, suggesting that other lymphocyte subsets, such as CD8 cytotoxic T lymphocytes and B cells, were concurrently undergoing apoptosis. de Oliveira Pinto et al<sup>46</sup> noted a significant persistence of lymphocyte apoptosis in over 70% of their chronically treated (up to 55 months) HAART patients. The persistence of lymphocyte apoptosis in this cohort may be attributed to mt toxicity via NRTI-induced (d4T and 3TC) mtDNA depletion, which is however predominant during chronic therapy.<sup>23,24,48</sup> NRTI-induced lymphocyte mtDNA depletion is, however, most prevalent in LDS patients compared with patients without LDS,<sup>24,49</sup> the latter which comprised our study subjects. Furthermore, PBMCs (lymphocytes and monocytes) intrinsically lack cytosolic TK1 but

retain mt expression of TK2.<sup>27</sup> Hence, in peripheral lymphocytes, the initial monophosphorylation step of AZT and d4T (thymidine analogues), as catalyzed by TK1/2, is localized to the mitochondrion, thus ultimately restricting the incorporation of these triphosphorylated NRTIs into nuclear DNA. In addition, the triphosphates of d4T and 3TC are poor inhibitors of  $\alpha$ ,  $\beta$ , and  $\epsilon$  nuclear DNA polymerases.<sup>12</sup> When analyzed as a separate group, the lymphocyte apoptosis and  $\Delta\psi_m^{low}$  parameters of the 4 AZT-treated patients were not statistically different from those of cohorts 1a and 1b (data not shown). These factors therefore preclude the induction of peripheral lymphocyte apoptosis in our acutely treated subjects via NRTI-induced mtDNA and nuclear DNA damage.

Mean lymphocyte  $\Delta\psi_m^{low}$  was significantly higher in the regimen 1a cohort (EFV) than in the regimen 1b cohort (NVP); however, we noted a nonsignificant time-dependent decrease in  $\Delta\psi_m^{low}$  lymphocytes in the former between 4 and 18 months of treatment but a significant time-dependent

TABLE 3. Analysis of Lymphocyte Apoptosis and  $\Delta\psi m^{low}$  According to Duration of HAART

Group	Duration (months)	Annexin-V-FLUOS Assay		JC-1 Assay
		Total Lymphocyte Apoptosis (%)	CD4 <sup>+</sup> T <sub>H</sub> Apoptosis (%)	Total Lymphocyte $\Delta\psi m^{low}$ (%)
Regimen 1a				
4–6 months (n = 3)	5 ± 0.3	11.16 ± 3.72*¶	10.78 ± 5.05†	35.28 ± 12.03‡
7–12 months (n = 5)	10 ± 1.0	31.66 ± 4.61¶	25.41 ± 2.69	29.05 ± 9.31
13–18 months (n = 2)	17 ± 1.0	34.96 ± 8.27	15.42 ± 1.39§	13.52 ± 2.40
19–24 months (n = 2)	21 ± 1.0	33.86 ± 14.63	21.18 ± 8.94	24.44 ± 8.75
Regimen 1b				
4–6 months (n = 10)	5 ± 0.2	29.37 ± 2.45*	23.29 ± 2.24†	9.11 ± 2.66‡
7–12 months (n = 7)	10 ± 0.8	28.19 ± 4.50	23.49 ± 2.43	21.21 ± 5.02#
13–18 months (n = 2)	15 ± 1.0	34.44 ± 1.19	28.35 ± 1.18§	33.12 ± 6.61**
19–24 months (n = 1)	24	15.45	13.01	35.01

All values are reported as mean ± SEM.  
\**P* = 0.004; †*P* = 0.027; ‡*P* = 0.006; §*P* = 0.019 (Difference between regimens 1a and 1b).  
¶*P* = 0.026; ††*P* = 0.029 (Difference between 4 and 6 months and 7 and 12 months of regimen 1a treatment).  
#*P* = 0.036; \*\**P* = 0.005 (Difference from months 4 to 6 of treatment).

increase in  $\Delta\psi m^{low}$  lymphocytes in the latter group. Furthermore, there was a positive significant correlation between T<sub>H</sub> apoptosis and lymphocyte  $\Delta\psi m^{low}$  in the regimen 1b cohort (Fig. 2A), which suggests that NVP induces apoptosis in peripheral lymphocytes via the collapse of  $\Delta\psi m$  in vivo at therapeutic concentrations. Interestingly, we noted that neither total lymphocyte nor T<sub>H</sub> apoptosis correlated with lymphocyte  $\Delta\psi m^{low}$  in the regimen 1a cohort. Although the plasma concentrations of EFV achieved during therapy may be sufficient to disrupt  $\Delta\psi m$  in peripheral lymphocytes, only a small percentage of these cells may develop mt permeabilization with the subsequent release of cytochrome c and apoptosome formation, thereby committing them to apoptosis. In addition, immune activation has been shown to persist, although at a lower level, during HAART.<sup>49</sup> Activated lymphocytes express high levels of surface Fas and Fas ligand, rendering them susceptible to apoptosis by activation-induced cell death. Apoptosis in these cells may occur via the type 1 Fas pathway,<sup>50</sup> which bypasses mitochondria. These 2 factors provide a plausible explanation for the lack of correlation between lymphocyte  $\Delta\psi m^{low}$  and apoptosis in the EFV cohort. de Oliveira Pinto et al<sup>46</sup> reported the highest levels of apoptosis in peripheral lymphocytes of HAART patients following Fas receptor ligation in vitro, a finding that supports our theory.

In contrast to our findings for EFV-treated patients, Pilon et al<sup>30</sup> reported concentration-dependent increases in apoptosis and  $\Delta\psi m^{low}$  in EFV-treated Jurkat cells and PBMCs in vitro. However, the concentrations of EFV assayed by Pilon et al exceed the peak plasma levels achieved by a daily dose of 600 mg EFV (*C*<sub>min</sub> = 5.6 μM; *C*<sub>max</sub> = 12.9 μM). Furthermore, in circulation, EFV is 99% bound to plasma albumin (compared with 60% for NVP), thereby reducing the availability of EFV to peripheral-circulating lymphocytes.

Uncoupling proteins are proton transporters, present in the inner mt membrane, that mediate a regulated dissipation of the  $\Delta\psi m$ .<sup>51</sup> Rodriguez de la Concepcion et al<sup>52</sup> reported a significant induction of uncoupling protein 1 expression in brown adipocytes mediated by both NVP and d4T in vitro, with the

degree of induction by NVP being significantly higher than that of d4T. The collapse in PBMC  $\Delta\psi m$  could be mediated by NVP in vivo, via the induction of other uncoupling protein isoforms in PBMCs. This effect could be exacerbated during cotreatment with d4T, as in the case with our regimen 1b-treated patients. A decrease in ATP synthesis via oxidative phosphorylation will lead to an increase in  $\Delta\psi m^{low}$ .

Notably, lymphocyte  $\Delta\psi m^{low}$  in EFV-treated patients was approximately 4 times higher than in NVP-treated patients within the 4–6 months period of treatment. However, this difference could be attributed to the patient numbers of the EFV cohort being considerably lower than that of the NVP cohort at this period of treatment. It must be noted that the varying degrees of toxicity in patients treated with EFV and NVP may be attributed to interpatient variations in the biotransformation of these drugs, due to polymorphic variants of the hepatic cytochrome P<sub>450</sub> 2B6 (*CYP 2B6*) gene.<sup>53,54</sup> The influence of NRTIs sulfamethoxazole and trimethoprim on the induction or inhibition of hepatic *CYP 2B6* is negligible because the NRTIs are not metabolized by the hepatic *CYP*<sub>450</sub> system, whereas trimethoprim and sulfamethoxazole are selective inhibitors of only *CYP 2C8* and *2C9*, respectively, at concentrations achievable in vivo.<sup>55</sup>

This pilot study provides evidence that the NNRTIs, EFV, and NVP are potential inducers of mt toxicity at concentrations achieved in vivo during clinical therapy. This mechanism of toxicity has important implications in the etiology of NNRTI-induced adverse effects including central nervous system toxicity, Stevens–Johnson syndrome, and toxic epidermal necrolysis. Because neither EFV nor NVP are inhibitors of PoI-γ, the exact mechanism by which EFV and NVP induce mt toxicity requires further investigation. The foremost limitation of this study is that the in vivo toxicity of each NNRTI drug could not be determined alone, as triple-drug therapy is now standard of care for the treatment of HIV-1 infection. We have however provided plausible explanations that exclude the involvement of NRTIs in the induction of lymphocyte apoptosis and mt depolarization in our treatment

groups. Furthermore, because both EFV and NVP are extensively metabolized by the CYP<sub>450</sub> system, the time-dependent lymphocyte  $\Delta\psi_m$  trends could indeed be influenced by the polymorphic variability of this enzyme system in individual patients. Finally, patients on EFV showed increased mt toxicity as compared with patients on NVP. This may indicate a synergism between d4T and NVP in mt toxicity induction and warrants further investigation.

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## ***CONCLUSION***

The mechanisms by which the NRTIs and PIs modulate lymphocyte mitochondrial function, both *in vivo* and *in vitro*, are documented extensively in the literature. By contrast, literature pertaining to the potential of the NNRTIs to mediate lymphocyte mitochondrial toxicity is scarce. The data from this novel pilot study strongly implicates the NNRTIs, EFV and NVP, in promoting lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  at concentrations achieved *in vivo* during clinical management of HIV-1 infected patients. This is supported by the persistence of lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  in both the EFV and NVP patient cohorts, in the absence of detectable viral replication. Furthermore this study demonstrates that NVP promotes lymphocyte apoptosis through the induction of lymphocyte  $\Delta\psi\text{m}^{\text{low}}$ . Evidence in support of this includes the significant direct correlation between  $\text{CD4}^+$   $\text{T}_{\text{H}}\text{L}$  apoptosis and  $\Delta\psi\text{m}^{\text{low}}$  and a significant therapy duration-dependant increase in lymphocyte  $\Delta\psi\text{m}^{\text{low}}$  in the NVP patient cohort.

These findings have two important clinical implications. Firstly, the NNRTIs, especially NVP, may exacerbate the mitochondrial toxicity of NRTIs and thereby hasten onset of LDS in patients treated with these drug class combinations. Secondly, considering the mechanisms of HIV-1 – mediated lymphocyte mitochondrial toxicity, the NNRTIs may inadvertently promote the decline of  $\text{CD4}^+$   $\text{T}_{\text{H}}\text{L}$  cells in patients that develop NNRTI-resistant viral mutants as a result of poor adherence to HAART. In contrast to the NRTIs, NNRTI drugs do not inhibit mt Pol- $\gamma$ , which suggests that the NNRTIs employ a different mechanism of mt toxicity to the NRTIs. Previous studies reported the induction of mt UCP expression by NVP. This presents a plausible explanation of the observed increase in NVP-induced collapse of lymphocyte  $\Delta\psi\text{m}$  in this study, however further investigations into this potential mechanism of action are now warranted.

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