Combination of Tenofovir and Emtricitabine with Efavirenz Does Not Moderate Inhibitory Effect of Efavirenz on Mitochondrial Function and Cholesterol Biosynthesis in Human T Lymphoblastoid Cell Line

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ABSTRACT Efavirenz (EFV), the most popular nonnucleoside reverse transcriptase inhibitor, has been associated with mitochondrial dysfunction in most *in vitro* studies. However, in real life the prevalence of EFV-induced mitochondrial toxicity is relatively low. We hypothesized that the agents given in combination with EFV moderate the effect of EFV on mitochondrial function. To test this hypothesis, we cultured a human T lymphoblastoid cell line (CEM cells) with EFV alone and in combination with emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) to investigate the effects on mitochondrial respiration and function and cholesterol biosynthesis. There was a statistically significant concentration- and time-dependent apoptosis, reduction in mitochondrial membrane potential, and increase in production of reactive oxygen species in cells treated with either EFV alone or in combination with TDF plus FTC. Compared to dimethyl sulfoxide-treated cells, EFV-treated cells had significant reduction in oxygen consumption rate contributed by basal mitochondrial respiration and decreased protein expression of electron transport chain complexes (CI, CII, and CIV). Treatment with EFV resulted in a decrease in mitochondrial DNA content and perturbation of more coding genes ($n = 13$); among these were 11 genes associated with lipid or cholesterol biosynthesis. Our findings support the growing body of knowledge on the effects of EFV on mitochondrial respiration and function and cholesterol biosynthesis. Interestingly, combining TDF and FTC with EFV did not alter the effects of EFV on mitochondrial respiration and function and cholesterol biosynthesis. The gap between the prevalence of EFV-induced mitochondrial toxicity in *in vitro* and *in vivo* studies could be due to individual differences in the pharmacokinetics of EFV.

KEYWORDS efavirenz, antiretroviral toxicity, mitochondria, respiration, cholesterol biosynthesis

Combination antiretroviral therapy (ART) has resulted in a decrease in AIDS-related morbidity and mortality (1), although the therapeutic benefit of ART is often limited by delayed toxicity (2). Although contemporary ART regimens are less toxic than early ART regimens (3, 4), toxicity is still pervasive and affects a significant number of people living with HIV (4, 5). *In vitro* studies demonstrated that inhibition of polymerase gamma (Pol- γ), the enzyme responsible for mitochondrial DNA replication, by nucleoside reverse transcriptase inhibitors (NRTIs), leads to depletion of mitochondrial DNA (mtDNA) and subsequent mitochondrial dysfunction (6, 7), the so-called Pol- γ inhibition hypothesis. However, there is a growing body of knowledge to suggest that ART-associated mitochondrial dysfunction cannot be explained solely by Pol- γ inhibition (8,

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FIG 1 Effect of efavirenz on cell viability and apoptosis in CEM cells. A human T lymphoblastoid cell line (CEM cells) was treated with multiplicities of the plasma peak concentrations ($1\times$, or $2\times C_{max}$) of efavirenz (EFV), tenofovir (TDF), emtricitabine (FTC), TDF+FTC, and TDF+FTC+EFV. (A) Each day, aliquots of the cell culture were collected and live cells were counted with trypan blue staining using a hemocytometer, and percentage of live cells was compared to results with DMSO-treated control cells. (B) Apoptosis was assessed by staining with FITC annexin V apoptosis detection kit I. Bar graphs represent the proportion of apoptotic cells after 1 and 2 days of treatment compared to DMSO-treated cells. Data represent at least 3 independent experiments and are plotted as means $\pm$ SD. *P* values are two sided and considered significant at values of <0.05 (*), <0.01 (**), or <0.001 (***).

9). For instance, other classes of ART, such as protease inhibitors (PIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs), do not inhibit Pol- γ and yet cause side effects akin to mitochondrial dysfunction (8, 10). Taken together, there must be alternative or additional mechanisms by which ART impairs mitochondrial function.

Efavirenz (EFV), the most popular NNRTI and a key component of several ART regimens, has been associated with metabolic disorders (11), hepatic toxicity (12, 13), diminished bone density (14), neuropsychiatric symptoms (15, 16), and neurocognitive impairment (17). Although the underlying cellular and molecular mechanisms of EFV-induced toxicity are still not well understood, several *in vitro* and *in vivo* studies have implicated mitochondrial dysfunction as the underlying mechanism. EFV effects on mitochondria include decrease in mitochondrial membrane potential, inhibition of OXPHOS complex I enzymes, decrease in oxygen consumption, and increased production of mitochondrial reactive oxygen species (ROS) (8, 18, 19).

With this litany of effects of EFV on mitochondria, one would have expected a much higher incidence of EFV-associated toxicity in patients. The incidence of severe EFV-associated neuropsychiatric symptoms is less than 2% of patients (15, 20), and severe hepatic toxicity is up to 8% of patients (12, 13). In individuals infected with HIV, EFV is given in combination with other antiretroviral agents. We therefore hypothesized that the agents given in combination with EFV moderate the effect of EFV on mitochondrial function, hence the relatively low incidence of EFV-induced mitochondrial toxicity in patients. To test this hypothesis, we cultured a human T lymphoblastoid cell line (CEM cells) with EFV, tenofovir disoproxil fumarate (TDF), or emtricitabine (FTC) alone and in combination (TDF+FTC or TDF+FTC+EFV) to investigate their effects on mitochondrial function and cholesterol biosynthesis.

RESULTS

EFV treatment decreased CEM cell growth. We treated CEM cells with EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV at multiples of their respective maximum concentration of drug in serum (C_{max} ; i.e., $1\times$ or $2\times C_{max}$). Growth of cells treated with antiretroviral (ARV) drugs compared to that of dimethyl sulfoxide (DMSO)-treated cells was monitored. EFV and TDF+FTC+EFV treatments ($1\times$ - and $2\times C_{max}$) reduced cell growth in a dose- and time-dependent manner (Fig. 1A). At day 2, the effect of treatment with EFV alone on cell growth was comparable to that of treatment with the TDF+FTC+EFV combination. Compared to that of DMSO treatment, TDF, FTC, and TDF+FTC treatments did not have a significant effect on cell growth.

FIG 2 Effect of efavirenz treatment on mitochondrial membrane potential, ROS production, and mitochondrial DNA content. A human T lymphoblastoid cell line (CEM cells) was treated with multiplicities of the plasma peak concentrations ($1\times$ - or $2\times C_{max}$) of EFV, TDF, FTC, TDF+FTC, and TDF+FTC+EFV. (A) At days 1 and 2 the cells were harvested and interrogated for mitochondrial membrane potential ($\Delta\Psi$) using a TMRE-mitochondrial membrane potential assay kit with flow cytometry. Inactive mitochondria had decreased membrane potential and failed to sequester TMRE. The bar graph represents the relative decrease of mitochondrial $\Delta\Psi$ in cells treated with antiretroviral drugs compared to DMSO-treated control cells. (B) Production of ROS was determined using MitoSOX assay. The cells were washed once with wash buffer before incubation with $5\ \mu\text{M}$ MitoSOX reagent working solution at room temperature for 10 min. The cells were then washed once before running on an LSRII flow cytometer (Beckman Coulter). The bar graph represents the fold change in ROS production in cells treated with antiretroviral drugs compared to results for DMSO-treated control cells. (C) The relative mtDNA content was determined on day 2 of culture as the ratio of mtDNA to nuclear DNA using quantitative PCR. Data represent at least 3 independent experiments and are plotted as means $\pm$ SD. *P* values are two sided and considered significant at values of <0.05 (*), <0.01 (**), and <0.001 (***).

EFV treatment increased proportion of apoptotic cells. Mitochondria are central to the process of cell apoptosis. We therefore investigated the effect of exposure of CEM cells to EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV on apoptosis. We determined cell death/apoptosis using propidium iodide/annexin V flow cytometry at day 1 and day 2 (21). Figure 1B illustrates the fold change in apoptosis in ARV-treated cells compared to DMSO-treated cells. We observed a statistically significant dose- and time-dependent apoptosis in cells treated with either EVF alone or the TDF+FTC+EFV combination. There was no statistically significant difference between cells treated with TDF, FTC, or TDF+FTC at the two concentrations and cells treated with DMSO.

EFV treatment decreased mitochondrial membrane potential in cell culture. We next investigated the effect of EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV treatment on mitochondrial membrane potential ($\Delta\Psi$) of CEM cells using TMRE (tetramethylrhodamine, ethyl ester). Compared to cells treated with DMSO, cells treated with both concentrations of EFV or TDF+FTC+EFV showed a statistically significant higher proportion of cells with decreased mitochondrial $\Delta\Psi$ in a dose- and time-dependent manner (Fig. 2A). We did not observe any statistically significant difference between cells treated with TDF, FTC, or TDF+FTC at the two concentrations compared to cells treated with DMSO.

EFV treatment increased mitochondrial ROS production. Loss of mitochondrial $\Delta\Psi$ is associated with oxidative stress and, therefore, increased mitochondrial production of ROS (22). We next investigated whether the decrease in mitochondrial $\Delta\Psi$ in cells treated with EFV translated to an increase in production of ROS. ROS production was determined using MitoSOX Red mitochondrial superoxide indicator. As illustrated in Fig. 2B, cells treated with EFV and TDF+FTC+EFV produced significantly more ROS than cells treated with DMSO in a dose- and time-dependent manner. There was no statistically significant production of ROS in cells treated with TDF, FTC, TDF+FTC, or TDF+FTC+EFV.

EFV treatment reduced mtDNA content. With the effect of EFV treatment alone and in combination with TDF+FTC on cell growth, apoptosis, mitochondrial $\Delta\Psi$, and

ROS production presented above, we investigated whether these findings are associated with changes in mitochondrial DNA (mtDNA) content. We determined the mtDNA content on day 2 of CEM cells treated with EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV compared to cells treated with DMSO (Fig. 2C). We observed a statistically significant decrease in the mtDNA of CEM cells treated with $1 \times C_{\max}$ of EFV and TDF+FTC+EFV. There was no significant change in mtDNA in cells treated with TDF, FTC, or TDF+FTC compared to cells treated with DMSO.

EFV altered mitochondrial respiratory function of CEM cells. We next used a more sensitive extracellular flux analysis to investigate the effect of EFV on mitochondrial respiratory parameters in CEM cells (23). Addition of inhibitor of ATP synthase (2 mM oligomycin) and electron transport chain (ETC) inhibitors [0.6 mM carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and a mixture of rotenone and antimycin A, namely, 1 mM rotenone plus 1 mM antimycin A] allowed for the determination of basal respiration rate, ATP production-linked rate, proton leakage, maximum respiration capacity (MRC), spare respiratory capacity (SRC), and respiratory control ratio (RCR). The relative oxygen consumption rate (OCR) of each component of mitochondrial respiration over time is illustrated in Fig. 3A (adapted and modified from Funes et al. [18], with permission). Relative contribution of OCR by basal, ATP-linked, maximal, proton leak, and nonmitochondrial respirations was not affected by any of the treatments (Fig. 3B). However, EFV treatment had a significant effect on basal mitochondrial respiration. TDF+FTC+EFV at $1 \times C_{\max}$ reduced spare respiration capacity, although not in a dose-dependent manner. We computed the parameters in comparison to basal respiration, expressed as a percentage of basal respiration (Fig. 3C), where basal respiration is the combination of mitochondrial and nonmitochondrial respirations. $2 \times C_{\max}$ of TDF+FTC+EFV significantly reduced the relative participation of respiration that was linked to maximal respiration. Moreover, TDF+FTC+EFV reduced the respiration linked to spared respiration capacity in a non-dose-dependent manner. Interestingly, both concentrations of EFV significantly increased the relative participation of respiration linked to nonmitochondrial respiration compared to that of DMSO treatment. The RCR is a sensitive indicator of mitochondrial dysfunction that is influenced by all components of ETC (18). RCR was computed as the ratio between the maximal respiration and the respiration rate detected in the presence of oligomycin (i.e., ATP production-linked respiration). All of the treatment conditions tended to decrease RCR compared to DMSO treatment; however, only treatment with $2 \times C_{\max}$ of TDF+FTC+EFV resulted in a statistically significant decrease in RCR (Fig. 3D).

Further, we investigated whether the effect of EFV on mitochondrial respiration translated to protein expression of the mitochondrial respiratory complexes (I to V) using Western blot analysis. Figure 3E illustrates a representative Western blot image of subunits of ETC complexes. The density of the band for each complex was normalized to that of tubulin. The number below each band represents the ratio of complex expression to tubulin compared to that for DMSO-treated cells multiplied by 100 (data were from an average of three experiments). Although the efficiencies of the antibodies varied, all of the treatments decreased protein expression of CI and, to a lesser extent, CII. Moreover, treatment with EFV and TDF+FTC+EFV decreased protein expression of CIV. There was no significant effect of treatment on protein expression of CIII and CV.

Gene expression profiles of CEM cells treated with EFV, TDF, FTC, and TDF+FTC. We investigated the differential gene expression profiles of CEM cells treated with EFV ($1 \times C_{\max}$), TDF ($4 \times C_{\max}$), FTC ($4 \times C_{\max}$), and TDF+FTC ($4 \times C_{\max}$) using Affymetrix GeneChip human gene 2.0 ST arrays. We used $4 \times C_{\max}$ of TDF and FTC, because at lower concentrations there was no significant differential gene expression. The numbers of genes expressed with at least a 2-fold difference over cells treated with DMSO are illustrated in Fig. 4A. There were no genes expressed at the 2-fold threshold with FTC-treated cells. The total number of coding genes perturbed by TDF+FTC treatment was 3. EVF treatment led to perturbation of more coding genes ($n = 13$); among these were 11 genes associated with lipid or cholesterol biosynthesis.

FIG 3 Effect of efavirenz treatment on mitochondrial respiratory function. A human T lymphoblastoid cell line (CEM cells) was treated with multiplicities of the plasma peak concentrations ($1\times$ or $2\times C_{max}$) of EFV, TDF, FTC, TDF+FTC+EFV, and TDF+FTC+EFV for 2 days. The cells were counted and washed with the calibration medium, and 1.2×10^5 cells were added to each of 1% gelatin-coated 96-well plates in triplicate. Sequential addition of inhibitor of the ATP synthase (2 mM oligomycin) and electron transport chain (ETC) inhibitors (0.6 mM FCCP and a mixture of rotenone and antimycin A, 1 mM rotenone plus 1 mM antimycin A) allowed determination of basal respiration rate, ATP production-linked rate, proton leakage, maximum respiratory capacity (MRC), spare respiratory capacity (SRC), and respiratory control ratio (RCR) in CEM cells. (A) Schematic representation of the oxygen consumption rate (OCR) measurement over time. Figure adapted and modified from Funes et al. (18), with permission. To estimate the proportion of OCR coupled to ATP synthesis, oligomycin (an inhibitor of ATP synthase complex V) was injected. To determine the maximal OCR, the proton ionophore (uncoupler) FCCP was injected. Finally, to determine mitochondrial respiration, the high-affinity ETC inhibitors rotenone and antimycin A were added to inhibit the electron flux through complex I and complex III, respectively. The remaining OCR corresponds to the nonmitochondrial respiration. (B) Quantification of the mean OCR in CEM cells exposed to different antiretroviral treatments is shown for basal respiration (mitochondrial and nonmitochondrial respiration), ATP-linked respiration (after the addition of oligomycin), maximal respiration capacity (after the addition of FCCP), proton leak respiration (mitochondrial respiration minus ATP-linked respiration), spare respiratory capacity (maximal respiration capacity minus mitochondrial respiration), and nonmitochondrial respiration (recorded after the addition of rotenone plus antimycin A). (C) OCR as a function of basal respiration. Data for each parameter were expressed as the percentage of basal respiration (mitochondrial respiration [Mito. Res.] plus nonmitochondrial respiration). (D) Graph of the mean RCR. The RCR was calculated as the ratio between the maximal uncoupled mitochondrial respiration and the respiration rate detected in the presence of oligomycin. (E) Representative Western blot image of subunits of the electron transport chain (ETC) complexes: complex V (CV-APT5A), complex III (CIII-UQCRC2), complex IV (CIV-MTCO1), complex II (CII-SDH8), and complex I (CI-NDUF8). The density of the band for each complex was normalized to that of tubulin. The number below each band represents the ratio of complex expression to tubulin compared to that for DMSO-treated cells multiplied by 100. Data are from an average of 3 independent experiments. Data (means $\pm$ SD; $n = 3$) were compared to results for DMSO-treated cells and were analyzed by Student's *t* test. *P* values are two sided and considered significant at <0.05 (*), <0.01 (**), or <0.001 (***).

Figures 4B and C illustrate the genes that were either down- or upregulated by EFV and TDF+FTC treatment, respectively.

EFV treatment was associated with downregulation of genes associated with cholesterol biosynthesis. With the finding of downregulation of genes of cholesterol biosynthesis by EFV and a previously reported effect of EFV on the lipogenic transcription factor sterol regulatory element binding protein-1c (SREBP-1c) (24), we sought to validate our microarray data with quantitative PCR (qPCR) (Fig. 5). Relative gene expression in cells treated with antiretroviral drugs was compared to gene expression in cells treated with DMSO. Cells treated with EFV had a significant reduction in expression of 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) synthase, HMGCS (*P* value of <0.01), and 3-hydroxy-3-methylglutaryl-CoA reductase, HMGCR (*P* value of <0.05) (Fig. 5A). TDF+FTC+EFV treatment also decreased the expression of HMGCS (*P* value of <0.01) and HMGCR (*P* value of <0.05). TDF+FTC treatment did not have any significant effect on the expression of HMGCS and HMGCR. All treatment conditions had significant effects on insulin-induced gene-1 (INSIG-1) but not insulin-induced gene-2 (INSIG-2) (Fig. 5B). EFV treatment reduced the expression of SREBP1 (*P* value of <0.05)

FIG 4 Effect of efavirenz treatment on global gene expression. A human T lymphoblastoid cell line (CEM cells) was treated with $1 \times C_{max}$ of EFV, $4 \times C_{max}$ of TDF, or FTC alone or in combination (TDF+FTC) for 2 days. The cells were collected and total RNA was extracted using TRIzol reagent. One hundred ng of total RNA was amplified and labeled using the whole-transcript sense target labeling protocol. Affymetrix GeneChip human gene 2.0 ST arrays were hybridized and scanned for signals. Each experiment was repeated on at least three occasions. Affymetrix TAC 3.1 was used to analyze the result. One-way ANOVA was used, and significance was achieved when the fold change was ≥ 2 or the ANOVA P value was < 0.05 . (A) The number of differentially expressed genes. (B) Genes perturbed by treatment of CEM cells with EFV. (C) Genes perturbed by treatment of CEM cells with TDF+FTC.

(Fig. 5C), low-density lipoprotein receptor (LDLR) (P value of < 0.05), and squalene epoxidase (SQLE) (P value of < 0.05) (Fig. 5D). Compared to DMSO treatment, none of the treatments had significant effects on the expression of AMP-activated protein kinase (AMPK), namely, PRKAA1, PRKAA2, and PRKAB (Fig. 5E).

Further, we investigated whether the effect of EFV on these cholesterol biosynthesis genes translated to protein expression using Western blot analysis (Fig. 5F). The density of the band for each gene was normalized to that of tubulin. The number below each band represents the ratio of gene expression to tubulin compared to that for DMSO-treated cells multiplied by 100 (data were from an average of three experiments). We observed a dose-dependent decrease in HMGCR, SQLE, HMGCS-1, and SREBP-1 in cells treated with EFV and TDF+FTC+EFV (Fig. 5F). Moreover, there was a dose-dependent decrease in expression of LDLR with all treatments.

DISCUSSION

In this study, we sought to investigate whether combining EFV with TDF+FTC moderates reported EFV-associated mitochondrial dysfunction (8, 18, 25–27) and perturbation of cholesterol biosynthesis (8, 28, 29). Most of the reported EFV-induced toxicities are from *in vitro* studies where cells were treated with EFV alone. However, in HIV therapy, EFV is used in combination with other ARVs, such as TDF and FTC. TDF+FTC+EFV combination is one of the most popular ART regimens globally. We conducted comprehensive assessment of mitochondrial function from mtDNA content to mitochondrial respiration using extracellular flux analysis, the gold standard for analyzing basal mitochondrial respiration in intact cells (18). Compared to DMSO-treated cells, EFV-treated cells had significant reduction in OCR, contributed by mitochondrial respiration, and decreased protein expression of ETC complexes (CI, CII, and CIV). EFV treatment increased apoptosis and led to a high proportion of cells with reduced mitochondrial $\Delta\Psi$, increased production of ROS, decreased mtDNA content, downregulation of mRNA, and decreased protein expression of enzymes involved in cholesterol biosynthesis. In general, treatment of cells with TDF+FTC+EFV had effects on mitochondrial function and cholesterol biosynthesis similar to those of treatment with EFV alone. Furthermore, we did not observe such effects when CEM cells were treated with TDF, FTC, or TDF+FTC. Thus, our findings do not support our hypothesis that agents given in combination with EFV moderate the effect of EFV on mitochondrial function, leading to less prevalence of EFV-induced mitochondrial dysfunction in real

FIG 5 Effect of efavirenz treatment on the expression of cholesterol biosynthesis. A human T lymphoblastoid cell line (CEM cells) was treated with multiplicities of the plasma peak concentrations ($1 \times C_{max}$ or $2 \times C_{max}$) of EFV, TDF, FTC, TDF+TFC, and TDF+FTC+EFV. At day 2, the cells were harvested and RNA were extracted. Real-time RT-PCR of selected genes was performed. The expression levels of the genes were normalized to the expression of the housekeeping gene GAPDH. Data (means $\pm$ SD; $n = 3$) are represented as fold change in expression compared to results for cells treated with DMSO. (A) Fold change in expression of HMGCS and HMGCR. (B) Fold change in expression of INSIG-1 and INSIG-2. (C) Fold change in expression of SREBP1 and SREBP2. (D) Fold change in expression of LDLR and SQLE. (E) Fold change in expression of PRKAA1, PRKAA2, and PRKAB. (F) Representative Western blot image of expression of HMGCR, SQLE, HMGCS-1, SREBP-1, INSIG1, and LDLR. The density of the band for each gene was normalized to that of tubulin. The number below each band represents the ratio of cholesterol biosynthesis gene expression to tubulin compared to that for DMSO-treated cells multiplied by 100 from an average of 3 independent experiments. Data represent at least 3 independent experiments and are plotted as means $\pm$ SD. *P* values are two sided and considered significant at <0.05 (*), <0.01 (**), or <0.001 (***).

time. If any, the effect of TDF and FTC on EFV-induced mitochondrial dysfunction was marginal.

The threshold at which mitochondrial dysfunction will translate into a toxic phenotype in a cell is not well understood; it depends on the type and energy requirement of the cell (30). In CEM cells treated with EFV, we observed a reduction in mitochondrial respiration and SRC (Fig. 3B). These findings are consistent with effects of EFV on mitochondrial respiratory parameters in human glioma, neuroblastoma, and HepG2 cell lines (18, 31) and primary cultures of rat neurons and astrocytes (25) reported in previous studies. In these studies, EFV was cultured with the cells for 1 h to 24 h, while we extended treatment for 48 h. The SRC of a cell translates into the ability of the cell to survive and function under high-energy-demanding conditions (18). Decrease in SRC has been associated with heart diseases, neurodegenerative disorders, and aging (32). Interestingly, the prevalence of these conditions is on the rise in HIV treatment-experienced individuals (33, 34). We also observed an increase in nonmitochondrial respiration with EFV treatment (Fig. 3B). Nonmitochondrial respiration accounts for other oxygen consumption processes in the cell that do not involve the ETC. Is the increase in nonmitochondrial respiration caused by EFV a compensatory mechanism? Takemoto et al. recently reported that HIV-infected youth with insulin resistance had lower levels for mitochondrial respiratory parameters and concluded that disordered mitochondrial respiration may be a mechanism of insulin resistance in HIV-infected youth (35).

Moreover, we investigated the effect of EFV on other functions of mitochondria. CEM cells treated with either EFV alone or in combination with TDF+FTC showed a higher proportion of nonviable cells (Fig. 1A) and increased apoptosis (Fig. 1B) in dose- and time-dependent manners. These findings are consistent with other reports. EFV has been reported to decrease cell viability and increase apoptosis in different cell types, such as primary human hepatocytes and the HepG2 cell line (8, 36), pancreatic cancer cell lines (37), human endothelial cell lines (38), human T lymphocytes (Jurkat cell lines) (39), and peripheral blood mononuclear cells (39). Mitochondrial function depends on intact mitochondrial membrane (40). The loss of mitochondrial $\Delta\Psi$ is believed to be the first and crucial step in mitochondrial dysfunction, which triggers a cascade of events ultimately leading to cell death (27, 41). However, there are reports that complex I is inhibited first, followed by the decline of $\Delta\Psi$ (42). It is therefore not surprising that the effect of EFV on mitochondrial $\Delta\Psi$ (Fig. 2A) paralleled the effect of EFV on apoptosis (Fig. 1B). This is consistent with other studies that reported that treatment with EFV led to reduced mitochondrial $\Delta\Psi$ in several cell types (25, 26). Mitochondrial dysfunction and compromise of ETC lead to increased production of ROS. Treatment with EFV alone and in combination with TDF+FTC resulted in a time-dependent increase in ROS production. This finding is consistent with the effect of EFV on the production of ROS in hepatocytes (8). Treatment with EFV and TDF+FTC+EFV resulted in about 50% and 25% reduction in mtDNA content compared to treatment with DMSO. Although EFV does not inhibit Pol- γ , we observed mtDNA depletion in CEM cells; this is consistent with a previous report using Huh 7.5 human hepatoma cells (27). Thus, EFV can affect mitochondrial function through a Pol- γ -independent mechanism(s). In a study comparing Hep3B cells with functional mitochondria (rho⁺) and Hep3B cells lacking functional mitochondria (rho⁻), cells with functional mitochondria were more sensitive to the toxic effects of EFV (43).

Mitochondria play a role in cholesterol and lipid metabolism (44). In a microarray analysis, we observed a downregulation of the expression of cholesterol biosynthesis genes in CEM cells treated with EFV. We validated these data using qPCR assay to investigate the expression of genes involved in synthesis (HMGCS and HMGCR), regulation (SREBP1, SREBP2, INSIG-1, INSIG-2, PARKAA1, PRKAA2, and PRKAB), and uptake (LDLR and SQLE) of cholesterol. EFV treatment resulted in downregulation of HMGCS, HMGCR, INSIG-1, SREBP1, LDLR, and SQLE. We confirmed that EFV treatment led to a decrease in protein expression of HMGCS, HMGCR, SREBP1, LDLR, and SQLE as well (Fig. 5F). Intracellular cholesterol is tightly regulated by cholesterol uptake, *de novo* synthesis, and efflux out of the cell (45). Is it possible that EFV treatment results in accumulation of intracellular cholesterol? Our findings are consistent with the findings of Feeney et al. (45) in a case-control study of expression of cholesterol biosynthesis genes in monocytes of HIV treatment-experienced, treatment-naïve, and uninfected individuals. Blas-Garcia et al. also reported that EFV treatment of Hep3B cells and primary human hepatocytes resulted in intracellular accumulation of lipids (8). They concluded that EFV-induced mitochondrial dysfunction resulted in the activation of AMPK, leading to intracellular accumulation of lipids. In our study, EFV did not have a significant effect on AMPK (PRKAA1, PRKAA2, and PRKAB) (Fig. 5D). Moreover, we also observed that cells treated with EFV had decreased INSIG-1 RNA and protein expression (Fig. 5B and F). Under normal regulation of cholesterol homeostasis, depletion of intracellular cholesterol leads to increase expression of INSIG-1 (46). Further studies are needed to determine whether EFV treatment could lead to intracellular accumulation of cholesterol. In contrast, Hadri et al. reported that treatment of human adipocytes with EFV resulted in depletion of intracellular triglycerides and at the same time a decrease in the expression of SREBP-1c (24). This differential effect of EFV on intracellular lipids may be due to the different types of cells or duration of treatment used in these studies. The $C_{\max}$ of EFV used in our experiments is about 12.7 μM , which is within the range of the plasma concentration of EFV in individuals receiving a daily dose of 600 mg of EFV (3.17 to 12.67 μM) (47). There is marked individual variability in plasma concentration of EFV; certain individuals can achieve plasma concentration over and above the $2 \times C_{\max}$ used in our experiments. Individuals with certain polymorphisms in the cytochrome P450

(CP450) gene could have plasma concentrations ranging from 30 to 80 μM (48–51). The individual differences in EFV plasma concentration may partly explain why certain patients on EFV-based therapy do not experience effects of EFV on mitochondrial function. Ganta et al. detected EFV by high-performance liquid chromatography (HPLC) in purified mitochondrial lysate from Huh 7.5 and HepaRG cells after 12 h of treatment with 6.25 μM (27). The investigators explained that since EFV is hydrophilic, it can pass freely through the outer mitochondrial membrane and initiate depolarization of the inner mitochondrial membrane and subsequent mitochondrial dysfunction (27). This theory may be consistent with the individual differences in the mitochondrial effects of EFV.

Our study, like all *in vitro* studies, has several limitations. First, we could not possibly mimic or take into account all the complex biological pathways and processes that occur *in vivo*. Second, the experiments were conducted using a cancer cell line whose cellular bioenergetics may not be fully comparable to those of primary cells (18). We chose CEM cells because they can be infected with HIV. Therefore, they can be used to tease out the relative contributions of HIV infection and ART in mitochondrial dysfunction and cholesterol biosynthesis in future studies. Third, one might argue that 2 days of culture is not adequate to tease out the delayed effect of EFV on mitochondria in patients on lifelong ART. However, our duration is consistent with the duration used in several *in vitro* studies of ART toxicity (52, 53), and the decrease in viability of CEM cells with EFV treatment did not allow us to go beyond 48 h of culture.

In conclusion, EFV treatment resulted in reduction in mitochondrial respiration. Moreover, we observed increased apoptosis, a high proportion of cells with reduced mitochondrial $\Delta\Psi$, increased production of ROS, decreased mtDNA content, and down-regulation of genes involved in cholesterol biosynthesis in cells treated with EFV. Interestingly, combining TDF+FTC with EFV did not alter the effects of EFV on mitochondrial function. The main rationale of our study was to understand the gap between reported *in vitro* and *in vivo* EFV-induced toxicity. This gap may be due to individual differences in the pharmacokinetics of EFV. EFV can diffuse into the mitochondria and initiate mitochondrial dysfunction (27). Thus, individuals who achieve higher tissue concentrations of EFV may have a predilection for EFV-induced mitochondrial dysfunction. Further studies are needed to elucidate the underlying mechanisms.

MATERIALS AND METHODS

Antiretroviral agents. EFV was obtained from the NIH AIDS Reagent Program (Germantown, MD, USA). FTC and TDF were purchased from Selleckchem (Houston, TX, USA). All drugs were dissolved in dimethyl sulfoxide (DMSO). DMSO was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and cell viability. A human T lymphoblastoid cell line (CEM cells) was cultured in RPMI 1640 supplemented with 10% dialyzed fetal calf serum (Thermo Fisher Scientific, NY, USA). Cells were incubated at 37°C in a 5% CO_2 humidified environment. A total of $3 \times 10^4/\text{ml}$ cells (total volume of 40 ml) were incubated with EFV, FTC, TDF, TDF+FTC, or TDF+FTC+EFV at multiples of the plasma peak concentration, e.g., $1 \times C_{\text{max}}$ or $2 \times C_{\text{max}}$. The cells were collected for cell count and the experiments described below each day. Aliquots of cells were stained with Trypan blue to distinguish live cells from dead cells, and the number of live cells was counted using a hemocytometer. For each treatment condition at least three cell culture experiments were conducted on different occasions.

Apoptosis assay. Apoptosis was assessed by staining with a fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit I (BD, San Jose, CA) according to the manufacturer's instructions. In brief, the cells cultured with various concentrations of EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV ($1 \times$ - or $2 \times C_{\text{max}}$) were harvested at days 1 and 2, and then 1×10^6 cells were washed with ice-cold phosphate-buffered saline (PBS). The early apoptotic cells (annexin V^+/PI^-) or late apoptotic cells (annexin V^+/PI^+) were evaluated by double staining with annexin V-FITC and PI and then run on a BD LSR II flow cytometer and analyzed with BD FACSDiva software (San Jose, CA). Each experiment was done in triplicates and repeated on at least three occasions.

Mitochondrial $\Delta\Psi$. Mitochondrial function depends on intact mitochondrial membranes that can maintain an electrochemical potential generated by the electron transport chain; therefore, mitochondrial membrane potential ($\Delta\Psi$) is a good measure of mitochondrial functional capacity. Mitochondrial $\Delta\Psi$ was analyzed using a TMRE-mitochondrial membrane potential assay kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. TMRE is a cell-permeant, positively charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. At days 1 and 2 of treatment, cells were collected and stained with 200 nM TMRE, respectively, for 20 min at room temperature. The reaction was stopped by adding 300 μl of PBS and immediately analyzed on a BD LSR

II flow cytometer (BD, San Jose, CA) with BD FACS Diva software. Each experiment was done in triplicates and repeated on at least three occasions.

Mitochondrial reactive oxygen species production. Production of ROS by mitochondria was determined using MitoSOX Red mitochondrial superoxide indicator, a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells, according to the manufacturer's instructions. Treated cells were washed once with wash buffer before incubation with 5 μ M MitoSOX reagent working solution at room temperature for 10 min. The cells were then washed and run on an LSRII flow cytometer (Beckman Coulter). Each experiment was done in triplicates and repeated on at least three occasions.

Mitochondrial DNA quantification. To assess mtDNA content, genomic DNA was extracted from CEM cells treated with EFV, TDF, FTC, TDF+FTC, or TDF+FTC+EFV using TRIzol Reagent according to the manufacturer's instructions. Fragments of MT-TL1 gene (encodes tRNA leucine 1) and 18S rRNA nuclear gene were amplified using quantitative reverse transcription-PCR as previously described (54). The primers for mitochondrial fragment (MIT) and 18S were MIT forward (5' AGGACAAGAGAAATAAGGCC) and MIT reverse (5' TAAGAAGAGGAATTGAACCTCTGACTGTAA) as well as 18S forward (5' TAC CTG GTT GAT CCT GCC AGT) and 18S reverse (5' GAT CCT TCC GCA GGT TCA CCT AC). Each experiment was done in duplicate and repeated on at least three occasions. The relative mtDNA content was determined as the ratio of mtDNA to nuclear DNA. Final data for each treatment condition represent the means and standard deviations (SD) from at least three cell culture experiments.

Mitochondrial respiratory function. Mitochondrial respiratory function was determined using the Seahorse XF-96 extracellular flux analyzer (Agilent Technologies, Inc., Wilmington, DE, USA) according to the manufacturer's instructions [[www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/basic-procedures-to-run-an-xf-assay](http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/basic-procedures-to-run-an-xf-assay)] and as previously published (18, 35). In brief, the Agilent Seahorse uses modulators of respiration that target components of the electron transport chain. Sequential addition of inhibitor of the ATP synthase (2 mM oligomycin) and electron transport chain (ETC) inhibitors (0.6 mM FCCP and a mixture of rotenone and antimycin A, namely, 1 mM rotenone plus 1 mM antimycin A) allowed determination of basal mitochondrial respiration rate, ATP production-linked rate, proton leakage, maximum respiratory capacity (MRC), spare respiratory capacity (SRC), and respiratory control ratio (RCR) in CEM cells. Treated CEM cells were seeded at a density of 1.2×10^5 cells per well in triplicate in a 1% gelatin-treated 96-well plate. The sensor cartridge then was loaded with oligomycin, FCCP, and a mixture of rotenone and antimycin A in ports A, B, and C, respectively. The cell culture plate was then inserted into the analyzer and programed to ensure a homogenous environment. For each phase, measurements were performed in triplicate, totaling 12 points per trace. To allow for comparison between different experiments, the data obtained for each condition were normalized to the cell number per well and expressed as the oxygen consumption rate (OCR) in picomoles per minute. The experiment was repeated three times. No significant interassay differences were detected between the three repeats with regard to appearance and viability of cell or the values of OCR that were obtained.

Western blot analysis. Western blot analysis was performed as described previously (55) using total cell protein extracts from CEM cells treated with EFV, TDF, FTC, TDF+FTC, TDF+FTC+EFV, and DMSO. Tubulin was used as the housekeeping gene. Primary antibodies, including complex V (CV-APT5A), complex III (CIII-UQCRC2), complex IV (CIV-MTCO1), complex II (CII-SDH8), and complex I (CI-NDUFB8), were used at 1:500 (Abcam, Cambridge, MA); HMGCR, SQLE, SREBP-1, INSIG1, and LDLR were used at 1:1,000 (Abcam, Cambridge, MA) and HMGCS-1 at 1:2,000 (Cell Signaling Technology, Danvers, MA); secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies at 1:2,000 (Cell Signaling Technology, Danvers, MA) and HRP-conjugated anti-mouse antibodies at 1:7,500 (GE, Pittsburg, PA). Enhanced chemiluminescence substrate was used for signal development (PerkinElmer, Shelton, CT).

Microarray gene expression assay. Total RNA was extracted from cell pellets using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer's instructions. Contaminated genomic DNA was removed by treating RNA samples with Ambion RNase-free DNase (Thermo Fisher Scientific) for 20 min at 37°C. RNA quantity and quality were assessed by microvolume spectrophotometry on an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland) and by on-chip capillary electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), respectively. The absorbance ratio at 260 and 280 nm was ≥ 1.9 , and the RNA integrity number (RIN) was >8 for all samples. One hundred ng of total RNA was amplified and labeled using the whole-transcript sense target labeling protocol by Affymetrix (Santa Clara, CA) without rRNA reduction. Affymetrix GeneChip human gene 2.0 ST arrays were hybridized with 11 μ g of labeled sense DNA, washed, stained, and scanned on an Affymetrix 7G scanner according to the manufacturer's protocols. Each experiment was repeated on at least three occasions. Affymetrix transcriptome analysis console (TAC) 3.1 was used to analyze the results. One-way analysis of variance (ANOVA) was used, and significance was achieved when the fold change was ≥ 2 and the *P* value was <0.05 .

Quantitative real-time PCR for expression of cholesterol biosynthesis genes. RNA was extracted from aliquots of EFV-, TDF-, FTC-, TDF+FTC-, or TDF+FTC+EFV-treated cells using TRIzol Reagent (ThermoFisher Scientific, Carlsbad, CA) according to the manufacturer's instructions and used in quantitative real-time PCR as previously described (56). Melting curve analysis was performed after the completion of PCR to assess the possibility of false-positive results. All of the samples were run in duplicate in at least three independent experiments. The genes of interest encoded HMGCS, HMGCR, SREBP-1 and -2, INSIG-1 and -2, LDLR, SQLE, and AMPK (PRKAA1, PRKAA2, and PRKAB). The primers are listed in Table S1 in the supplemental material. The gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for all reactions. The cycle threshold (*C_t*) values of the genes were determined for each treatment condition. The fold change in gene expression was

calculated as $2^{\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(\text{treated})} - \Delta C_{T(\text{control})}$, $\Delta C_{T(\text{treated})} = (C_{T(\text{gene of interest})} - C_{T(\text{GAPDH})})$, and $\Delta C_{T(\text{control})} = (C_{T(\text{gene of interest})} - C_{T(\text{GAPDH})})$.

Data and statistical analysis. All statistical analyses were performed with GraphPad Prism software with the Student *t* test. Data are expressed as means $\pm$ SD, and significance was achieved when the *P* value was <0.05 .

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00691-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicts of interest to declare.

REFERENCES

- HIV-CAUSAL Collaboration, Ray M, Logan R, Sterne JA, Hernandez-Diaz S, Robins JM, Sabin C, Bansi L, van Sighem A, de Wolf F, Costagliola D, Lanoy E, Bucher HC, von Wyl V, Esteve A, Casbona J, del Amo J, Moreno S, Justice A, Goulet J, Lodi S, Phillips A, Seng R, Meyer L, Perez-Hoyos S, Garcia de Olalla P, Hernan MA. 2010. The effect of combined antiretroviral therapy on the overall mortality of HIV-infected individuals. *AIDS* 24:123–137. <https://doi.org/10.1097/QAD.0b013e3283324283>.
- Gardner K, Hall PA, Chinnery PF, Payne BA. 2013. HIV treatment and associated mitochondrial pathology: review of 25 years of in vitro, animal, and human studies. *Toxicol Pathol* 42:811–822. <https://doi.org/10.1177/0192623313503519>.
- Di Biagio A, Cozzi-Lepri A, Prinapori R, Angarano G, Gori A, Quirino T, De Luca A, Costantini A, Mussini C, Rizzardini G, Castagna A, Antinori A, d'Arminio Monforte A, ICONA Foundation Study Group. 2016. Discontinuation of initial antiretroviral therapy in clinical practice: moving toward individualized therapy. *J Acquir Immune Defic Syndr* 71:263–271. <https://doi.org/10.1097/QAI.0000000000000849>.
- Gonzalez-Serna A, Chan K, Yip B, Chau W, McGovern R, Samji H, Lima VD, Hogg RS, Harrigan R. 2014. Temporal trends in the discontinuation of first-line antiretroviral therapy. *J Antimicrob Chemother* 69:2202–2209. <https://doi.org/10.1093/jac/dku112>.
- Cicconi P, Cozzi-Lepri A, Castagna A, Trearichi EM, Antinori A, Gatti F, Cassola G, Sighinolfi L, Castelli P, d'Arminio Monforte A, ICoNA Foundation Study Group. 2010. Insights into reasons for discontinuation according to year of starting first regimen of highly active antiretroviral therapy in a cohort of antiretroviral-naïve patients. *HIV Med* 11:104–113. <https://doi.org/10.1111/j.1468-1293.2009.00750.x>.
- Lewis W, Dalakas MC. 1995. Mitochondrial toxicity of antiviral drugs. *Nat Med* 1:417–422. <https://doi.org/10.1038/nm0595-417>.
- Chen CH, Cheng YC. 1989. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2',3'-dideoxycytidine. *J Biol Chem* 264:11934–11937.
- Blas-Garcia A, Apostolova N, Ballesteros D, Monleon D, Morales JM, Rocha M, Victor VM, Esplugues JV. 2010. Inhibition of mitochondrial function by efavirenz increases lipid content in hepatic cells. *Hepatology* 52:115–125. <https://doi.org/10.1002/hep.23647>.
- Apostolova N, Blas-Garcia A, Esplugues JV. 2011. Mitochondrial interference by anti-HIV drugs: mechanisms beyond Pol-gamma inhibition. *Trends Pharmacol Sci* 32:715–725. <https://doi.org/10.1016/j.tips.2011.07.007>.
- Deng W, Baki L, Yin J, Zhou H, Baumgarten CM. 2010. HIV protease inhibitors elicit volume-sensitive Cl⁻ current in cardiac myocytes via mitochondrial ROS. *J Mol Cell Cardiol* 49:746–752. <https://doi.org/10.1016/j.jmcc.2010.08.013>.
- Haubrich RH, Riddler SA, DiRienzo AG, Komarow L, Powderly WG, Klingman K, Garren KW, Butcher DL, Rooney JF, Haas DW, Mellors JW, Havlir DV, AIDS Clinical Trials Group (ACTG) A5142 Study Team. 2009. Metabolic outcomes in a randomized trial of nucleoside, nonnucleoside and protease inhibitor-sparing regimens for initial HIV treatment. *AIDS* 23:1109–1118. <https://doi.org/10.1097/QAD.0b013e328332b4377>.
- Rivero A, Mira JA, Pineda JA. 2007. Liver toxicity induced by non-nucleoside reverse transcriptase inhibitors. *J Antimicrob Chemother* 59:342–346. <https://doi.org/10.1093/jac/dkl524>.
- Neukam K, Mira JA, Ruiz-Morales J, Rivero A, Collado A, Torres-Cornejo A, Merino D, de Los Santos-Gil I, Macias J, Gonzalez-Serrano M, Camacho A, Parra-Garcia G, Pineda JA, SEGURIDAD HEPÁTICA Study Team of the Grupo HEPAVIR de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). 2011. Liver toxicity associated with antiretroviral therapy including efavirenz or ritonavir-boosted protease inhibitors in a cohort of HIV/hepatitis C virus co-infected patients. *J Antimicrob Chemother* 66:2605–2614. <https://doi.org/10.1093/jac/dkr357>.
- Dave JA, Cohen K, Micklesfield LK, Maartens G, Levitt NS. 2015. Antiretroviral therapy, especially efavirenz, is associated with low bone mineral density in HIV-infected South Africans. *PLoS One* 10:e0144286. <https://doi.org/10.1371/journal.pone.0144286>.
- Cavalcante GI, Capistrano VL, Cavalcante FS, Vasconcelos SM, Macedo DS, Sousa FC, Woods DJ, Fonteles MM. 2010. Implications of efavirenz for neuropsychiatry: a review. *Int J Neurosci* 120:739–745. <https://doi.org/10.3109/00207454.2010.520541>.
- Kenedi CA, Goforth HW. 2011. A systematic review of the psychiatric side-effects of efavirenz. *AIDS Behav* 15:1803–1818. <https://doi.org/10.1007/s10461-011-9939-5>.
- Wang Z, Zheng Y, Liu L, Shen Y, Zhang R, Wang J, Lu H. 2013. High prevalence of HIV-associated neurocognitive disorder in HIV-infected patients with a baseline CD4 count $\leq$ 350 cells/ μ L in Shanghai, China. *Biosci Trends* 7:284–289.
- Funes HA, Blas-Garcia A, Esplugues JV, Apostolova N. 2015. Efavirenz alters mitochondrial respiratory function in cultured neuron and glial cell lines. *J Antimicrob Chemother* 70:2249–2254. <https://doi.org/10.1093/jac/dkv098>.
- Panel on Antiretroviral Guidelines for Adult and Adolescents. 2018. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. U.S. Department of Health and Human Services, Washington, DC.
- Scourfield A, Zheng J, Chinthapalli S, Waters L, Martin T, Mandalia S, Nelson M. 2012. Discontinuation of Atripla as first-line therapy in HIV-1 infected individuals. *AIDS* 26:1399–1401. <https://doi.org/10.1097/QAD.0b013e328353b047>.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J Immunol Methods* 184:39–51.
- Ly JD, Grubb DR, Lawen A. 2003. The mitochondrial membrane potential ($\Delta\psi$) in apoptosis: an update. *Apoptosis* 8:115–128. <https://doi.org/10.1023/A:1022945107762>.
- Brand MD, Nicholls DG. 2011. Assessing mitochondrial dysfunction in cells. *Biochem J* 435:297–312. <https://doi.org/10.1042/BJ20110162>.

24. El Hadri K, Glorian M, Monsempes C, Dieudonne MN, Pecquery R, Giudicelli Y, Andreani M, Dugail I, Feve B. 2004. In vitro suppression of the lipogenic pathway by the nonnucleoside reverse transcriptase inhibitor efavirenz in 3T3 and human preadipocytes or adipocytes. *J Biol Chem* 279:15130–15141. <https://doi.org/10.1074/jbc.M312875200>.
25. Funes HA, Apostolova N, Alegre F, Blas-Garcia A, Alvarez A, Marti-Cabrera M, Esplugues JV. 2014. Neuronal bioenergetics and acute mitochondrial dysfunction: a clue to understanding the central nervous system side effects of efavirenz. *J Infect Dis* 210:1385–1395. <https://doi.org/10.1093/infdis/jiu273>.
26. Apostolova N, Gomez-Sucerquia LJ, Moran A, Alvarez A, Blas-Garcia A, Esplugues JV. 2010. Enhanced oxidative stress and increased mitochondrial mass during efavirenz-induced apoptosis in human hepatic cells. *Br J Pharmacol* 160:2069–2084. <https://doi.org/10.1111/j.1476-5381.2010.00866.x>.
27. Ganta KK, Mandal A, Chaubey B. 2017. Depolarization of mitochondrial membrane potential is the initial event in non-nucleoside reverse transcriptase inhibitor efavirenz induced cytotoxicity. *Cell Biol Toxicol* 33: 69–82. <https://doi.org/10.1007/s10565-016-9362-9>.
28. Gibellini L, De Biasi G, Nasi M, Carnevale G, Pisciotto A, Bianchini E, Bartolomeo R, Polo M, De Pol A, Pinti M, Cossarizza A. 2015. Different origin of adipogenic stem cells influences the response to antiretroviral drugs. *Exp Cell Res* 337:160–169. <https://doi.org/10.1016/j.yexcr.2015.07.031>.
29. Rodriguez de la Concepcion ML, Yubero P, Domingo JC, Iglesias R, Domingo P, Villarroya F, Giral M. 2005. Reverse transcriptase inhibitors alter uncoupling protein-1 and mitochondrial biogenesis in brown adipocytes. *Antivir Ther* 10:515–526.
30. Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, Darley-Usmar VM. 2013. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Lab Invest* 93:690–700. <https://doi.org/10.1038/labinvest.2013.53>.
31. Wang R, Novick SJ, Mangum JB, Queen K, Ferrick DA, Rogers GW, Stimmel JB. 2015. The acute extracellular flux (XF) assay to assess compound effects on mitochondrial function. *J Biomol Screen* 20: 422–429. <https://doi.org/10.1177/1087057114557621>.
32. Desler C, Hansen TL, Frederiksen JB, Marcker ML, Singh KK, Juel Rasmussen L. 2012. Is there a link between mitochondrial reserve respiratory capacity and aging? *J Aging Res* 2012:192503. <https://doi.org/10.1155/2012/192503>.
33. Weber R, Ruppik M, Rickenbach M, Spoerri A, Furrer H, Battegay M, Cavassini M, Calmy A, Bernasconi E, Schmid P, Flepp M, Kowalska J, Ledergerber B, Swiss HIV Cohort Study (SHCS). 2013. Decreasing mortality and changing patterns of causes of death in the Swiss HIV Cohort Study. *HIV Med* 14:195–207. <https://doi.org/10.1111/j.1468-1293.2012.01051.x>.
34. Neuhaus J, Angus B, Kowalska JD, La Rosa A, Sampson J, Wentworth D, Mocroft A, Insight S, INSIGHT SMART and ESPRIT Study Groups. 2010. Risk of all-cause mortality associated with nonfatal AIDS and serious non-AIDS events among adults infected with HIV. *AIDS* 24:697–706. <https://doi.org/10.1097/QAD.0b013e3283365356>.
35. Takemoto JK, Miller TL, Wang J, Jacobson DL, Geffner ME, Van Dyke RB, Gerschenson M, Pediatric HIV/AIDS Cohort Study. 2017. Insulin resistance in HIV-infected youth is associated with decreased mitochondrial respiration. *AIDS* 31:15–23. <https://doi.org/10.1097/QAD.0000000000001299>.
36. Bumpus NN. 2011. Efavirenz and 8-hydroxyefavirenz induce cell death via a JNK- and BimEL-dependent mechanism in primary human hepatocytes. *Toxicol Appl Pharmacol* 257:227–234. <https://doi.org/10.1016/j.taap.2011.09.008>.
37. Hecht M, Erber S, Harrer T, Klinker H, Roth T, Parsch H, Fiebig N, Fietkau R, Distel LV. 2015. Efavirenz has the highest anti-proliferative effect of non-nucleoside reverse transcriptase inhibitors against pancreatic cancer cells. *PLoS One* 10:e0130277. <https://doi.org/10.1371/journal.pone.0130277>.
38. Faltz M, Bergin H, Pilavachi E, Grimwade G, Mabley JG. 2017. Effect of the anti-retroviral drugs efavirenz, tenofovir and emtricitabine on endothelial cell function: role of PARP. *Cardiovasc Toxicol* 17:393–404. <https://doi.org/10.1007/s12012-016-9397-4>.
39. Pilon AA, Lum JJ, Sanchez-Dardon J, Phenix BN, Douglas R, Badley AD. 2002. Induction of apoptosis by a nonnucleoside human immunodeficiency virus type 1 reverse transcriptase inhibitor. *Antimicrob Agents Chemother* 46: 2687–2691. <https://doi.org/10.1128/AAC.46.8.2687-2691.2002>.
40. Ricci JE, Waterhouse N, Green DR. 2003. Mitochondrial functions during cell death, a complex (I-V) dilemma. *Cell Death Differ* 10:488–492. <https://doi.org/10.1038/sj.cdd.4401225>.
41. Gottlieb E, Armour SM, Harris MH, Thompson CB. 2003. Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ* 10:709–717. <https://doi.org/10.1038/sj.cdd.4401231>.
42. Apostolova N, Blas-Garcia A, Galindo MJ, Esplugues JV. 2017. Efavirenz: what is known about the cellular mechanisms responsible for its adverse effects. *Eur J Pharmacol* 812:163–173. <https://doi.org/10.1016/j.ejphar.2017.07.016>.
43. Polo M, Alegre F, Funes HA, Blas-Garcia A, Victor VM, Esplugues JV, Apostolova N. 2015. Mitochondrial (dys)function—a factor underlying the variability of efavirenz-induced hepatotoxicity? *Br J Pharmacol* 172: 1713–1727. <https://doi.org/10.1111/bph.13018>.
44. Ribas V, Garcia-Ruiz C, Fernandez-Checa JC. 2016. Mitochondria, cholesterol and cancer cell metabolism. *Clin Transl Med* 5:22. <https://doi.org/10.1186/s40169-016-0106-5>.
45. Feeney ER, McAuley N, O'Halloran JA, Rock C, Low J, Satchell CS, Lambert JS, Sheehan GJ, Mallon PW. 2013. The expression of cholesterol metabolism genes in monocytes from HIV-infected subjects suggests intracellular cholesterol accumulation. *J Infect Dis* 207:628–637. <https://doi.org/10.1093/infdis/jis723>.
46. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. 2002. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* 110:489–500. [https://doi.org/10.1016/S0092-8674\(02\)00872-3](https://doi.org/10.1016/S0092-8674(02)00872-3).
47. Staszewski S, Morales-Ramirez J, Tashima KT, Rachlis A, Skiest D, Stanford J, Stryker R, Johnson P, Labriola DF, Farina D, Manion DJ, Ruiz NM. 1999. Efavirenz plus zidovudine and lamivudine, efavirenz plus indinavir, and indinavir plus zidovudine and lamivudine in the treatment of HIV-1 infection in adults. Study 006 Team. *N Engl J Med* 341:1865–1873. <https://doi.org/10.1056/NEJM199912163412501>.
48. Kwara A, Lartey M, Sagoe KW, Court MH. 2011. Paradoxically elevated efavirenz concentrations in HIV/tuberculosis-coinfected patients with CYP2B6 516TT genotype on rifampin-containing antituberculous therapy. *AIDS* 25:388–390. <https://doi.org/10.1097/QAD.0b013e3283427e05>.
49. Carr DF, la Porte CJ, Pirmohamed M, Owen A, Cortes CP. 2010. Haplotype structure of CYP2B6 and association with plasma efavirenz concentrations in a Chilean HIV cohort. *J Antimicrob Chemother* 65:1889–1893. <https://doi.org/10.1093/jac/dkq260>.
50. Burger D, van der Heiden I, la Porte C, van der Ende M, Groeneveld P, Richter C, Koopmans P, Kroon F, Sprenger H, Lindemans J, Schenk P, van Schaik R. 2006. Interpatient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. *Br J Clin Pharmacol* 61: 148–154. <https://doi.org/10.1111/j.1365-2125.2005.02536.x>.
51. Gounden V, van Niekerk C, Snyman T, George JA. 2010. Presence of the CYP2B6 516G>T polymorphism, increased plasma Efavirenz concentrations and early neuropsychiatric side effects in South African HIV-infected patients. *AIDS Res Ther* 7:32. <https://doi.org/10.1186/1742-6405-7-32>.
52. Lund KC, Peterson LL, Wallace KB. 2007. Absence of a universal mechanism of mitochondrial toxicity by nucleoside analogs. *Antimicrob Agents Chemother* 51:2531–2539. <https://doi.org/10.1128/AAC.00039-07>.
53. Birkus G, Hitchcock MJ, Cihlar T. 2002. Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* 46:716–723. <https://doi.org/10.1128/AAC.46.3.716-723.2002>.
54. McComsey GA, Kang M, Ross AC, Lebrecht D, Livingston E, Melvin A, Hitti J, Cohn SE, Walker UA. 2008. Increased mtDNA levels without change in mitochondrial enzymes in peripheral blood mononuclear cells of infants born to HIV-infected mothers on antiretroviral therapy. *HIV Clin Trials* 9:126–136. <https://doi.org/10.1310/hct0902-126>.
55. Paintsil E, Dutschman GE, Hu R, Grill SP, Wang CJ, Lam W, Li FY, Ghebremichael M, Northrup V, Cheng YC. 2011. Determinants of individual variation in intracellular accumulation of anti-HIV nucleoside analog metabolites. *Antimicrob Agents Chemother* 55:895–903. <https://doi.org/10.1128/AAC.01303-10>.
56. Lu L, Katsaros D, Wiley A, Rigault de la Longrais IA, Puopolo M, Yu H. 2007. Expression of MDR1 in epithelial ovarian cancer and its association with disease progression. *Oncol Res* 16:395–403. <https://doi.org/10.3727/000000006783980892>.