Inhibition of Telomerase Activity by Human Immunodeficiency Virus (HIV) Nucleos(t)ide Reverse Transcriptase Inhibitors: A Potential Factor Contributing to HIV-Associated Accelerated Aging

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Background. Human immunodeficiency virus (HIV)-infected patients on combination active antiretroviral therapy (cART) are at increased risk of age-related complications. We hypothesized that nucleos(t)ide reverse transcriptase inhibitors (NRTI) may contribute to accelerated aging in HIV-infected individuals on cART via inhibition of telomerase activity.

Methods. Telomerase activity and telomere length (TL) were measured by quantitative polymerase chain reaction in vitro in activated peripheral blood mononuclear cells (PBMCs) cultured with NRTI and ex vivo in PBMCs from uninfected patients exposed to NRTI and from HIV-infected patients on NRTI-containing cART.

Results. Lamivudine, abacavir, zidovudine, emtricitabine, and tenofovir significantly inhibited telomerase activity in activated PBMCs in vitro. Tenofovir was the most potent inhibitor of telomerase activity and caused greatest shortening of TL in vitro at the therapeutic concentration of 0.3 µM. PBMCs from HIV-infected patients receiving NRTI-containing cART (n = 39) had significantly lower telomerase activity than HIV-uninfected patients (n = 47; $P = .011$) and HIV-infected patients receiving non-NRTI–containing cART (n = 11; $P < .001$). TL was significantly inversely associated with age ($P = .009$) and the total duration on any NRTI ($P = .01$).

Conclusions. NRTIs and, specifically tenofovir at therapeutic concentrations, inhibit telomerase activity leading to accelerated shortening of TL in activated PBMCs. The relationship between NRTI, reduced telomerase activity, and accelerated aging requires further investigation in HIV-infected individuals on cART.

Keywords. HIV infection; antiretroviral therapy; telomerase; telomere; aging.

Despite the great success of combination antiretroviral therapy (cART) for human immunodeficiency virus (HIV) infection [1], patients are at increased risk for a range of non-AIDS–defining illnesses that are associated with aging, including malignancy, cardiovascular disease, and bone disease [2, 3]. A primary mechanism underlying cellular aging, or senescence, is the progressive

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shortening of telomeres with cell division. Telomeres, which are located at the ends of chromosomes, are short, tandem, G-rich hexanucleotide repeats that repeat TTAGGG sequences that are 5–20 kilobases long [4, 5]. During mitotic cellular division, telomeric DNA is not duplicated by DNA polymerase, and telomere length (TL) is progressively shortened until a critical length is reached and cells then enter replicative senescence [5]. Retrospective secondary analyses using samples from large, randomized studies have shown a strong association between shortened TL in peripheral blood mononuclear cells (PBMCs) and diseases of aging, including increased cardiovascular risk [6–9] and dementia [10–12].

TL is maintained via the enzyme telomerase [13], which is a ribonucleoprotein enzyme complex containing a critical telomerase reverse transcriptase (TERT) subunit that is required for addition of hexameric nucleotides to the telomeric regions [14]. The nucleos(t)ide reverse transcriptase inhibitors (NRTIs) zidovudine (AZT), didanosine (ddI), and abacavir (ABC) have been shown to inhibit telomerase activity in replicating cell lines in vitro, leading to accelerated shortening of TL [15–22]. This is largely thought to be due to inhibition of TERT via chain length termination, similar to the mechanism of inhibition of the HIV reverse transcriptase (RT). However, other mechanisms might be at work because these in vitro assays only measure overall telomerase activity and not TERT activity specifically [23]. The effects on telomerase activity and TL of the more commonly used NRTIs such as lamivudine (3TC), emtricitabine (FTC), and tenofovir disoproxil fumarate (TDF) have not been investigated.

We therefore hypothesized that NRTIs inhibit telomerase activity, leading to enhanced shortening of TL in HIV-infected patients on NRTI-containing cART. The main objective of this study was to determine the effects of the more commonly used NRTIs on telomerase activity and TL in vitro in activated PBMCs and ex vivo in PBMCs from both HIV-uninfected and HIV-infected individuals receiving NRTIs.

METHODS

Participants

HIV-infected patients were recruited from the Alfred's Infectious Diseases Outpatient Clinic (Melbourne, Australia), Gold Coast Sexual Health Clinic (Miami, Australia), and St Vincent's Hospital (Sydney, Australia). Inclusion criteria included the following: patients were HIV antibody positive, patients were receiving cART (either NRTI-containing or non-NRTI–containing cART), plasma HIV RNA was <50 RNA copies/mL for at least 12 months prior to recruitment, and there was no history of AIDS-defining illness. Sex-matched, healthy HIV-uninfected individuals were recruited via advertisement within the Burnet Institute (Melbourne, Australia). HIV-uninfected individuals who received nonoccupational postexposure prophylaxis

(NPEP) were recruited at the Alfred's Emergency and Trauma Centre and the Melbourne Sexual Health Centre. NPEP was administered for 4 weeks to HIV-negative patients who had recent significant risks of HIV exposure. Blood was collected prior to initiation of NPEP and then after 1, 4, and 12 weeks. The Health Human Research Ethics Committee at each site approved the study, and all participants provided written informed consent.

Culture of PBMCs In Vitro in the Presence of NRTIs

PBMCs from whole blood of uninfected and HIV-infected patients were isolated by density gradient centrifugation using Ficoll-Pague PLUS (GE Healthcare). In some experiments, PBMCs were stained with anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE-Cy7 Biosciences (BD) and sorted using a FACSAria flow cytometer (BD). For the in vitro assays, PBMCs from HIV-negative healthy donors were cultured with phytohemagglutinin (PHA; 10 μg/mL; Sigma) and human recombinant interleukin-2 (IL-2; 10 U/mL; Roche) or anti-CD3 (10 μg/mL) with anti-CD28 (10 μg/mL). NRTIs (National Institutes of Health AIDS Research and Reference Reagent Program) were added, and IL-2 and NRTIs were replenished every 48 hours. Toxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) dye reduction assay according to the manufacturer's protocol (Non-Radioactive Cell Proliferation Assay, Promega).

Measurement of Telomerase Activity

Telomerase-containing extracts were prepared from PBMCs, sorted cells, or after in vitro cell culture [24]. Telomerase activity was measured using the standard real-time quantitative telomerase repeats amplification protocol (RQ-TRAP) as previously described [23]. Standard curves were generated from 2-fold serial dilutions of telomerase-positive 1301 T-cell leukemia cells (kind gift from Dr Anthony Jaworowski, Burnet Institute, Melbourne, Australia). Relative telomerase activity (RTA) was expressed relative to 1301 cells and calculated using the formula: $[2_t^{C_t 1301}/2_t^{C_t \text{sample}}] \times 1000$. Change in telomerase activity following cell culture was expressed relative to telomerase activity at baseline.

Measurement of TL

TL was measured using real-time quantitative polymerase chain reaction (qPCR) where TL was expressed as a ratio to that of a single (S) copy housekeeping gene *36B4* as previously described [25]. TL was expressed relative to the single copy housekeeping gene (T/S ratio) and calculated using the T/S ratio = $[2_t^{C_t \text{telomere}}/2_t^{C_t 36B4}]^{-1}$, where C_t denotes the threshold cycle. Change in relative TL (RTL) following cell culture was expressed relative to RTL at baseline.

Statistical Analyses

Categorical variables were summarized using frequency and percentage. Continuous variables were first assessed for skew and summarized using mean and SD. Significant differences in independent samples were assessed using the χ^2 test or Fisher exact test for categorical variables and the t test or Mann–Whitney U test for continuous variables as appropriate. The Wilcoxon signed rank test was used to determine significance between paired samples. Comparison of telomerase activity and TL using different concentrations of the same NRTI was determined using the Friedman nonparametric paired test followed by the Wilcoxon signed rank test. Generalized estimating equations were used to investigate associations between telomere length and telomerase activity over time in HIV-uninfected patients who received NPEP. TERT was $\log_{10}$ transformed in order to meet the normal distribution assumption. Significant differences in the rates of change of TL and TERT against age by HIV status were assessed by adding an interaction term between HIV status and age in the linear regression. Telomerase activity and TL in PBMCs from patients on NRTI-containing cART were compared using a univariable linear regression analysis adjusted for age. Multivariable linear regression was used to further adjust these associations for potential confounders identified as priori ($P < .2$ in the univariable analysis). P values $< .05$ were considered significant. Statistical analyses were carried out using Prism v5 (GraphPad) or Stata v11 software (StataCorp).

RESULTS

Upregulation of Telomerase Activity in PHA-Stimulated PBMCs In Vitro

Following addition of PHA/IL2 to PBMCs, there was a significant increase in telomerase activity, peaking on day 7 poststimulation before returning to basal activity by 21 days poststimulation; however, there was no change in TL (Figure 1A). Upregulation in telomerase activity in PBMCs was also observed following stimulation with anti-CD3 and anti-CD28 (mean ± SD fold change in RTA at day 3 compared with resting cells = 31.8 ± 23.7, n = 4), which was of similar magnitude to PHA/IL2 (56.4 ± 37.5). All NRTIs were noncytotoxic at concentrations ranging from 0.1 µM to 300 µM, except for TDF, which exhibited cytotoxicity at a concentration >3 µM (Supplementary Figure 1).

NRTIs Inhibit Telomerase Activity In Vitro in PHA-Stimulated PBMCs

All NRTIs tested significantly inhibited telomerase activity in PHA-activated PBMCs with varying potency (Figure 1B), but no NRTIs inhibited telomerase activity in resting, unstimulated PBMCs (data not shown). TDF was the only NRTI tested that significantly inhibited telomerase activity both at therapeutic concentrations of 0.3 µM [median interquartile range (IQR), inhibition = 65.1 (64.5%–75.6%), $P = .043$] and 1 µM [71.3 (52.0%–82.1%), $P = .043$; all n = 5]. Due to cytotoxicity, we did not assess concentrations greater than 3 µM (Figure 1B).

FTC significantly inhibited telomerase activity only at 30 µM [38.6 (12.8%–58.4%), $P = .028$] and 300 µM [54.9 (25.8%–59.8%), $P = .046$, both n = 6; Figure 1B]. Inhibition of telomerase by ABC occurred at concentrations >30 µM [30 µM = 33.5 (21.0%–60.1%); 100 µM = 58.1 (36.0%–62.7%), and 300 µM = 81.1 (53.7%–84.9%); all n = 5, $P = .043$; Figure 1B]. AZT inhibited telomerase activity at 100 µM [31.7 (20.3%–50.9%)] and 300 µM [63.8 (52.5%–78.1%)] (all n = 6, $P = .028$; Figure 1B). 3TC significantly inhibited telomerase activity overall (Friedman $P = .029$); however, we did not detect statistical significance in pairwise comparisons of individual concentrations (n = 4; Figure 1B). Finally, we examined the combination of TDF (at 0.1 µM and 0.3 µM) and 3TC (at 3.0 µM and 100 µM) because it is commonly used in clinical practice and both NRTIs were previously shown to inhibit telomerase activity in vitro (Figure 1). We found no evidence of synergistic inhibition of telomerase activity (data not shown).

We also observed enhanced shortening of TL following incubation with 3TC, ABC, and TDF, consistent with inhibition of telomerase activity mediated by these NRTIs (Figure 1C). Again, TDF was the only NRTI tested that enhanced shortening of TL at therapeutic concentrations, which is in agreement with the potent inhibition of telomerase activity (Figure 1B). AZT and FTC did not enhance shortening of TL at all tested concentrations in PHA-activated PBMCs (Figure 1C).

No Change in Telomerase and TL in HIV-Uninfected Individuals Exposed to NRTI

Next, we investigated the effects of short-term NRTI exposure in vivo on TL and telomerase activity in PBMCs, CD4+, and CD8+ T-cell subsets from 29 healthy, HIV-uninfected people [all males, mean (SD) age = 34 (8.6) years] who had recently been exposed to HIV and had received NRTIs as part of NPEP. The NRTIs administered included the combination of FTC and TDF (n = 27) or 3TC and AZT (n = 2), with or without lopinavir/ritonavir for 4 weeks. Cell sorting and TERT and TL analyses were only performed on patients who had attended on at least 1 occasion during NPEP as well as following NPEP cessation (n = 11). There was no significant change in TL and telomerase activity (log-transformed) at baseline (week 0), during NPEP (week 1 and week 4), and 8 weeks after NPEP cessation in total PBMCs (week 12; Figure 2A) or in CD4+ and CD8+ T cells (data not shown). RTA in both CD4+ and CD8+ T cells was significantly lower than RTA in PBMCs [mean (SD)]. RTA pre-NRTI was 1.82
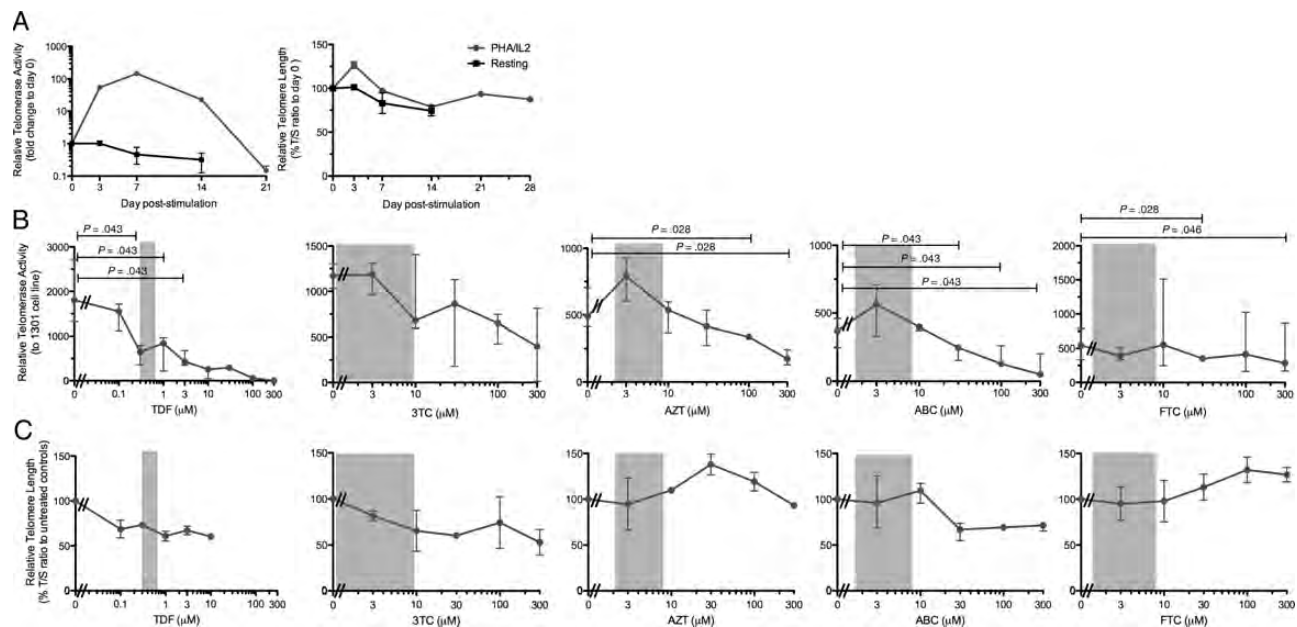
Figure 1. The effect of nucleos(t)ide reverse transcriptase inhibitors (NRTIs) on telomerase activity and telomere length (TL) in mitogen-activated peripheral blood mononuclear cells (PBMCs). *A*. Freshly isolated PBMCs were cultured in media alone (black lines and square symbols) and with 10 μg/mL phytohaemagglutinin (PHA) supplemented with 10 U/mL interleukin-2 (IL-2) every 48-hours (black line and round symbols). Then 1–2×10^5 cells were harvested at indicated time points, and telomerase-containing extracts and genomic DNA were prepared. Relative telomerase activity and TL were measured using real-time quantitative telomerase repeats amplification protocol and quantitative polymerase chain reaction, respectively. Telomerase activity was expressed relative to the telomerase-positive 1301 cell line, and TL was expressed relative to that of the single copy housekeeping gene *36B4*. Telomerase activity and TL are shown as relative to day 0. Freshly isolated PBMCs were stimulated with 10 μg/mL PHA and supplemented with 10 U/mL IL-2. Cells were harvested after 3 days of stimulation for telomerase activity (*B*) or after 21 days for TL (*C*). Effect of lamivudine (3TC), abacavir (ABC), zidovudine (AZT), emtricitabine (FTC), and tenofovir disoproxil fumarate (TDF) on telomerase activity and TL was determined in PHA-activated PBMCs. For telomerase activity (*B*), the median (closed circles) and interquartile ranges (error bars) are shown for 3TC (n = 4), ABC, TDF (n = 5) or AZT, FTC (n = 6). For TL (*C*), the median (closed circle) and range (error bars) are shown (n = 2 experiments for all NRTIs). Gray bars represent the expected steady-state plasma levels in vivo for each NRTI. Statistical significance for telomerase activity was calculated using the Friedman test followed with Wilcoxon signed rank test.
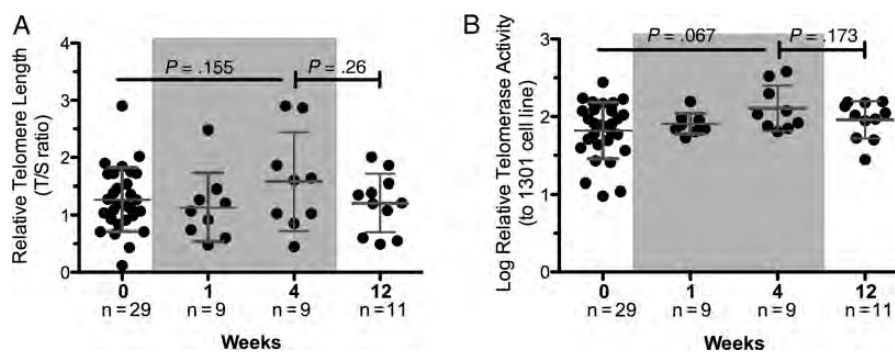

Figure 2. Short-term exposure to nucleos(t)ide reverse transcriptase inhibitors (NRTIs) did not significantly affect telomerase activity and telomere length (TL) in HIV-uninfected individuals. TL was determined by quantitative polymerase chain reaction and expressed relative to the single copy gene 36B4 (*A*), and telomerase activity was measured using real-time quantitative telomerase repeats amplification protocol and expressed relative to that of telomerase-positive 1301 cell line (*B*) in activated peripheral blood mononuclear cells (PBMCs) from HIV-uninfected individuals receiving NRTI-containing nonoccupational postexposure prophylaxis (NPEP). Horizontal lines represent the mean and error bars represent the standard deviation. Statistical significance over weeks 0, 1, and 4 was determined using a generalized estimating equations method, and significance between week 4 and week 12 was determined using the Wilcoxon signed rank test for paired data. Gray bars represent the period when subjects were on NPEP.

(0.36) for PBMCs; 1.11 (0.38, $P = .0004$) for CD4+; and 1.21 (0.39, $P = .001$) for CD8+ T cells.

Lower Telomerase Activity in HIV-Infected Patients Receiving NRTI-Containing cART

We then examined telomerase activity and TL in PBMCs from HIV-infected patients (n = 39) who were receiving NRTI-containing cART with HIV RNA < 50 copies/mL (see Table 1 for patient details). Telomerase activity (log-transformed) in HIV-infected patients (n = 36) was significantly decreased when compared with uninfected controls (n = 42) after adjusting for age ($P = .011$; Figure 3A). There was a trend to shorter TL in PBMCs from HIV-infected patients when compared with uninfected controls using a linear regression analysis on HIV status and adjusted for age; however, this did not reach statistical significance ($P = .061$; Figure 3B).

Using a linear regression analysis, we found that TL negatively correlated with age in HIV-infected patients ($\beta = -0.02$, $P = .009$), but we did not find significant correlation between TL and age in uninfected controls ($\beta = -0.004$, $P = .430$). When we compared HIV-infected patients with uninfected controls, we found no significant difference in the rate of change of TL ($P = .111$; Figure 3C) and telomerase activity ($P = .659$, data not shown) against age.

Next we compared HIV-infected patients receiving NRTI-containing cART ("on" NRTI; n = 39) and patients receiving non-NRTI–containing cART ("off" NRTI; n = 11). These patients had been switched to non-NRTI–containing cART by their physicians for reasons other than virological failure and had been off NRTIs for a median (IQR) of 48 (45–51) weeks (see Table 1 for clinical details). Telomerase activity in PBMCs was significantly lower in patients who were still receiving NRTI ($P < .001$; Figure 3A), and there was no significant difference in TL ($P = .840$; Figure 3B) and no difference in TL from uninfected controls ($P = .162$; Figure 3B).

Six of the 11 patients who had ceased NRTIs had matched samples available from when they were on NRTI and after 36–60 weeks off NRTI (but still on suppressive cART), but there were no significant differences in telomerase (Figure 3D) or TL (Figure 3E) when on and off NRTI.

In a univariate analysis of all HIV-infected patients on NRTI (n = 39), TL was inversely associated with age ($P = .009$) and with duration of NRTI-containing cART ($P = .01$; Table 2). There were no significant associations between telomerase activity and any clinical parameters (Table 2). In a multivariate analysis for TL and telomerase, no parameter remained statistically significant.

DISCUSSION

Accelerated aging remains a significant problem in HIV-infected patients on cART [2, 3]. In this study we asked whether NRTIs play a role in accelerated aging in HIV-infected individuals by inhibition of telomerase activity. We assessed the effects of commonly used NRTIs on telomerase activity and TL in vitro using PHA-activated PBMCs, which we believe are more physiologically relevant than cell lines. Similar to previous studies of cell lines [16–22], we showed that all NRTIs significantly inhibited telomerase activity in vitro. TDF was the only NRTI that significantly inhibited telomerase activity and TL at concentrations that are therapeutic in vivo. Furthermore, we showed that HIV-infected patients receiving NRTI-containing cART had lower telomerase

Table 1. Clinical Details of Study Participants

Clinical details	HIV-	HIV+ "on" NRTI	HIV+ "off" NRTI	P Value[a]
N	47	42	11	ND
Age, years	37.2 (14.6)	47.2 (11.9)	54.5 (7.5)	.06
Sex male, n (%)	45 (95.7)	40 (95.2)	10 (90.9)	.51
CD4 count, cells/µL	ND	579.9 (226.5)	513.4 (266.1)	.41
Nadir CD4 count, cells/µL	ND	182.2 (22.7)	150.3 (34.1)	.51
Length of time on NRTI, months	ND	121.5 (66.2)	139.6 (74.6)	.43
Received TDF, n (%)	NA	22 (52.4)	7 (63.6)	.74
Received AZT, n (%)	NA	29 (69.1)	4 (36.4)	.08
Received NNRTI, n (%)	NA	34 (87.2)	0 (0)	ND
Received PI, n (%)	NA	28 (71.8)	11 (100)	ND
Received other ART drugs,[b] n (%)	NA	10 (25.6)	11 (100)	ND

Abbreviations: ART, antiretroviral therapy; AZT, zidovudine; HIV, human immunodeficiency virus; NA, not applicable; ND, not done; NNRTI, non-nucleoside HIV-1 RT inhibitors; NRTI, nucleos(t)ide reverse transcriptase inhibitor; PI, protease inhibitors; TDF, tenofovir disoproxil fumarate.

[a] Comparison of HIV+ "on" NRTI and HIV+ "off" NRTI.

[b] Other ART drugs included fusion and integrase inhibitors. Mean and SD shown for all parameters unless otherwise specified.
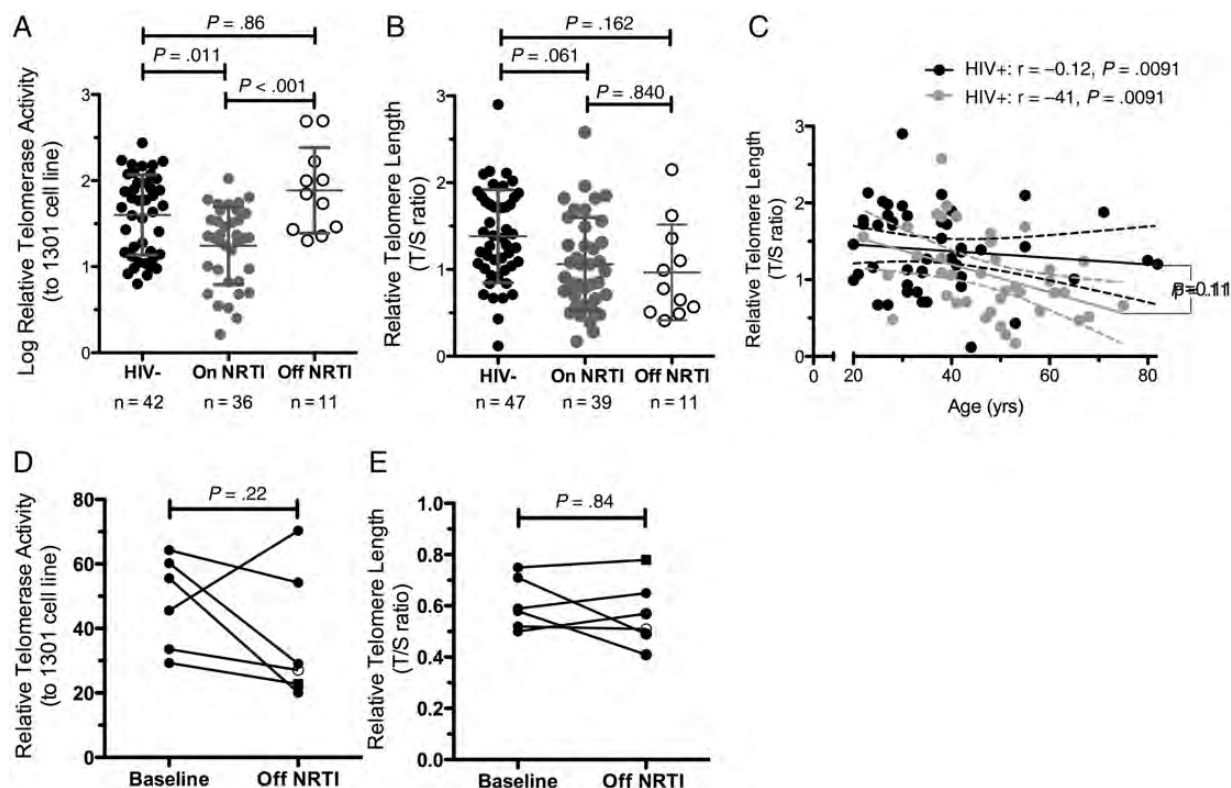
Figure 3. Telomerase activity and telomere length (TL) in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus (HIV)-infected patients receiving nucleos(t)ide reverse transcriptase inhibitors (NRTI)-containing and non-NRTI–containing combination active antiretroviral therapy (cART). Genomic DNA and telomerase-containing extracts were prepared from PBMCs from uninfected individuals, HIV-infected patients who were receiving NRTI-containing cART, and HIV-infected patients who had discontinued NRTI and were receiving non-NRTI–containing cART. *A,* Relative telomerase activity was measured using real-time quantitative telomerase repeats amplification protocol and expressed relative to that of telomerase-positive 1301 cell line. *B,* TL was determined by quantitative polymerase chain reaction and expressed relative to the single copy gene 36B4. Horizontal lines represent the mean and error bars represent the standard deviation. Significance was determined using a linear regression analysis adjusted for age. *C,* Correlation between TL and age in uninfected (black symbols and black line) and HIV-infected patients (gray symbols and gray line) was determined using a simple linear regression analysis. Relative telomerase activity (*D*) and TL(*E*) were measured in HIV-infected patients with HIV RNA < 50 copies/mL while on NRTI-containing cART (baseline) and after stopping NRTI for 36 weeks (open circle), 48 weeks (closed circles), and 60 weeks (closed square). Statistical significance was determined using the Wilcoxon signed rank test.

activity when compared with both HIV-uninfected controls and HIV-infected patients receiving non-NRTI–containing cART.

Multiple previous studies using a range of cell lines have shown that some NRTIs, including AZT [16–18], ddI [18], and ABC [20], reduce telomerase activity and TL in vitro. However, the effects of the more commonly used NRTI, namely, TDF, 3TC, and FTC, have not been evaluated in vitro to date. We demonstrate for the first time that NRTIs inhibit telomerase activity in PHA-activated PBMCs and that TDF was the most potent inhibitor of telomerase, leading to accelerated shortening of TL. It is important to note that we only measured RTA and TL in total PBMCs and therefore are currently unable to determine from these experiments whether the main effects of NRTI were on CD4+ or CD8+ T cells, or specific subsets of T cells, or even secondary to effects on non-

T–cell populations such as monocytes. This will be important to understand for further studies.

TDF is a prodrug of tenofovir. In vivo, TDF is converted to an acyclic nucleoside phosphate, which is an analog of adenosine 5′ monophosphate called tenofovir diphosphate [26]. One explanation for the effects of TDF on telomerase could be that TDF has a much higher affinity toward the mammalian TERT subunit than other NRTIs tested in this study. TDF is a very potent inhibitor of HIV RT, with an EC_{50} value of 5 nM in PBMCs, and it has a long intracellular half-life of >60 hours in the form of its active metabolite tenofovir diphosphate [27]. In contrast, the other NRTIs have far higher EC_{50} values, including AZT (60 nM), 3TC (45 nM), FTC (58 nM), and ABC (260 nM) [26, 28–30]. The significantly higher EC_{50} with RT inhibition by TDF may explain TDF's potent effect on telomerase via potential inhibition of TERT.

Table 2. Univariate and Multivariate Analysis of Telomere Length and Telomerase Activity in Human Immunodeficiency Virus–Infected Patients on Nucleos(t)ide Reverse Transcriptase Inhibitor–Containing Combination Active Antiretroviral Therapy

Predictor	Level	Telomere Length		Log(Telomerase Activity)	
		Unadjusted Coefficient, (95% CI) P Value	Adjusted Coefficient, (95% CI) P Value	Unadjusted Coefficient, (95% CI) P Value	Adjusted Coefficient, (95% CI) P Value
Sex	Female	1.000		1.000	
	Male	−0.262 (−1.060, .536) .510		0.003 (−1.559, 1.564) .997	
Age	(…)	**−0.018 (−.032, −.005) .009**	−0.012 (−.028, .003) .108	−0.010 (−.040, .019) .475	
Current CD4	(…)	−0.0001 (−.0009, .0007) .842		0.0002 (−.002, .002) .829	
Nadir CD4	(…)	0.0008 (−.0004, .0019) .200		−0.0003 (−.003, .002) .808	
Length of time on NRTI, months	(…)	**−0.003 (−.006, −.001) .010**	−0.002 (−.005, .001) .128	0.0004 (−.005, .006) .897	
Length of time on TDF, months	(…)	−0.002 (−.007, .004) .484		0.001 (−.008, .010) .893	
Length of time on AZT, months	(…)	−0.004 (−.004, .003) .828		−0.002 (−.009, .004) .522	

Abbreviations: AZT, zidovudine; CI, confidence interval; NRTI, nucleos(t)ide reverse transcriptase inhibitor; TDF, tenofovir disoproxil fumarate.; Bold type: Statistically significant (p<.05)

We were surprised to demonstrate in vitro that FTC and 3TC inhibited telomerase activity only at high concentrations. Telomeres are made up of TTAGGG repeat sequences. If NRTIs inhibit telomerase via inhibition of TERT leading to chain termination, then a cytosine analogue (such as 3TC, 2′-deoxy-3′-thiacytidine or FTC, 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3- oxathiolan-5-yl]cytosine) would not be expected to inhibit TERT. These data raise the possibility that NRTI, or potentially 3TC and FTC, inhibit telomerase activity by an alternative mechanism to TERT inhibition, such as inhibition of cell proliferation [18]. In agreement with our observation, some of the NRTIs that we tested have been shown to induce cell cycle arrest in human cancer cells (TDF and FTC [31]) and to inhibit cellular proliferation in activated PBMCs (AZT, 3TC, and TDF [32]).

Changes in TL and telomerase activity have previously been described in cells from HIV-infected patients, in the absence of NRTI treatment. It has been shown in HIV-infected patients not receiving cART that TL is reduced in total CD8+ T cells [33], HIV-specific CD8+T cells [34], naive CD4+ T cells [35], and monocytes [36]. In vitro HIV infection of activated CD4+ T-cells, in the absence of any NRTI, led to a reduction in telomerase activity [37]. In contrast, in cells from HIV-infected patients, both an increase and decrease in telomerase activity in different T-cell subsets have been reported [33, 34], and no prior studies have examined telomerase activity in total PBMCs.

Given these known mixed effects of HIV infection on telomerase activity and TL, we first assessed the effects of NRTI on telomerase and TL in HIV-uninfected individuals who had received NRTI and were unable to demonstrate any significant change in telomerase or TL. These findings could be explained by the very short administration of the NRTIs, that is, only 4 weeks. Indeed, a recent study showed that relatively short-term exposure to NRTI during pregnancy did not significantly affect TL in both HIV-infected mothers and uninfected infants [38]. In future studies, it may be of interest to assess the effects of NRTIs such as TDF or 3TC/FTC in patients with chronic hepatitis B virus (HBV) infection. Patients with chronic HBV do not have the abnormalities in T-cell turnover seen in HIV infection; however, they frequently receive long-term NRTI treatment to control HBV replication [39].

A limited number of studies have assessed telomerase activity and TL in HIV-infected individuals receiving cART. No studies have assessed these parameters in PBMCs, and most studies have enrolled very few patients [22, 33]. In 1 study, the authors demonstrated an increase in TL in CD4+ T cells and a decrease in TL in CD8+ T cells in 7 HIV-discordant monozygotic twins who were only receiving NRTIs and had persistent HIV replication [33]. In another study, telomerase activity in both CD4+ and CD8+ T cells from HIV-infected patients was similar to that in HIV-negative controls; however, telomerase activity decreased in both CD4+ and CD8+ T cells from HIV-infected patients following introduction of NRTI monotherapy with either ddI or AZT [22].

In our study we chose to measure TL and telomerase activity in total PBMCs given the large population-based studies that demonstrate an association between clinical outcomes such as cardiovascular disease and TL in PBMCs [6, 7]. In addition, we hypothesized that if NRTI inhibited telomerase activity, this should occur in all mononuclear cells that express telomerase, not just CD4+ T cells where NRTIs are active against HIV. Although we detected significant reduction of telomerase activity in patients on NRTI-containing cART, it is possible that we

did not detect significant shortening of TL in PBMCs from HIV-infected subjects, given the potentially divergent changes in TL in CD4+ and CD8+ T cells and/or the small sample size [33]. Finally, telomerase-independent alternative lengthening of telomeres has been described in multiple human cell types [40–42] and would be of interest to explore in future studies. To better understand the differences in findings from our study and previous studies, future studies should assess TL and telomerase activity in sorted T-cell subsets.

We found that the duration of NRTI-containing cART was inversely associated with TL and that there was no association with telomerase activity. The association with duration of NRTI-containing cART demonstrated that for every year on NRTI-containing cART, there was a 0.036-unit reduction in TL, and for a 1-year increase in age, there was a 0.018-unit reduction in TL. However, in a multivariate analysis, both parameters were no longer statistically significantly related to TL. Potential explanations for this finding could include the small sample size or an interaction between these 2 parameters. The small sample size also did not allow us to take into account other factors that could potentially confound our findings, such as chronic immune activation, cytomegalovirus coinfection, cigarette smoking, levels of physical activity, and the use of statins and other ART drugs [7] [43–48]. Saquinavir has been demonstrated to upregulate telomerase activity in vitro [44, 45]. However, despite the possibility of saquinavir and therefore other PIs antagonizing NRTI-mediated telomerase inhibition in vivo, we still observed significantly lower telomerase activity in patients receiving NRTI-containing cART, suggesting that any upregulation of telomerase activity by PIs was not sufficient to overcome the inhibitory effect of NRTIs on telomerase activity.

There were several limitations in our study. First, we were unable to prove that NRTIs inhibited telomerase activity in vivo because we only performed a cross-sectional study comparing patients on NRTI, off NRTI, and HIV-uninfected controls. We did not include a control group of HIV-infected patients naive to cART given the potential confounder of HIV itself inhibiting telomerase activity as demonstrated previously in vitro [37]. Second, our comparison of patients on and off NRTI was largely cross sectional, the number of samples that were matched was small, and the length of time off NRTI was relatively short (median of 48 weeks). Any effects of NRTIs on telomerase and TL may take many years to become apparent following initiation of NRTI and/or may reverse following cessation of NRTI. However, in the HIV-infected patients on NRTI-containing cART in this study, the median time on NRTI was 123 months (over a 10-year period), and we therefore believe this to be adequate time to see any significant changes in telomerase activity and TL.

In summary, we have demonstrated that all NRTIs inhibit telomerase activity and TL in activated PBMC in vitro, with the greatest effect observed with TDF. Reduced telomerase activity was greatest in HIV-infected patients on NRTI-containing cART. An important future clinical question will be to determine whether any inhibition of telomerase by NRTIs in vivo is reversible following cessation of NRTI. The long-term effects in HIV-infected patients of NRTI exposure on telomerase activity, TL, and accelerated aging warrant further investigation.

Notes

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