# Differential Effects of Tenofovir, Abacavir, Emtricitabine, and Darunavir on Telomerase Activity In Vitro

*Natalia Stella-Ascariz, MS,* Rocío Montejano, MD,† Laura Pintado-Berninches, MS,‡ Susana Monge, MD,§ José I. Bernardino, MD,† Ignacio Pérez-Valero, MD,† María L. Montes, MD,† Jesús Mingorance, PhD,* Rosario Perona, PhD,‡ and José R. Arribas, MD†*

**Abstract:** In vitro, tenofovir and abacavir induced a significant dose-dependent inhibition of telomerase activity at therapeutic concentrations in peripheral blood mononuclear cells of healthy subjects. Median inhibition of telomerase activity by tenofovir at 0.5 and 1 μM was 29% [Interquartile range (IQR) 29%–34%, $P = 0.042$] and 28% (IQR 28%–41%, $P = 0.042$), respectively. Abacavir inhibition was 12% (IQR 9%–13%, $P = 0.043$) at 3 μM and 14% (IQR 10%–29%, $P = 0.043$) at 10 μM. Tenofovir and abacavir did not change human telomerase reverse transcriptase (hTERT) levels or mRNA levels of other telomerase complex genes. Exposure to emtricitabine or darunavir did not affect telomerase activity, hTERT protein levels, or mRNA levels of telomerase/shelterin genes.

**Key Words:** HIV infection, antiretroviral therapy, telomerase, telomere, hTERT

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## INTRODUCTION

There is growing concern about the issue of aging of HIV-infected patients. It is well established that HIV-infected patients have an increased risk for several "non-AIDS" complications (cardiovascular disease, malignancy, liver disease, kidney disease, bone disease, and neurocognitive decline) that are classically associated with the normal aging process.[1] It remains unclear if the higher risk of these complications is expression of an "accelerated" aging process, complications occurring at earlier ages, or of an "accentuated" aging process—higher prevalence of complications at every age strata.[2,3] It is also unknown if this accentuated or accelerated aging is caused by the proinflammatory state associated with even well-controlled HIV infection, traditional risk factors (such as smoking) that are more prevalent among HIV-infected people, or other still unknown causes.[4]

Another potential cause of accelerated or accentuated aging in HIV-infected patients could be telomere shortening caused by antiretroviral drugs.[5] There is a close association between shortened telomere length (TL) in peripheral blood mononuclear cells (PBMCs) and diseases of aging, including increased cardiovascular diseases and dementia.[6,7]

Telomerase is a ribonucleoprotein enzyme complex with a RNA template (TERC), a human telomerase reverse transcriptase (hTERT) subunit, and other regulatory proteins that together with the shelterin complex maintains telomere structure.[8] Telomerase adds repetitive TTAGGG sequences to the ends of chromosomes, compensating for the progressive telomeric loss occurring at each cell division. Because of structural and mechanistic similarity to HIV reverse transcriptase, nucleoside/nucleotide reverse transcriptase inhibitors [N(t)RTI] can inhibit telomerase.[9] Zidovudine (AZT), stavudine (d4T), didanosine (ddI), and abacavir (ABC) can inhibit telomerase activity in replicating cell lines in vitro, leading to accelerated shortening of TL.[10,11] This inhibition is not observed with nonnucleoside reverse transcriptase inhibitors.[12] Recently, Leeansyah and collaborators reported that tenofovir (TFV)[13] at therapeutic concentrations is a potent inhibitor of telomerase

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From the *Microbiology Service, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain; †HIV Unit, Internal Medicine Service, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain; ‡Instituto de Investigaciones Biomédicas CSIC/UAM, IdiPAZ, Biomarkers and New Therapies and CIBER de Enfermedades Raras (CIBERER), Madrid, Spain; and §Universidad de Alcalá de Henares, Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain.

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Correspondence to: José R. Arribas, MD, PhD, Consulta Medicina Interna 2, Hospital La Paz, IdiPAZ, Castellana 261, 28046 Madrid, Spain (e-mail: joser.arribas@salud.madrid.org).

activity, causing telomere shortening in vitro. In Leeansyah et al study, lamivudine (3 TC) and emtricitabine (FTC) also inhibited telomerase activity although only at high concentrations. This finding is surprising because, being cytidine analogues, 3 TC and FTC should not act as chain terminators and therefore other mechanism of telomerase inhibition by 3 TC and FTC could be operating.

Two studies have reported that saquinavir, a protease inhibitor (PI), is able to increase telomerase activity in PBMCs[14,15] and demonstrated that telomerase up-regulation appeared to be the result of enhanced expression of hTERT in human T leukemia cells in vitro.[16] Saquinavir is no longer a preferred protease inhibitor in expert guidelines. Darunavir (DRV) boosted with ritonavir is the recommended protease inhibitor in the majority of expert guidelines. The impact of DRV on telomerase activity is currently unknown.

The main objective of our study was to confirm if ABC, FTC and especially TFV, at therapeutic concentrations, inhibit telomerase activity in vitro in activated-PBMCs. Secondly, we assayed if DRV is able to increase telomerase activity in vitro. Finally, we wanted to evaluate the possible impact of these antiretrovirals on mechanisms of telomerase inhibition different from chain termination such as expression of telomerase genes.

## METHODS

### Culture of PBMCs In Vitro With Antiretrovirals

PBMCs from healthy volunteers were isolated from whole blood by density gradient centrifugation using Ficoll-Pague PLUS (GE Healthcare). PBMCs were cultured in RPMI medium with 10% FBS supplemented with phytohemaglutinin, M form (PHA-M) (1% vol/vol; Gibco—Life Technologies), and human recombinant interleukin 2 (IL-2) (5 ng/mL; Gibco—Life Technologies) for 72 hours. Subsequently, $4 \times 10^6$ activated PBMCs were treated with increased concentrations of TFV, ABC, FTC or DRV (National Institute of Health AIDS Research and Reference Reagent Program) in fresh medium plus PHA-M/IL-2 for another 72 hours. Concentrations used for ABC and FTC were 0; 1; 3; 10; 50; and 100 μM, for TFV were 0; 0.1; 0.5; 1; 3; and 5 μM and for DRV were 0; 0.5; 1; 5; 10; 50 μM. Concentrations were calculated based on the pharmacokinetic parameters summarized in http://www.hiv-druginteractions.org/fact_sheets website of University of Liverpool.

### Measurement of Telomerase Activity

We determined telomerase activity according to the Telomeric Repeat Amplification Protocol (TRAP) by TRAPeze Telomerase Detection kit (EMD Millipore, Billerica, MA) using radioisotopic detection according to the manufacturer's instructions with following modifications: radioactive end-labeling of the TS Primer[17] was incubated 30 minutes at 37°C and 10 minutes at 85°C, telomerase extension reaction was performed with 3 serial dilutions of cell extract at 30°C for 30 minutes followed by 5 minutes denaturation at 94°C, and amplification of the telomeric repeats was done in 30 cycles. The reaction was visualized by autoradiography and was analyzed using the image-processing program ImageJ (http://imagej.nih.gov/ij/). Telomerase activity was normalized using the internal control provided in the kit and expressed relative to untreated PBMCs.

### Western Blot Analysis of hTERT Expression

20 μg of total cellular protein was subjected to 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Membranes were then blocked with 5% milk and incubated with rabbit anti-hTERT antibody (1:1000) (cat no. sc-7212; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by incubation with a secondary anti-rabbit antibody (1:1000) conjugated with horseradish peroxidase at room temperature for 30 minutes. Membranes were rehybridized with α-tubulin antibody as a housekeeping expressed control protein. Membrane antibody binding was detected by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). Image quantifications were performed using ImageJ software.

### Measurement of Gene Expression of the Telomerase/Shelterin Complex

Total RNA was isolated from cells using Tri Reagent (Sigma) and was quantified spectrophotometrically. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcriptase in a volume of 20 μL containing 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega), 1X M-MLV 5X reaction buffer (Promega), 20 U of RNase OUT (Invitrogen), 0.5 μg random primer (Promega), and 0.5 mM dNTPs. The reaction was performed for 60 minutes at 37°C and cDNA was stored at −80°C until use.

The mRNA expression level of the genes coding for the different telomerase or shelterin complex subunits (*hTERT*, *TERC*, *DKC1*, *TINF2*, *TRF1* and *TRF2*) was quantified by quantitative real-time PCR (qPCR) using TaqMan Gene Expression Assay (Applied Biosystem). To normalize the amount of total mRNA present in each reaction, we amplified an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). qPCR was performed with 100 ng of cDNA in a total volume of 20 μL containing 1X TaqMan Universal Master Mix II, no UNG and 1X TaqMan Gene Expression Assay of each gene (Applied Biosystem) (see Table 1, Supplemental Digital Content, http://links.lww.com/QAI/A880). Samples were performed in duplicate using a Real Time Stratagene MX3000P. The thermal cycling conditions included preincubation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

### Statistical Analysis

Statistic evaluation was performed with STATA v12 (Stata Corporation, College Station, Texas). Statistical significance for telomerase activity, levels of hTERT protein, and expression of telomerase complex subunits genes were calculated using

Wilcoxon signed-rank test. $P$ values $< 0.05$ were considered statistically significant.

The institutional review board of Hospital La Paz approved the study and samples were obtained after written informed consent.

## RESULTS

### TFV and ABC Inhibit Telomerase Activity In Vitro

We analyzed the effect of 3 N(t)RTIs (TFV, ABC and FTC) and 1 PI (DRV) on telomerase activity in PHA-activated PBMCs from healthy volunteers after 72 hours of treatment. We performed 5 independent experiments with all N(t)RTIs and 6 independent experiments with DRV. Telomerase activity was expressed relative to untreated PBMCs.

Of the N(t)RTIs tested, only TFV and ABC induced a significant dose-dependent decrease of telomerase activity within the therapeutic concentration range. Median inhibition induced by TFV at 0.5 and 1 μM was 29% (IQR: 29%–34%; range: 12%–39%; $P = 0.042$) and 28% (IQR: 28%–41%; range: 25%–47%; $P = 0.042$), respectively. For ABC at 3 and 10 μM, median inhibition was 12% (IQR: 9%–13%; range: 8%–17%; $P = 0.043$) and 14% (IQR: 10%–29%; range: 7%–40%; $P = 0.043$), respectively. Exposure to FTC or DRV did not affect telomerase activity even at concentrations above the therapeutic plasma level range (Fig. 1A).

### No Changes in Levels of hTERT Protein or Expression of the Telomerase/Shelterin Complex Genes.

Experiments were performed to establish whether reduction of telomerase activity mediated by TFV and ABC was the consequence of a decrease in the amount of hTERT protein. We determined the levels of hTERT after treatment for 72 hours with ABC, FTC and DRV (4 experiments each) and TFV (5 experiments). We did not find differences in the amount of hTERT between untreated and treated PBMCs (Fig. 1B). In addition, we measured the expression levels of the genes coding for the different telomerase and shelterin complex subunits (*hTERT*, *TERC*, *DKC1*, *TINF2*, *TRF1*, and *TRF2*). We measured the levels of mRNA after treatment for 72 hours with TFV, ABC, and DRV (6 experiments each) and FTC (5 experiments). We did not detect changes in expression of the genes that code for the catalytic subunit hTERT of the telomerase complex (Fig. 1C) or other telomerase subunits.

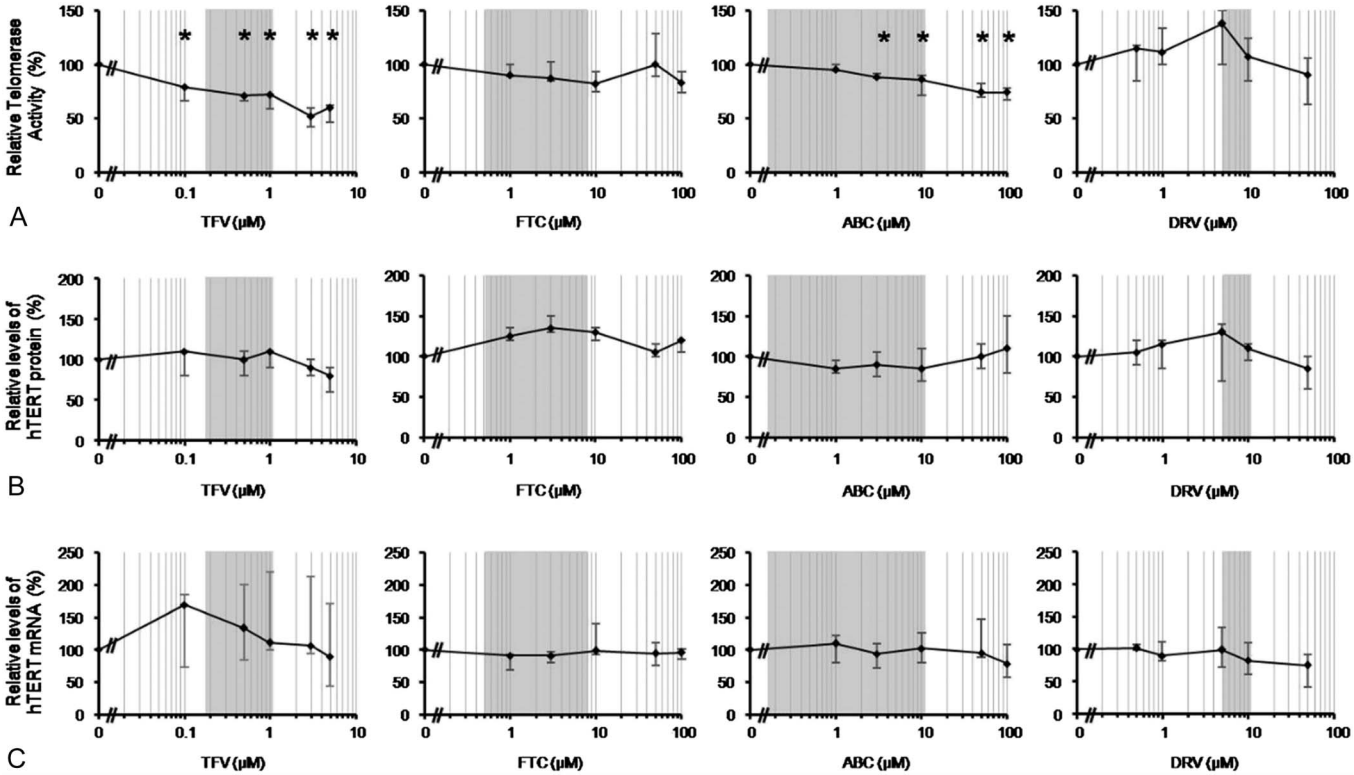


**FIGURE 1.** The effect of nucleos(t)ide reverse transcriptase inhibitors and darunavir on telomerase activity (A), levels of hTERT protein (B), and hTERT mRNA (C) in activated PBMCs. Closed circles represent the median, error bars represent interquartile range, and gray area represents the expected plasma levels in vivo for each antiretroviral (boosted DRV therapeutic range was assumed for DRV). The data are represented as percentage telomerase activity, levels of hTERT protein, and amount of hTERT mRNA of untreated cells. For telomerase activity (A), results are shown for TFV, ABC, and FTC (n = 5) and for DRV (n = 6). For levels of hTERT protein (B), results are shown for TFV (n = 5) and ABC, FTC, and DRV (n = 4). For levels of hTERT, mRNA results are shown for TFV, ABC, and DRV (n = 6) and FTC (n = 5). *$P < 0.05$.

## DISCUSSION

In our study, we explored if N(t)RTIs could play a role in the aging process of HIV-infected patients by means of inhibition of telomerase activity and whether DRV could have a similar effect. We have found that TFV and ABC, but not FTC, produced a significant dose-dependent decrease of telomerase activity in PHA-activated PBMCs within the therapeutic concentration range in vivo. After 72 hours of treatment, telomerase inhibition caused by TFV was more than double the inhibition caused by ABC: 29% and 12%, respectively. The observed decrease in telomerase activity caused by TFV and ABC was not associated with a decrease in hTERT protein level, or a change in the expression of *hTERT* gene or the other genes that code for the subunits of the telomerase/shelterin complexes. Furthermore, we have shown that DRV did not affect telomerase activity, *hTERT* gene expression, or hTERT protein levels.

The active forms of TFV, ABC, and FTC compete with the intracellular dATP, dGTP and dCTP pools, respectively, and the incorporation of these nucleotide analogs causes viral DNA chain termination. Telomeres are made up of hexamer repeat TTAGGG sequences. Telomerase binds to the 3' end of the telomeres through its own RNA template and adds TTAGGG polynucleotides to the extreme, whereas the complementary strand is filled in by DNA polymerase later on. Consequently, inhibition of the reverse transcriptase activity of hTERT by the chain termination mechanism would be possible with TFV and ABC but not with FTC. Leeansyah and collaborators[13] have previously shown that TFV was the only N(t)RTI that at therapeutic concentrations in vitro significantly inhibited telomerase activity and enhanced shortening of TL. However, in their study, ABC and FTC were also able to inhibit telomerase activity at concentrations above the therapeutic range, but only ABC enhanced shortening of TL. Probably, our study underestimates the inhibition of activity telomerase with ABC and FTC because we performed measurements of telomerase activity after 72 hours of treatment with a single dose of each drug, in contrast to Leeansyah study in which N(t)RTIs were replenished every 48 hours. This difference could account for the lack of effect of FTC on telomerase activity observed in our study. Moreover, another limitation of our study is that we cannot determine whether the main effects of NRTIs were on $CD4^+$ or $CD8^+$ T cells, or specific subsets of T cells or non–T-cell population.

The in vivo relevance of the differential effects of TFV, ABC, FTC, and DRV on telomerase activity remains to be elucidated. Although Leeansyah and colleagues reported that in vivo telomere length was significantly inversely associated with the total duration of treatment with any N(t)RTI, other studies have not found this association. In a substudy of the MONET clinical trial[18] comparing darunavir/ritonavir monotherapy versus darunavir/ritonavir and 2 N(t)RTIs for maintenance of virological suppression, there were not significant differences between 2 arms after 3 years of follow-up in telomerase activity or mean change per year of telomere length.

Our results provide more evidence about the inhibition caused by some NRTI on telomerase activity. We have confirmed that TFV and ABC inhibit telomerase activity in activated PBMCs in vitro at therapeutic concentrations and that TFV is the most potent inhibitor. Telomerase activity inhibition caused by N(t)RTIs is probably due to inhibition of hTERT activity leading to chain termination and does not involve changes in expression levels of telomerase genes or hTERT protein. In addition, we did not find that DRV affects telomerase activity, *hTERT* gene expression, or hTERT protein levels. To our knowledge, ours is the first study showing a lack of implication of hTERT proteins levels and mRNA expression. The in vivo relevance of these findings remains to be elucidated.

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