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Exposure to zidovudine adversely affects mitochondrial turnover in primary T cells

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ABSTRACT

Zidovudine (ZDV) is a widely used component of antiretroviral therapy (ART) in resource-limited settings, despite its known adverse effects, which include mitochondrial toxicity in muscle, liver and adipose tissue. It has also been associated with impaired immunological recovery. We hypothesised that ZDV might impair mitochondrial health and survival of primary T cells. We performed a cross-sectional analysis of mitochondrial function, mitophagy and susceptibility to apoptosis in healthy donor primary T cells after exposure to ZDV in vitro, together with T cells from patients who were virologically suppressed on ZDV-containing ART regimens for ≥1 year and age-matched subjects receiving non-ZDV ART regimens. The proportion of T cells expressing mitochondrial reactive oxygen species (mtROS) was significantly higher after in vitro ($CD4^+$ T cells and $CD8^+$ T cells) and in vivo ($CD4^+$ T cells) exposure to ZDV than other antiretroviral agents. We did not detect any effect of ZDV on mitophagy, as indicated by change in autophagic flux. However, spontaneous apoptosis, indicated by upregulation of caspase-3 was greater in ZDV-exposed T cells. In conclusion, ZDV exposure was associated with impaired mitochondrial turnover and increased susceptibility to apoptosis in T cells. These mechanisms could contribute to sub-optimal immune reconstitution.

Zidovudine (ZDV), a thymidine analogue, was the first approved agent for treatment of HIV infection and for prevention of mother-to-child transmission (Connor et al., 1994; Fischl, 1989). Within the class of nucleoside reverse transcriptase inhibitors (NRTIs), the thymidine analogues ZDV and stavudine (d4T) formed the backbone of the first combination antiretroviral therapy (ART) regimens and ZDV is still widely used in resource-limited settings. A spectrum of metabolic toxicities emerged as long-term adverse effects of these NRTIs, including lipoatrophy, myopathy, lactic acidosis and hepatic steatosis (Brinkman et al., 1998). This has been largely explained by mitochondrial toxicity, with depletion of mitochondrial DNA (mtDNA) due to inhibition of mtDNA polymerase-γ being the best established mechanism underlying myopathy. However, there is a lack of correlation between Pol-γ inhibitory activity among NRTIs and their clinical toxicity (Apostolova et al., 2011; Benbrik et al., 1997; Gardner and Hall, 2013; Johnson et al., 2001). Recent studies have shown that ZDV and d4T inhibit autophagic flux in hepatocytes and adipocytes in vitro, resulting in the accumulation of defective mitochondria and reactive oxygen species (mtROS), increased apoptosis and decreased proliferative capacity (Stankov et al., 2013, 2012). These mechanisms have been proposed to explain the hepatotoxicity and lipoatrophy associated with these drugs.

Although NRTIs have been shown to induce mtDNA depletion, it is unclear whether this alone explains their negative effect on the proliferative capacity of primary human lymphocytes (Setzer et al., 2005). A recent large cohort study in South Africa showed that ZDV-containing ART regimens were associated with significantly impaired immunological recovery, defined by $CD4^+$ cell count gains, compared with non-ZDV ART (Wandeler et al., 2013). Given these findings, we sought to investigate the effects of ZDV on mitochondrial turnover and autophagy (mitophagy) in primary T cells, following short-term exposure in vitro and long-term exposure in vivo.

Peripheral blood mononuclear cells (PBMC) from HIV-positive adults were obtained with approval from the Oxfordshire Research Ethics Committee, UK and with written informed consent from all participants. All subjects had received ART for ≥12 months and were virologically suppressed (HIV-1 RNA <50 copies/ml) at the time of sampling. The demographic and relevant clinical

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characteristics of the patients are shown in Table 1. Healthy HIV-uninfected donor PBMC were obtained from buffy coats supplied by the National Health Service Blood Transfusion Service, Bristol, UK or healthy laboratory donors. All PBMC were stored in vapour phase liquid nitrogen until use. Thawed cells were rested at 37°C for 2 h prior to use. In selected experiments, $CD8^-$ ($CD4^+$) or $CD8^+$ T-cells were enriched from PBMC by magnetic bead selection to achieve >97% purity (Miltenyi-Biotech). For assessment of in vitro exposure to antiretroviral agents, healthy donor PBMC were cultured for 6 h at 37 °C with ZDV (5 μM) and tenofovir disoproxil fumarate (TDF, 10 μM) or darunavir (DNV, 10 μM) as control antiretrovirals (all from NIH AIDS Reagents Program) or diluent (phosphate-buffered saline, PBS). Cells were stained with Aqua Live/Dead fixable stain (Life Technologies) and CD3-APC*Cy7, CD8-APC (BioLegend), CD4-PerCP (BD) antibodies, with either MitoSox (5 μM, Life Technologies) to assess mtROS or MitoTracker Green FM (150 nM, Life Technologies) to assess mtMass. Autophagy in ex vivo T cells was detected using an LC3-specific antibody after culture with Autophagy Reagent A (both Merck Millipore) or in R10 medium (RPMI supplemented with 10% foetal calf serum) alone for 2.5 h; in vitro T cells were cultured with ARA, ZDV (5 μM) or diluent control for 2–6 h before detection of LC3. The Autophagy Activity Factor was calculated from the mean fluorescence intensity (MFI) as follows: $AAF = (MFI\ LC3\text{-}II_{treatment} - MFI\ LC3\text{-}II_{basal})/(MFI\ LC3\text{-}II_{basal})$. Apoptosis was measured in PBMC that were exposed to ZDV in vitro or in vivo. Cells were fixed, permeabilized and stained with Aqua Live/Dead fixable stain and antibodies to CD3, CD4, CD8 as above and caspase-3 (PE-conjugated, BD). Samples were acquired on a CyAn flow cytometer (Beckman Coulter) and analysed using FlowJo, version 15.

Group differences were analysed using parametric tests, either paired or unpaired T-tests or 1-way ANOVA, using GraphPad Prism software, version 6. P values < 0.05 were considered significant.

To determine the effect of ZDV on mitochondrial turnover in primary T cells, we cultured PBMC from healthy donors ($n = 10$) with clinically relevant concentrations of ZDV, TDF and DNV for 6 h and then quantified total mtROS and mtMass production by flow cytometry (see Supplementary Fig. 1 for gating strategy) (Blas-Garcia et al., 2014; Stankov et al., 2012; Yang et al., 2016). Initial experiments were performed using cryopreserved PBMC to enable comparison with ZDV-treated patients, for whom only cryopreserved samples were available. Zidovudine exposure (5 μM) led to a significant increase (~2.5-fold) in the percentage of mtROS-positive cells among $CD8^+$ T cells [ZDV vs. control, mean (SD): 17.5% (9.7) vs. 7.1% (5.0); $p = 0.008$) and $CD4^+$ T cells (ZDV vs. control, mean (SD): 12.1% (10.1) vs. 4.7% (2.7); $p = 0.048$] (Fig. 1A, B). This effect was dose-dependent and the highest ZDV concentration (10 μM) caused excessive cell death. Furthermore, a fresh-frozen cell comparison showed that ZDV-induced mtROS production was even greater in freshly isolated PBMC (Supplementary Fig. 2). Neither TDF nor DNV had significant effects on mtROS production (Fig. 1A, B). Change in mtMass, assessed by % change in MFI upon drug exposure (MFI drug-treated − MFI control/MFI control), was slightly higher in ZDV-exposed than TDF- or DNV-exposed T cells (CD8+/CD4+ % difference ZDV vs. TDF vs. DNV: 22.3/16.6 vs. 7.6/0.8 vs. 0.6/0 respectively) but the differences were not statistically significant (Fig. 1C).

Next, we investigated the effects of ZDV in vivo: we assessed mtROS production and mtMass in T cells from 10 patients on a current ZDV-containing regimen and 10 patients matched for age and duration of ART with a non-ZDV regimen. The frequency of ROS-positive cells within the $CD4^+$ T cell subset was significantly higher in ZDV-treated group than the ZDV-spared group [ZDV + vs. ZDV-, mean (SD): 8.7 (3.4) vs. 6.00 (2.0); $p = 0.046$]. MtROS-positive cells were 2-fold more frequent among $CD8^+$ T cells [ZDV + vs. ZDV-, mean (SD): 23.6 (21.2) vs. 11.7 (12.0)] but this difference was not statistically significant (Fig. 1D). Based on the data obtained with in vitro-exposed PBMC (Supplementary Fig. 2), we speculate that the effect of ZDV in vivo may have been underestimated because only cryopreserved PBMC were tested. We hypothesised that ZDV exposure in vivo would result in accumulation of defective mitochondria, leading to an increase in mtMass. However, as pre-ART samples were unavailable, this was not assessed directly. Instead, we compared mtMass in patients on ZDV and non-ZDV ART regimens. Of note, we did not observe a distinct Mitotracker-high population, consistent with accumulation of defective mitochrondria (Petrovas et al., 2007) and thus were unable to confirm a ZDV effect on mtMass (Supplementary Figs. 1 and 3).

Table 1
Clinical information on the subjects in this study.

	Patient ID #	Age (years)	Gender	CD4 (cells/μl)	Duration of ART (years)	ART regimen
ZDV+	3	53	F	400	6	ZDV/TDF/R/DNV
	14	52	F	410	3	ZDV/*NRTI/NNRTI*
	26	49	M	400	2	ZDV/*NRTI/PI*
	27	42	M	1130	2	ZDV/*NRTI/PI*
	50	36	F	610	8	ZDV/3TC/NVP
	56	38	M	490	1	ZDV/3TC/NVP
	61	66	M	520	9	ZDV/3TC/NVP
	63	43	M	400	7	ZDV/3TC/TDF/R/LPV
	78	69	M	550	8	ZDV/*NRTI/NNRTI*
	97	49	M	450	14	ZDV/*NRTI/PI*
Mean (SD)		**50 (11)**		**536 (221)**	**6 (4)**	
ZDV−	28	40	F	560	7	*Dual NRTI/NNRTI*
	30	50	M	410	2	TDF/FTC/R/DNV
	32	47	F	790	2	ZDV/*NRTI/NNRTI*
	40	68	M	820	5	TDF/FTC/EFV
	62	39	F	380	8	ABC/3TC/NVP
	67	55	M	710	2	TDF/FTC/EFV
	81	64	M	600	10	ABC/3TC/NVP
	93	53	M	670	15	ABC/TDF/NVP
	113	37	M	420	1	*Dual NRTI/PI*
	123	54	F	600	6	*Dual NRTI//NNRTI*
Mean (SD)		**51 (10)**		**596 (156)**	**6 (4)**	

Abbreviations: ZDV – zidovudine; TDF – tenofovir disoproxil fumarate; 3TC – lamivudine; FTC – emtricitabine; ABC – abacavir; NVP – nevirapine; EFV – efavirenz; R – ritonavir (booster dose); DNV – darunavir; LPV – lopinavir. Where information on regimen composition was not available, this is indicated by italics.