

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

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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-1.¹ HAART results in a reduction in plasma viral load with a subsequent increase in circulating CD4 T-helper (T_H) lymphocytes^{2–4} and decreased lymphocyte apoptosis.^{5–7} 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.^{8,9}

NRTI-associated LDS is attributed to the mitochondrial (mt) toxicity of this drug class,^{9,10} which is mediated by the inhibition of the mt enzymes DNA polymerase gamma (Pol- γ),^{11,12} adenylate kinase,¹³ thymidine kinase (TK) type-2,¹⁴ and ADP/ATP translocator.¹⁵ The inhibition of Pol- γ , the enzyme responsible for mitochondrial DNA (mtDNA) replication, is however the most significant contributor to mt toxicity. The in vitro hierarchy of Pol- γ 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).¹² mtDNA depletion and deletion have been observed in vitro in NRTI-treated cell lines^{16–18} and in vivo in fat,^{19,20} skeletal muscle,²¹ and liver tissue²² of LDS patients.

In recent studies, human peripheral lymphocytes exposed in vivo^{23,24} and in vitro^{25,26} 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 lymphocytes²⁵ and peripheral blood mononuclear cells (PBMCs) of LDS patients, respectively.²⁴ Impairment of respiratory chain enzyme expression inhibits oxidative phosphorylation,²⁷ with possible induction of apoptosis via the mt pathway.²⁸ Conversely, PIs have been shown to prevent apoptosis, at the mt level by preventing collapse of the mt transmembrane potential ($\Delta\psi$ m).²⁹ 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.³⁰ 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

Background: Efavirenz (EFV) and nevirapine (NVP), unlike nucleoside reverse transcriptase inhibitor drugs, do not inhibit mitochondrial (mt) polymerase gamma (Pol- γ), 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

(*J Acquir Immune Defic Syndr* 2008;48:381–388)

Received for publication February 15, 2008; accepted April 2, 2008.

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Supported by the National Research Foundation toward this research is hereby acknowledged.

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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 \geq 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 1×10^6 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.³¹ PBMCs were incubated with 100 μ L Annexin-V-FLUOS reagent and 5 μ 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.³² PBMCs were incubated in 500 μ L JC-1 solution at 37°C (5% CO₂-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_H 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_H counts were also compared by parametric unpaired *t* tests (1-tailed *P* value). Differences between total lymphocyte and T_H 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

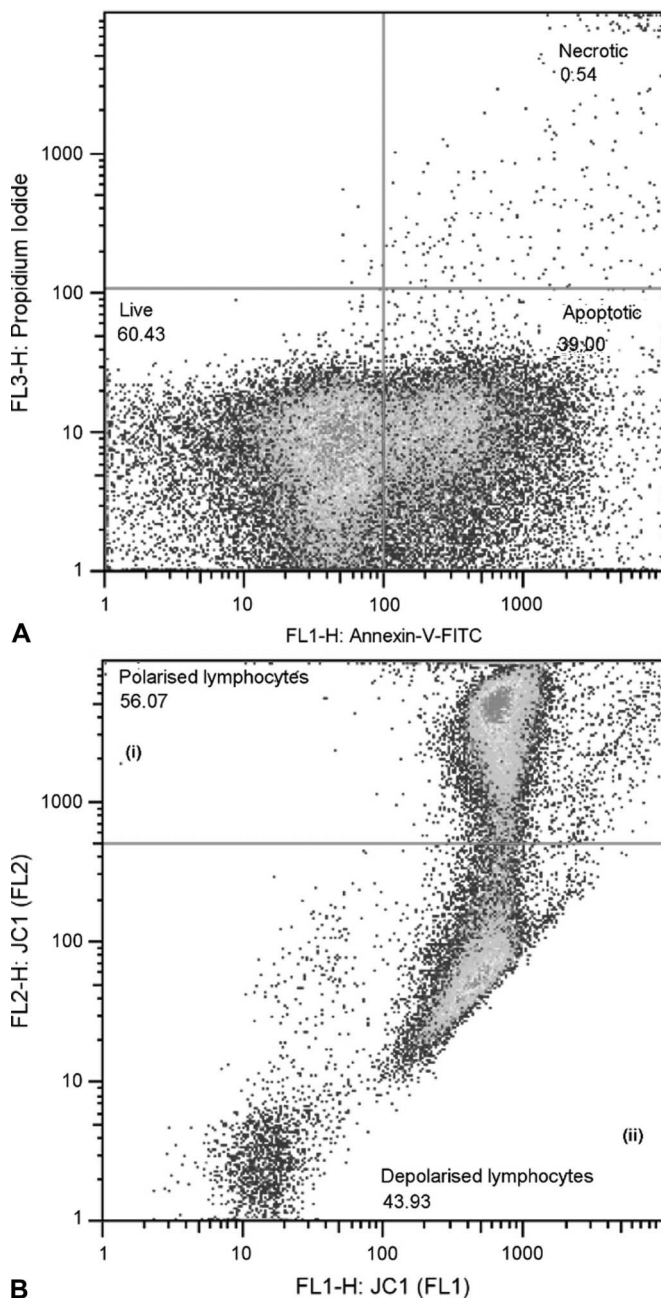


FIGURE 1. Flow cytometric scatter plots of HIV-1-infected patient lymphocytes stained with (A) Annexin-V-FLUOS and (B) JC-1 dye. A, Live, apoptotic, and necrotic lymphocytes were gated as live cells: Annexin-V-FITC log FL1-H < 10², Ppl log FL3-H < 10²; apoptotic cells: Annexin-V-FITC log FL1-H ≥ 10², Ppl log FL3-H < 10²; necrotic cells: Annexin-V-FITC log FL1-H ≥ 10², Ppl log FL3-H ≥ 10². B, JC-1 dimerizes in lymphocytes with an intact Δψ_m 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 Δψ_m 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_H counts than the HAART-naive cohort ($P = 0.014$ and $P = 0.0008$, respectively) (Table 1).

Apoptosis and Δψ_m^{low}

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_H 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_H 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_H 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_H apoptosis were also tested, with neither of these comparisons being significant.

Mean total lymphocyte Δψ_m^{low} 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 Δψ_m^{low} 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 Δψ_m^{low} 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 Δψ_m^{low} and apoptosis for all 3 groups. The only statistically significant correlation was observed between T_H apoptosis and lymphocyte Δψ_m^{low} for the regimen 1b cohort ($P = 0.039$; $r = 0.464$, Fig. 2A). Interestingly, the correlation between T_H apoptosis and lymphocyte Δψ_m^{low} in the HAART-naive cohort was not significant ($P = 0.105$).

Correlation of Apoptosis and Δψ_m^{low} With Duration of HAART

Regimen 1b (NVP) patients demonstrated a significant time-dependent increase in lymphocyte Δψ_m^{low} (Fig. 2B, $P = 0.0005$; $r = 0.704$). Such a correlation was not observed for regimen 1a (EFV) patients. Furthermore, total and T_H 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 sub-categories, 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 Δψ_m^{low} were tested. Between the initial 4–6 months of therapy, both mean total lymphocyte and T_H 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 ⁺ T _H count (cells/μL)	144 ± 23.25	254 ± 45.32*	315 ± 40.70†
Plasma viral load (log ₁₀ copies/mL)	4.40 ± 0.24	1.06 ± 0.02‡	1.22 ± 0.10‡
Duration on HAART (months)	—	12 ± 1.73	9 ± 1.11

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

However, during this period, mean total lymphocyte $\Delta\psi m^{\text{low}}$ 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_H 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_H 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_H apoptosis parameters were observed for regimen 1b. In regimen 1b, however, the only significant trend observed was a time-dependent increase in lymphocyte $\Delta\psi m^{\text{low}}$ 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 $\Delta\psi m^{\text{low}}$ up to 18 months of treatment. Notably, total lymphocyte apoptosis was consistently higher than T_H apoptosis in all subgroups of both regimens, a finding similar to the comparison of the means of these parameters (Table 1).

DISCUSSION

Elevated T_H and cytotoxic T-lymphocyte apoptosis is the primary mechanism of HIV-1-induced T-lymphocyte depletion^{33–35}; however, the majority of T_H that are committed to apoptosis are uninfected bystander cells.³⁶ Bystander T_H

apoptosis is primarily mediated by Fas ligand^{37–40} and/or tumor necrosis factor-related apoptosis-inducing ligand-dependent⁴¹ activation-induced cell death, whereas infected T_H 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.⁴² In the HAART-naive cohort, mean T_H apoptosis was higher than, although not significantly different from total lymphocyte apoptosis. Significant reductions in T_H apoptosis below total lymphocyte apoptosis and that of HAART-naive T_H apoptosis in both treatment cohorts suggest the reduction of apoptosis in bystander and directly infected T_H cells by HAART (Table 2). This is further supported by the absence of detectable plasma virus and significantly higher peripheral T_H counts in patients of both treatment cohorts.

Furthermore, the loss of $\Delta\psi m$ is a crucial event in T_H apoptosis during HIV-1 infection.⁴³ A mean lymphocyte $\Delta\psi m^{\text{low}}$ 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 $\Delta\psi m^{\text{low}}$ 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 $\Delta\psi m$ in both infected and uninfected cells by inducing the opening of the mt permeability transition pore complex.⁴⁴ Viral protein R, however, does not induce the release of apoptosis-inducing factor from the permeabilized mitochondria into the cytoplasm.⁴⁵ 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 $\Delta\psi m^{\text{low}}$ 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 ± 2.42ϕ ▼	27.45 ± 4.04§Δ	28.77 ± 2.08#¶⊖
CD4 ⁺ T _H apoptosis (%)	39.53 ± 4.04ϕ ▼	19.38 ± 2.62*§Δ	23.35 ± 1.51**¶⊖●
Total lymphocyte $\Delta\psi m^{\text{low}}$ (%)	44.22 ± 4.36	27.25 ± 5.05‡	17.04 ± 2.98‡‡●

All values are reported as mean ± 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_H apoptosis and $\Delta\psi m^{\text{low}}$).

ϕ*P* = 0.330; §*P* = 0.026; ¶*P* = 0.001 (Difference between total and T_H lymphocyte apoptosis).

▼*P* = 0.141; Δ*P* = 0.035; ⊖*P* = 0.0004 (Correlation between total and T_H lymphocyte apoptosis).

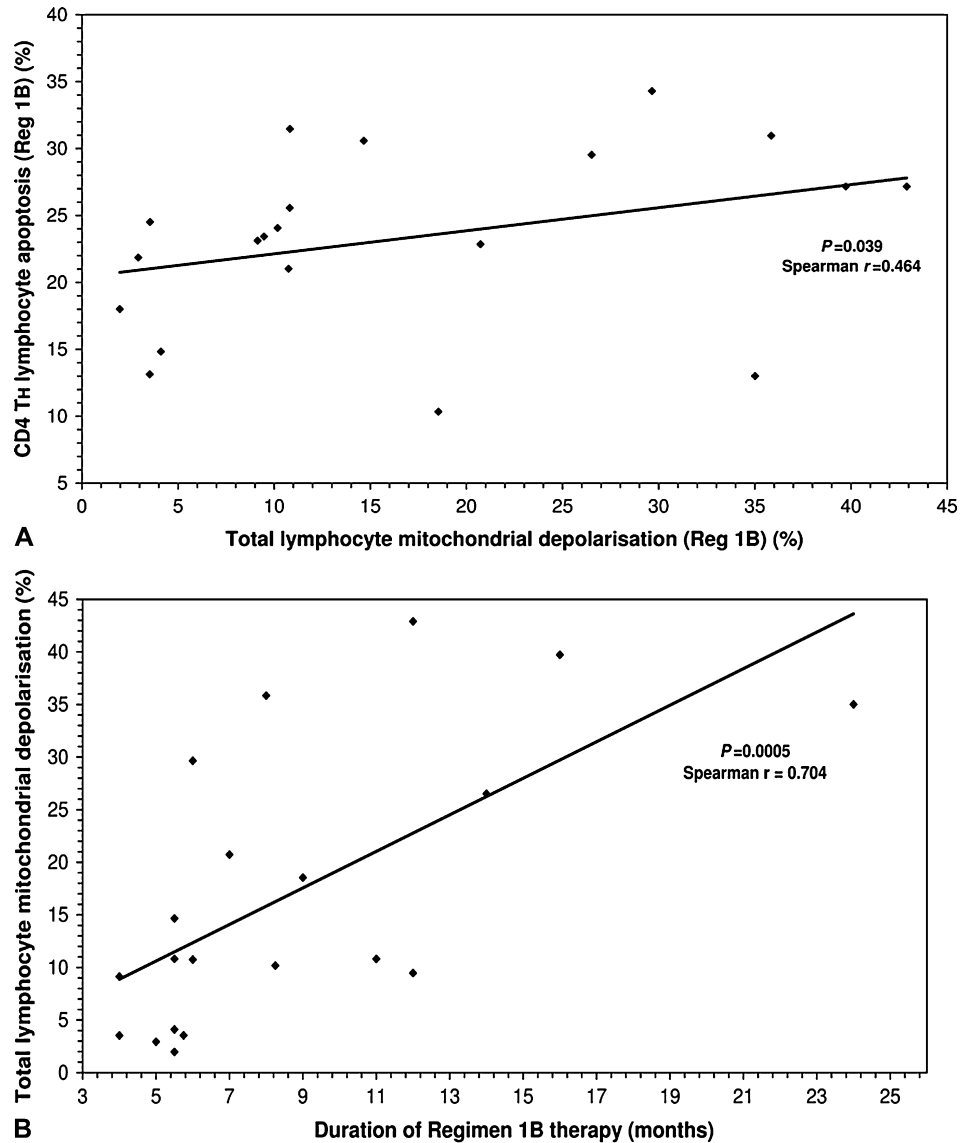


FIGURE 2. A, Spearman correlation between total lymphocyte $\Delta\psi m^{low}$ and T_H apoptosis in regimen 1b-treated patients; (B) Spearman correlation between duration of regimen 1b treatment and total lymphocyte $\Delta\psi m^{low}$.

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.^{46,47} 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 al⁴⁶ 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.^{23,24,48} NRTI-induced lymphocyte mtDNA depletion is, however, most prevalent in LDS patients compared with patients without LDS,^{24,49} the latter which comprised our study subjects. Furthermore, PBMCs (lymphocytes and monocytes) intrinsically lack cytosolic TK1 but

retain mt expression of TK2.²⁷ 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 α , β , and ϵ nuclear DNA polymerases.¹² 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 $\Delta\psi\text{m}^{\text{low}}$ According to Duration of HAART

Group	Duration (months)	Annexin-V-FLUOS Assay		JC-1 Assay
		Total Lymphocyte Apoptosis (%)	CD4 ⁺ T _H Apoptosis (%)	Total Lymphocyte $\Delta\psi\text{m}^{\text{low}}$ (%)
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 $\Delta\psi\text{m}^{\text{low}}$ lymphocytes in the latter group. Furthermore, there was a positive significant correlation between T_H apoptosis and lymphocyte $\Delta\psi\text{m}^{\text{low}}$ in the regimen 1b cohort (Fig. 2A), which suggests that NVP induces apoptosis in peripheral lymphocytes via the collapse of $\Delta\psi\text{m}$ in vivo at therapeutic concentrations. Interestingly, we noted that neither total lymphocyte nor T_H apoptosis correlated with lymphocyte $\Delta\psi\text{m}^{\text{low}}$ in the regimen 1a cohort. Although the plasma concentrations of EFV achieved during therapy may be sufficient to disrupt $\Delta\psi\text{m}$ 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.⁴⁹ 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,⁵⁰ which bypasses mitochondria. These 2 factors provide a plausible explanation for the lack of correlation between lymphocyte $\Delta\psi\text{m}^{\text{low}}$ and apoptosis in the EFV cohort. de Oliveira Pinto et al⁴⁶ 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 al³⁰ reported concentration-dependent increases in apoptosis and $\Delta\psi\text{m}^{\text{low}}$ 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 ($C_{\text{min}} = 5.6 \mu\text{M}$; $C_{\text{max}} = 12.9 \mu\text{M}$). 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 $\Delta\psi\text{m}$.⁵¹ Rodriguez de la Concepcion et al⁵² 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 $\Delta\psi\text{m}$ 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 $\Delta\psi\text{m}^{\text{low}}$.

Notably, lymphocyte $\Delta\psi\text{m}^{\text{low}}$ 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 P₄₅₀ 2B6 (*CYP 2B6*) gene.^{53,54} 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 CYP₄₅₀ system, whereas trimethoprim and sulfamethoxazole are selective inhibitors of only *CYP 2C8* and *2C9*, respectively, at concentrations achievable in vivo.⁵⁵

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 Pol- 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 CYP₄₅₀ 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.

ACKNOWLEDGMENTS

We acknowledge and thank the patients at the Family Health clinic, King Edward VIII Hospital, Durban, South Africa, for their contribution to this study and the nursing staff for their assistance during the study. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the National Research Foundation.

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