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Protective effect of the acyclic nucleoside phosphonate tenofovir toward human T-cell leukemia/lymphotropic virus type 1 infection of human peripheral blood mononuclear cells in vitro

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Abstract

9-(*R*)-[(2-Phosphonomethoxy)propyls]adenine (tenofovir), is an acyclic nucleoside phosphonate known to inhibit HIV replication in vitro and to reduce viremia in HIV-infected patients. Here we have investigated whether tenofovir is able to protect peripheral blood mononuclear cells (PBMCs) from healthy donors against human T-cell leukemia/lymphotropic virus type 1 (HTLV-1) infection in vitro. PBMCs were pre-treated with tenofovir and infected by exposure to an irradiated cell line chronically harbouring HTLV-1. Measurements of viral DNA, as well as viral gene and protein expression, at 4 weeks after infection, revealed that tenofovir at concentrations of 1 μM and higher completely protected PBMCs against HTLV-1; lower concentrations did not fully prevent HTLV-1 infection of the cultures. Nevertheless, in the long term, cell growth of infected PBMCs was inhibited in vitro even by 0.1 μM tenofovir. In addition, tenofovir directly inhibited HTLV-1 reverse transcriptase activity, in a cell-free assay that utilizes a crude preparation from HTLV-1 viral particles as a source of the enzyme. The selectivity index of tenofovir for HTLV-1, was about four times higher than that of azidothymidine. Taken together our results strongly encourage further studies to investigate the real impact of tenofovir towards HTLV-1 infection.

Keywords: Acyclic nucleoside phosphonates; Tenofovir; HTLV-1; Reverse transcriptase; Reverse transcriptase inhibitors

1. Introduction

Acyclic nucleoside phosphonates (ANPs) are nucleotide analogues possessing a phosphonate group attached to the acyclic nucleoside moiety through a P–C bond. This link assures relatively high stability to the phosphonate group that cannot be easily cleaved by esterases. ANPs have been found to inhibit different DNA and RNA (retro)viruses (De Clercq, 2003). In particular, one of these compounds, the 9-(*R*)-[2-(phosphonomethoxy)propyl] adenine (tenofovir) possesses an high antiviral potential, acting as a potent reverse-transcriptase inhibitor (RTI). The spectrum of antiviral activity of tenofovir includes retroviruses and hepadnaviruses (Balzarini and De Clercq, 1995; Balzarini et al., 1996; Heijtink et al., 1994). Tenofovir was found to inhibit HIV replication in vitro (Deeks et al., 1998) and tenofovir disoproxil fumarate, the orally bioavailable prodrug of tenofovir, caused a significant decline in plasma HIV-1 RNA levels in randomized, double blind placebo-controlled clinical trials in HIV-1 infected individuals (Deeks et al., 1998; Schooley et al., 2002). Tenofovir, like other ANPs, provides a long term antiviral response, leads poorly to the emergence of drug resistance and is well tolerated in vivo, in HIV-infected patients (Balzarini et al., 1996). In exceptional cases, renal failure was observed (Creput et al., 2003). Moreover, tenofovir has limited effects in vitro on the proliferation of renal proximal tubule epithelial cells, erythroid and myeloid progenitors (Cihlar et al., 2002). The low cytotoxic effect of tenofovir might be due to a low capability to produce mitochondrial dysfunction (Birkus et al., 2002b; Lee et al., 2003). This seems to be related: (i) to the poor capacity of tenofovir to act as a substrate for human cellular and mitochondrial α, β, γ and ε DNA polymerases in vitro (Birkus et al., 2002a), (ii) to the absence of influence on cellular expression of the mitochondrial cytochrome *C* oxidase complex (Birkus et al., 2002b; Lee et al., 2003) and on

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mitochondrial DNA levels (Biesecker et al., 2003). In addition to its anti-HIV activity, tenofovir was found to decrease RNA levels of SIV (Silvera et al., 2000) and to be effective in pre- and post-exposure prophylaxis of SIV infection (by different routes) in adult macaques (Tsai et al., 1995, 1997) as well as in the treatment of chronic SIV infection in infant rhesus (Van Rompay et al., 1997). Moreover, tenofovir has been shown to exert antiviral activity against FIV and murine leukaemia/sarcoma viruses (Naesens et al., 1997). Thus, due to its low toxic activity and to its broad spectrum of action against retroviruses, tenofovir represents a potential candidate for extending its use to human retroviruses other than HIV.

Adult T cell leukaemia/lymphoma (ATLL) and human myelopathy/tropic spastic paraparesis (HAM/TSP) are severe diseases caused by the human retrovirus termed human T-cell leukemia/lymphotropic virus type 1 (HTLV-1). It has been estimated that nowadays 10–20 million individuals are carriers of HTLV-1 in endemic areas of Japan and South America (Edlich et al., 2000). Nevertheless, therapeutic approaches to HTLV-1-related pathologies are still limited. Combination of classical anti-cancer chemotherapy with interferons and with monoclonal antibodies against the IL-2 receptor caused a limited and transient benefit in 50% of ATLL patients. It has also been shown that the combination of the nucleoside RTI azidothymidine (AZT) and interferon-α was able to ensure a partial response in ATLL patients (Gill et al., 1995). Furthermore, a transient response to therapy has been reported in HAM/TSP patients treated with lamivudine as a monotherapy (Taylor et al., 1999) or in combination with AZT (Machuca and Soriano, 2000). In vitro studies demonstrated that AZT can efficiently inhibit HTLV-1 transmission to human cells (Matsushita et al., 1987; Macchi et al., 1997) and that the active triphosphate forms of AZT, ddI, ddC and d4T can directly suppress HTLV-1 RT activity (Garcia-Lerma et al., 2001). Conversely, HTLV-1 has been found to present partial natural resistance to lamivudine in vitro (Garcia-Lerma et al., 2001; Balestrieri et al., 2002). Moreover, based on vitro experiments, a potential protective effect of tenofovir against HTLV-1 infection has been recently suggested (Hill et al., 2003).

In this study we have unequivocally established that tenofovir exerts a powerful, direct protective effect in vitro against de novo infection of human peripheral blood mononuclear cells (PBMCs) with HTLV-1.

2. Materials and methods

2.1. HTLV-1 infection in vitro and treatments

PBMCs were harvested from normal, adult donors who were seronegative for HIV and B/C hepatitis viruses. Mononuclear cells from heparinized blood were separated by Ficoll/Hypaque density gradient (Pharmacia, Uppsala, Sweden). The cells were then washed twice in RPMI-1640 medium (Gibco, Paisley, UK). HTLV-1 infection was performed by co-culturing PBMCs in toto with lethally irradiated MT-2 cells (120 Cy, from a caesium gamma cell irradiator 1000, Canada Atomic Energy Ltd, Canada) at a PBMCs/MT-2 ratio of 5:1. MT-2 is a HTLV-1 chronically infected cell line originally derived from human cord leukocytes co-cultured with leukemic T-cells from an ATLL patient (Miyoshi et al., 1981). Infected cultures were maintained in 6-well plates in 4 ml of medium containing 20 U/ml of recombinant interleukin 2 (IL-2; Hoffman La Roche, Basel, Switzerland), in the absence or presence of the acyclic nucleoside phosphonate analog of adenosine 5′-monophosphate, tenofovir (TFV, kindly provided by Dr Jan Balzarini, Rega Institute for Medical Research, Leuven, Belgium), at a final concentration ranging from 100 μM to 0.1 μM, and were split weekly. A concentration of 1 μM tenofovir corresponds approximately to a plasmatic peak of 326 ng/ml in vivo, i.e. a pharmacologically active concentration (Chapman et al., 2003). Cultures were treated according to two different modalities of treatment. One consisted of an overnight (about 13–14 h) pre-treatment before infection, while in the other case the drug was added immediately (about 3–5 min) before infection. Successively the drug was re-added at half concentration at 3, 7 and 10 days post-infection (p.i.), when half volume of the conditioned medium was replaced by fresh medium. For comparison the anti-HTLV-1 activity of AZT at the concentrations of 25, 1 and 0.1 μM was assayed. Cell growth of infected and uninfected cells in long-term culture, either treated with different concentrations of the drug or with control medium, was monitored weekly by evaluating living cells using the trypan blue dye exclusion test. Living cell number was calculated as the mean of two independent evaluations. After counting, the cell concentration was re-adjusted to 1×10^6/ml. Results of cell growth were expressed as total cell number (TCN) calculated from living cell counts as follows: for the first week, TCN was the actual number of cells, expressed as millions of viable cells, detected before the first adjustment, while in successive weeks, TCN was calculated theoretically as the TCN at the previous week multiplied by the cell concentration, expressed as millions of viable cells per milliliter, detected before weekly adjustment. Aliquots of irradiated MT-2 cells, utilized as donor cells for infection and kept separately in culture for the duration of the experiments, showed no evidence of growth.

2.2. Extraction of genomic DNA and PCR analysis

The antiviral effects of tenofovir and AZT at different concentrations were evaluated 4 weeks p.i. Before assaying the presence of proviral DNA, cells from cultures were subjected to centrifugation on density gradient to eliminate debris and dead cells. Cells were then incubated with proteinase K at 37 °C and DNA was extracted in phenol–chloroform–isoamylalcohol (50:49:1), according to standard procedures. One microgram of DNA was used as a template and was amplified in a standard PCR reaction mix: 1× PCR buffer, 0.2 mM dNTPs, 0.5 μM primer pair specific for the pol region of HTLV-1 (forward: SK54, reverse: SK55; Perkin Elmer, Boston, MA) and 1.25 U of Taq polymerase (Roche, Mannheim, Germany). As an internal control, the GAPDH gene was amplified using the same PCR reaction mix with a specific primer pair (forward primer, 5′ CCATGGAAAAAGGCTGGGG 3′; reverse primer, 5′ CAAAGTTGTCATGGATGACC 3′). Samples were subjected to 30 cycles of PCR amplification, each cycle consisting of 30 s at 94 °C, 30 s

at 55 °C and 45 s at 72 °C on a Cetus DNA thermal cycler 2400 (Perkin Elmer). Following the final cycle, samples were incubated at 72 °C for 7 min to ensure the completion of the final extension step.

2.3. Extraction of RNA and RT-PCR analysis

RNA isolation was performed using RNAzol (Life Technologies, Grand Island, NY), according to the Manufacturer's instructions. Before assaying the presence of viral RNA, cells from cultures were subjected to centrifugation on density gradient to eliminate debris and dead cells. Total RNA from infected cells was reverse-transcribed into cDNA in 25 μl reaction mix as follows. An amount of 2 μg of RNA was incubated with a mix containing a final dilution of 1× RT buffer, 1 mM dNTPs (Pharmacia), 1.5 μg oligo (dT) (New England Biolabs, Beverly, MA), 50 U recombinant RNAse inhibitor (Roche), 10 mM DTT (Sigma, St Louis, MO), 25 U Mu-MLV RT (New England Biolabs) for 1 h at 37 °C. The reaction mix was incubated at 95 °C for 5 min in order to inactivate RT and then chilled on ice. Three microliters of cDNA was amplified by PCR in a total volume of 50 μl. Amplifications with 0.5 μM of primer pairs specific for the Tax/Rex region of HTLV-1 (Kinoshita et al., 1989), or, as an internal control, with primers specific for GAPDH (forward primer, 5′ TGGTATCGTGAAAGGACT 3′; reverse primer, 5′ ATGCAAGTGAGCTTCCCGTTC 3′), were performed for 45 cycles and amplified using the program described above for DNA-PCR.

2.4. Liquid hybridisation

Amplified DNA was analysed by liquid hybridization as previously described and probed using the following specific [^{32}P]-end-labelled oligonucleotides: SK56 (Perkin Elmer) for HTLV-1 pol region, RPXPR-1 for HTLV-1 Tax/Rex region (Kinoshita et al., 1989), 5′ CTAAGCAGTTGGTGGTGCA 3′ for GAPDH DNA-PCR product, 5′ GAAACTGTGGCGTGATGGC 3′ for GAPDH RNA-PCR product. The samples were denatured for 5 min at 95 °C and annealed at 55 °C for 15 min. A running buffer was added to the samples, which were then loaded onto an 8% gel to detect the amplified products. Following electrophoresis the gel was dried and exposed to a Kodak XAR-5 film (Kodak Company, Rochester, NY) for autoradiography.

2.5. Western blot analysis

Five million cells from samples treated with different drug concentrations were subjected to centrifugation on density gradient to eliminate debris and dead cells, solubilized at 4 °C in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA pH 7.4, 1% Triton-X, NaCl 150 mM, 0.25% sodium deoxycholate, 1% NP-40 and, freshly added, 1 mM PMSF, 5 μM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM Na_3VO_4, 20 mM Na_3F) and centrifuged at 10,000 × *g*. An aliquot of the supernatant was saved for determination of protein concentration, and the rest was boiled in SDS sample buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). An amount of 60 μg of the proteins was loaded onto a 10% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred to PVDF membrane (NEN Life Science Products, Boston, MA), which was subsequently stained with 0.2% Ponceau red to ensure equal protein loading and transfer. After blocking the membrane in 10% non-fat dried milk and 3% BSA in TTBS (20 mM Tris–HCl pH 8.0, 0.9% NaCl, 0.03% Tween 20), the blots were incubated overnight at 4 °C with a 1:1000 dilution of anti-Tax monoclonal antibody (a generous gift from John Brady NCI, NIH, Bethesda, MD). Subsequently, the blots were washed and then incubated with anti-human IgG conjugated to peroxidase (Biorad, Hercules, CA). Binding of antibodies was detected by chemoluminescence staining using the ECL detection kit (NEN).

2.6. Cell-free assay of HTLV-1 reverse transcriptase inhibition

To test the inhibitory activity of tenofovir we have set up a novel HTLV-1-RT inhibition assay. This assay is a modified version of a method, that we have recently described and that utilizes commercially available RT enzymes from non-human retroviruses to screen the RT-inhibitory activity of new compounds (Chiacchio et al., 2004). As a template for reverse transcription, we utilized RNA isolated from transfectant cells ectopically expressing the glycoprotein D (gD) of herpes simplex virus type 1. These transfectant cells were previously generated and described for other purposes (Medici et al., 2003). The gD-transfectant cells were grown in D-MEM plus 12% foetal bovine serum (FBS), 400 μg/ml G418 (Gibco) and 30 μg/ml BrdU (Sigma). RNA isolation from 5×10^6 gD-transfectant cells, was performed using Trizol (Gibco), according to the Manufacturer's instructions. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated distilled water and RNA concentration was calculated by spectrophotometry at 260/280 nm. To remove possible DNA contamination, RNA was treated with 100 U/μg RNase free DNase (Roche) in the presence of 25 mM of $MgCl_2$ for 1 h at 37 °C. After DNase inactivation for 5 min at 95 °C, RNA was extracted in phenol–chloroform–isoamylalcohol (50:49:1), precipitated with 96% ethanol and dissolved in DEPC-treated water. Compounds to be assayed were activated through preincubation with a crude extract from previously stimulated PBMCs from healthy donors, serum negative for HIV and B/C hepatitis viruses. For preparation of the crude extract, 1×10^6 PBMCs, previously stimulated with 2 μg/ml PHA and 20 U/ml IL-2, for 72 h in RPMI plus 20%, were rinsed three times in cold PBS and then solubilized in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA pH 7.4, 0.05% Triton-X, NaCl 150 mM, 0.25% sodium deoxycholate, 0.1% NP-40 and, freshly added, 1 mM PMSF, 15 μM DTT, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM Na_3VO_4, 20 mM Na_3F, all from Sigma) on ice and centrifuged at 10,000 × *g*. The lysed extract was incubated with the drugs at different concentrations in a final volume of 10 μl for 15 min on ice and for a successive 45 min at 30 °C. After incubation the crude-extract/drug mixture was inactivated for 5 min at 95 °C. This

incubation condition was chosen to minimize the risk of DNA degradation of the final PCR product, on the basis of preliminary experiments showing that: (i) RT-inhibitory activity of the nucleoside drugs was not increased by pre-incubation at higher temperature, (ii) pre-incubation of the crude-extract/drug mixtures at higher temperature, such as 37 °C, resulted in a smear pattern of some PCR products, presumably due to residual DNA-degrading activity in the crude extract after inactivation. As a source of HTLV-1 RT, a viral lysate was prepared from 1 ml of supernatant from MT-2 cells according to the following procedures. The supernatant was clarified twice at 1000 × *g* for 8 min, and then ultracentrifuged at 30,000 × *g* for 1 h at 4 °C. The pellet was resuspended in 50 μl of lysing buffer (50 mM pH 7.4 Tris–HCl, 150 mM NaCl, 0.5%, NP 40, Sigma). Total DNase-treated RNA (1 μg) was reverse transcribed using 0.5 μM U_S6 reverse primer (5′ TGTCGTCATAGTGGGCCTCCAT 3′) in a reaction mix containing 1× RT buffer, 100 U RNase inhibitor, 1 mM dNTP, 10 mM DTT, (all from Promega, Madison, WI) plus, as a source of HTLV-1 RT, 10 μl of lysed viral particles. The reactions were performed in the presence or absence of activated substances, for 1 h at 37 °C. After incubation at 95 °C for 5 min, 5 μl of RT reaction were used for DNA PCR in a reaction mix containing 1× Taq Gold buffer (Promega), 0.5 μM primer pair (U_S6 reverse, see above, and U_S6 forward, 5′ AGACTTGTTGTAGGAGCATTCG 3′), 0.3 mM dNTP, 5 mM $MgCl_2$, 1.25 U Taq Gold (Promega), for 30 cycles (30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C) on a Cetus DNA thermal cycler 2400 (Perkin Elmer, Norwalk, CT). Following the final cycle, samples were incubated at 72 °C for 20 min to ensure the completion of the final extension step. Amplified DNA (350 bp) was visualized on 1% agarose gel containing 10 μg/ml ethidium bromide in TAE buffer 1×. The cycle number was optimally chosen on the basis of preliminary experiments, carried on using 45, 35, 30 and 25 cycles, to establish the linear range of the reaction useful for correlating the concentration of the obtained PCR product with that of the initial template.

2.7. Cytotoxic assays

The effect of tenofovir on cytotoxicity and growth of uninfected PBMCs in short-term culture was assessed by evaluating dead and living cells, respectively, using the trypan blue dye exclusion test, after 3 and 7 days of culture. PBMCs were cultured in the presence of IL-2 (20 U/ml), without additional stimuli, and tenofovir was either added or not at the onset of the cultures at concentrations of 0.5 μM, 2 μM, 8 μM, 32 μM, 128 μM, 512 μM and 2048 μM. For comparison, the effect of AZT on cell viability at the same concentrations as those used for tenofovir was assayed.

2.8. Data analysis and calculation of inhibitory concentrations

The results of different assays were utilized to calculate values of drug concentrations required to cause cytotoxicity in 20% of the cells (CC_{20}), to reduce cell growth by 20% (GIC_{20}) and to inhibit proviral DNA or viral RNA expression by 50% (DIC_{50} and RIC_{50}, respectively). Proviral DNA and viral RNA expression for DIC_{50} and RIC_{50} calculation was quantified by densitometric analysis of the bands of amplified DNA, produced and visualized as above described for DNA-PCR and RT-PCR analysis of HTLV-1 pol and Tax/Rex regions, respectively. Inhibitory concentrations were calculated according to the best-fit curve (y value versus $\log x$, where y is the value of the examined function and x is the drug concentration).The selectivity index (SI) for tenofovir and for AZT was defined as the ratio of GIC_{20} to RIC_{50}.The paired t test was used to compare inhibitory concentrations and SI values. Data analysis was performed using the SPSS statistical software system (version 10.0 for Windows; SPSS, Chicago, IL).

3. Results

3.1. Antiviral activity

The protective effect of tenofovir against HTLV-1 infection was assessed by analysing the inhibition of proviral DNA, viral RNA and viral protein expression in treated and untreated cultures of PBMCs exposed to HTLV-1, at 4 weeks following exposure. This time was chosen to exclude, without any doubt, the presence of viral material from MT-2 donor cells, as previously shown (Matteucci et al., 2004). Fig. 1 shows the results obtained in one representative experiment out of the three performed using PBMCs from different donors. The results show that pre-treatment with 100, 25, 5 and 1 μM tenofovir overnight, completely abolished the presence of proviral DNA (Fig. 1A, lanes 3–6), viral RNA (Fig. 1C, lanes 3–6) and tax protein expression (Fig. 1E, lanes 3–6 and F, columns 3–6) compared to untreated, infected controls (Fig. 1A, C and E, lanes 2; F, column 2). Conversely, 0.1 μM tenofovir did not exert an evident protective effect against HTLV-1 infection in the same assays. In fact, the pattern shown by treated samples (Fig. 1A, C and E, lanes 7; F, column 7) was similar to that obtained in untreated, infected samples (Fig. 1A, C and E, lanes 2; F, column 2). Detection of equal levels of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in different samples, as determined by densitometry analysis, showed that equal material both for DNA and RNA was analysed (Fig. 1B and D). On the other hand, when tenofovir was added immediately before infection, the drug did not exert any antiviral activity. In fact, in these experimental conditions proviral DNA, viral RNA and tax protein of HTLV-1 were detected without inhibition at all concentrations of tenofovir tested (data not shown). This finding is in contrast with what we previously observed and have confirmed in the present study for AZT, that exerted its antiviral activity in vitro even when added to PBMC immediately before HTLV-1 infection (data not shown).

3.2. Effect of tenofovir on HTLV-1 reverse transcriptase activity

In order to investigate in detail the mechanisms involved in the anti-HTLV-1 antiviral activity exerted by tenofovir, we

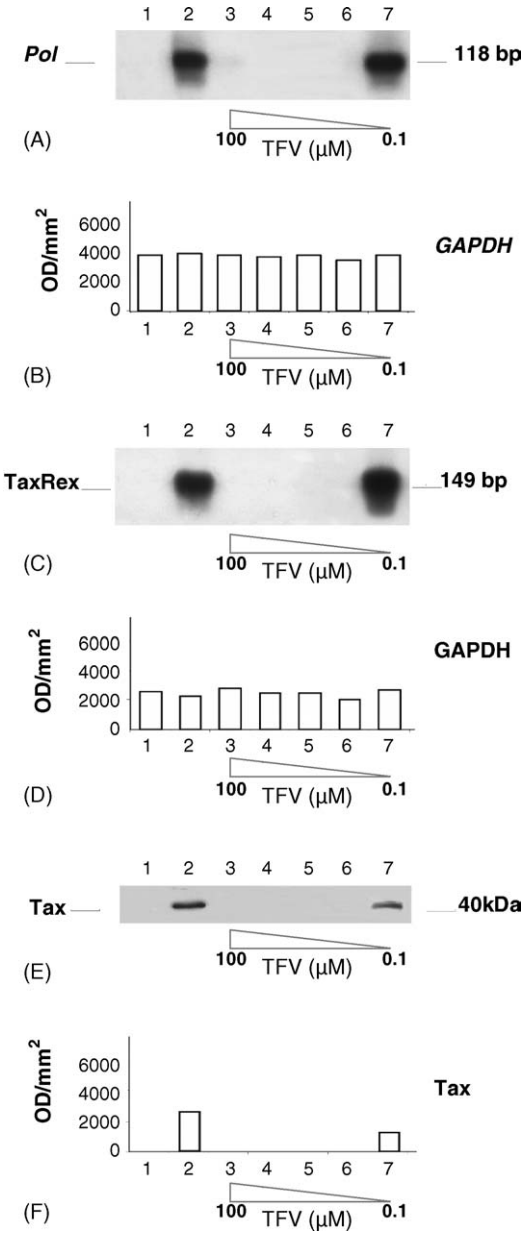

Fig. 1. Antiviral effect of tenofovir on HTLV-1 infection, evaluated at 4 weeks after infection in vitro of human PBMCs. Lane or column numbers for all panels of this figure: 1, uninfected control; 2, untreated HTLV-1 infected PBMCs; 3–7, PBMCs infected with HTLV-1 and treated with 100 μM, 25 μM, 5 μM, 1 μM and 0.1 μM tenofovir, respectively. (A) Presence of HTLV-1 proviral DNA in PBMCs exposed to HTLV-1 infection, left untreated or treated with tenofovir. (B) Levels of DNA-PCR-products following amplification with primers specific for GAPDH, in the same samples shown in panel A, quantified by densitometry. Results are expressed as optical density (OD)/mm^2. (C) HTLV-1 viral RNA expression revealed by RT-PCR in HTLV-1 infected cultures, treated or left untreated. (D) Levels of RT-PCR products following amplification with primers specific for GAPDH, in the same samples shown in panel C, quantified by densitometry. Results are expressed as OD/mm^2 values. (E) Expression of HTLV-1 protein Tax in PBMCs exposed to HTLV-1 infection, left untreated or treated with tenofovir, detected by western blotting analysis. (F) Levels of Tax protein detected by western blotting analysis, as shown in panel E, quantified by densitometry and expressed as OD/mm^2 values.

performed a cell-free assay to directly evaluate the inhibition of HTLV-1 reverse transcriptase activity. As a source of HTLV-1 RT, a viral lysate from supernatants of the MT-2 cell line was utilized. Based on preliminary experiments, activated tenofovir was tested at final concentrations, in PCR reaction mixtures, ranging from 1 nM to 0.1 nM, while activated AZT was tested at 10 nM and 1 nM. Non-activated drugs were tested at a ten-fold higher concentration, i.e. 10 nM for tenofovir and 100 nM for AZT. Moreover, as an unrelated negative control, the protease inhibitor indinavir was tested. In order to transform the drugs into their active forms, tenofovir and AZT were preventively activated through pre-incubation with a cell-free crude extract from stimulated PBMCs. Pre-incubation with the crude extract served to supply the necessary enzymes to transform the drugs to the tri-phosphate forms. Results in Fig. 2A show that the presence or the absence of the PBMC crude extract did not affect either the formation of the cDNA or the following PCR reaction, as revealed by the equal presence of the amplified 350 bp fragments in both experimental conditions (Fig. 2A, lanes 1 and 2). In addition, the singly absence of RNA template (Fig. 2A, lane 3), primer pair (Fig. 2A, lane 4) or HTLV-1-RT-containing MT-2 lysate (Fig. 2A, lane 5), respectively, resulted in the complete absence of any amplification products. Fig. 2B shows that non-activated tenofovir, at 10 nM, was not able to inhibit cDNA elongation as revealed by the RT-PCR reaction (Fig. 2B, lane 4), while pre-activated tenofovir at 1 nM completely inhibited the formation of cDNA (Fig. 2B, lane 5). Conversely, 0.1 nM tenofovir was not inhibitory (Fig. 2B, lane 6). Similarly, non-activated AZT 100 nM was not able to inhibit HTLV-1 RT activity (Fig. 2B, lane 1), while HTLV-1 RT was inhibited by activated AZT at a concentration of 10 nM AZT (Fig. 2B, lane 2), but not at 1 nM (Fig. 2B, lane 3). The unrelated control indinavir did not inhibit reverse transcriptase activity, as expected (Fig. 2B, lanes 7–9). Duplicate samples from two different in vitro reactions gave identical results. To further investigate the dose–response of tenofovir, we then utilized concentrations of the drug between 1 nM and 0.1 nM. Results in Fig 2C show that concentrations of tenofovir from 0.6 nM to 1 nM caused complete inhibition of RT activity (lanes 5–7), while 0.4 nM and 0.2 nM tenofovir caused an inhibition of around 70% (lane 4) and 14% (lane 3), respectively, compared to untreated control (lane 1), as revealed by densitometry analysis (Fig. 2D). Densitometry values were normalized versus the housekeeping GAPDH expression. It has to be emphasized that when activated tenofovir was added to control samples after cDNA formation instead of at the beginning of the reaction, no inhibitory effect was observed, confirming the specificity of the effect of this drug during reverse transcription and not during PCR amplification (data not shown).

3.3. Effect of tenofovir on growth of infected cells in the long term

In order to assess whether treatment with tenofovir could affect the growth curve of HTLV-1 infected cells in the long-term, cell growth was monitored weekly by evaluating living

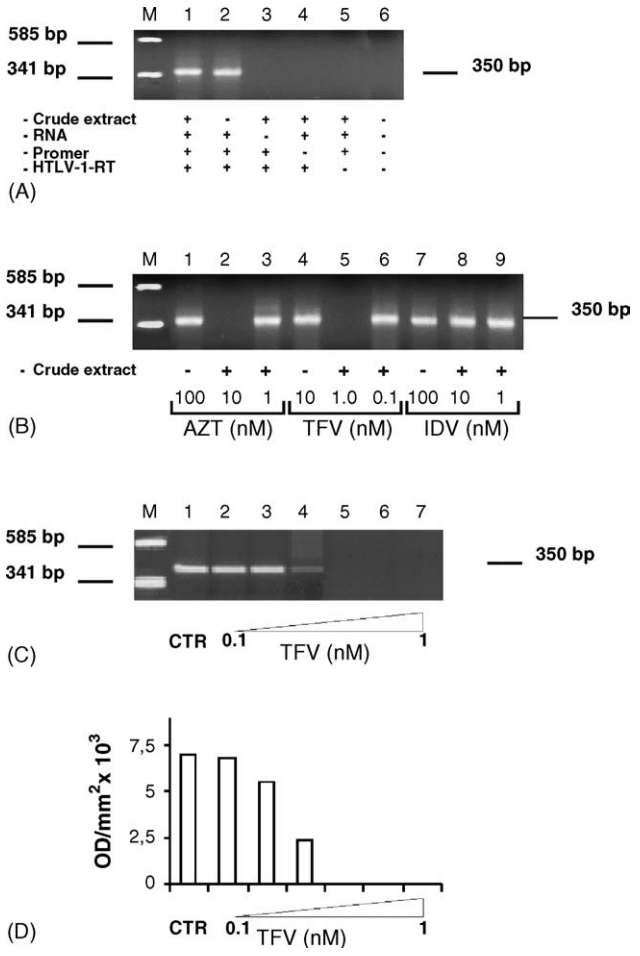

Fig. 2. Effect of tenofovir on HTLV-1 RT activity, measured by a cell-free assay. (A) RT-PCR products from different control samples, without addition of tenofovir, resolved in agarose gel. Lane 1: crude extract, HSV-1 gD specific primer pairs, RNA template of HSV-1 gD, HTLV-1 RT. Lane 2: no crude extract, HSV-1 gD specific primer pairs, RNA template of HSV-1 gD, HTLV-1 RT. Lane 3: crude extract, HSV-1 gD specific primer pairs, no RNA template of HSV-1 gD, HTLV-1 RT. Lane 4: crude extract, no HSV-1 gD specific primer pairs, RNA template of HSV-1 gD, HTLV-1 RT. Lane 5: crude extract, HSV-1 gD specific primer pairs, RNA template of HSV-I gD, no HTLV-1 RT. Lane 6: H_20. M: pUC 18 DNA Dpn I digest. (B) RT-PCR products of samples from cultures untreated or treated by exposure to different concentrations of tenofovir (TFV), AZT, and indinavir (IDV), activated by exposure to PBMC crude extract or not. Lane 1: no crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT, 100 nM AZT. Lanes 2 and 3: crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT, 10 nM and 1 nM AZT, respectively. Lane 4: no crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT, 10 nM TFV. Lanes 5 and 6: crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT, 1 nM and 0.1 nM TFV, respectively. Lanes 7–9: no crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT, 100 nM, 10 nM and 1nM indinavir, respectively. M: pUC 18 DNA Dpn I digest. (C) RT-PCR products of samples treated with crude extract, RNA template of HSV-1 gD, HSV-1 gD specific primer pairs, HTLV-1 RT. Lane 1: Untreated control. Lanes 2–7: 0.1, 0.2, 0.4, 0.6, 0.8, 1 nM TFV, respectively. M: pUC 18 DNA Dpn I digest. (D) Levels of RT-PCR products shown in panel C, quantified by densitometry and expressed as OD/mm^2. Numbers of columns correspond to lanes in panel C.

cells by means of the trypan blue dye exclusion test. Fig. 3 shows the growth curve of a culture representative of three that were treated with tenofovir at 100 μM, 25 μM, 5 μM, 1 μM and 0.1 μM (Fig. 3A–E). The results show that infected cultures treated with 100 μM and 25 μM tenofovir were consistently inhibited by 90% and 50%, respectively, in their growth and that they eventually ceased to grow at 8 weeks p.i. (Fig. 3A and B). Conversely, cultures treated with 5 μM, 1 μM and 0.1 μM tenofovir during the first 10 weeks showed a growth curve that was similar to that of control, untreated infected cells. Nevertheless, these cultures could not be maintained longer than 11–12 weeks, at which time they stopped to grow (Fig. 1C–E). In contrast, untreated infected cells were, as expected, after a transient growth crisis, still rapidly growing at 13 weeks p.i., when the experiment was interrupted. Thus, although treatment with 0.1 μM tenofovir did not cause prevention of HTLV-1 infection in PBMCs, as detected by our assays, it was still able to affect, in the long term, the HTLV-1-driven uncontrollable growth of PBMCs. This suggests the possibility of unidentified, adjunctive effects of the drug other than its RT-inhibitory activity. Parallel uninfected cultures, either treated or not with tenofovir, when kept in the same culture conditions as infected cells, survived for a limited period of time only, completely exhausting their growth, in the absence of stimulation caused by coculture with HTLV-1 donor MT-2 cells, at around 7 weeks from the onset of culture, as expected (data not shown).

3.4. Evaluation of the cytotoxic and antiproliferative effects of tenofovir versus its antiviral effect

In order to obtain further information on the cytotoxicity of tenofovir in our experimental system, in some experiments a portion of PBMCs utilized for experimental infection were left uninfected and assayed for their susceptibility to the cytotoxic action of tenofovir in 3 or 7 day short-term cultures. As a measure of the cytotoxic action of the drug, the capability to induce cell death in 20% of the cells and to inhibit living cell number by 20%, were calculated and expressed as CC_{20} and GIC_{20}, respectively. For comparison, the effects of AZT on parallel cell cultures were also tested. Results obtained after 3 days in culture were very similar to those obtained after 7 days in culture. Only the latter are summarized in Table 1. They represent the mean ± S.D. of three CC_{20} and GIC_{20} values, calculated from data obtained in three different experiments using PBMCs from different donors. The CC_{20} of tenofovir was higher than 2048, i.e. out of the range tested, showing that tenofovir had a very low cytotoxic effect. In contrast, CC_{20} of AZT was within the concentration range tested. The GIC_{20} values of tenofovir and AZT, as expected, were lower than the corresponding CC_{20} values, indicating that both the drugs were more powerful in their cytostatic rather than in their cytotoxic effects on PBMCs. However, the GIC_{20} value of tenofovir was more than three times higher than that of AZT, confirming, in our experimental system, a lower toxicity of tenofovir ($p < 0.05$). Tenofovir and AZT, when added to the infected cultures according to the pre-treatment protocol,

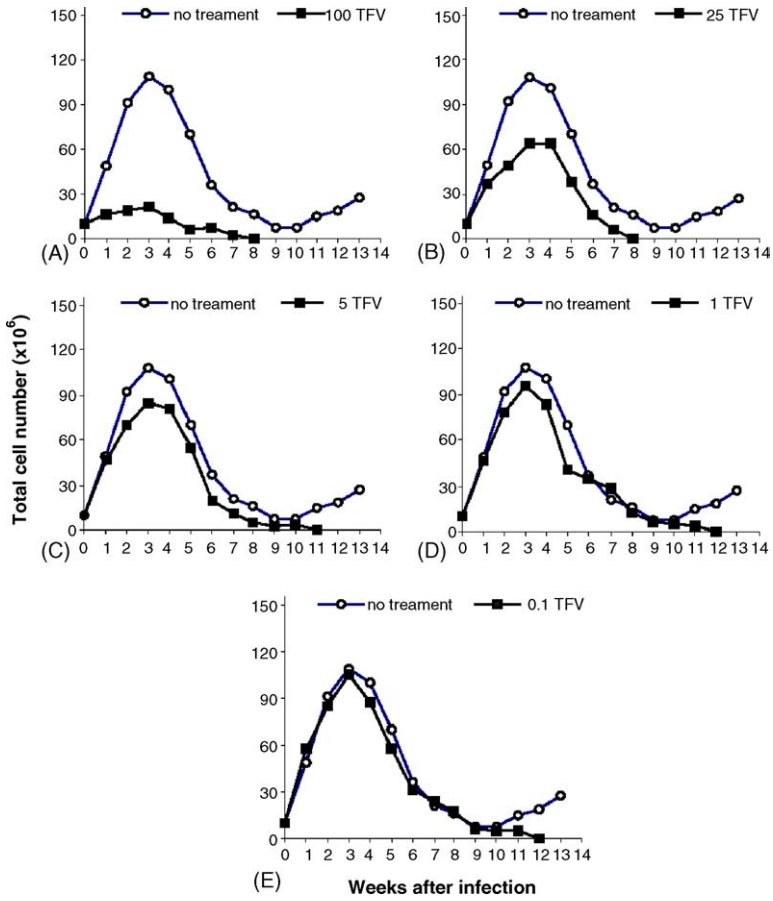

Fig. 3. Effect of tenofovir on cell growth of HTLV-1 infected cultures. Growth curves of HTLV-1 infected PBMCs, left untreated (open circle) or treated (solid squares) with tenofovir (TFV) 100 μM (A), 25 μM (B), 5 μM (C), 1 μM (D) and 0.1 μM (E). The growth curve refers to an experiment performed using PBMCs from one donor, representative of the three performed on PBMCs from three different donors.

Table 1
Cytotoxic and antiviral activity of tenofovir compared with AZT

Drug	CC_{20}[a] ± S.D. (μM)[b]	GIC_{20}[c] ± S.D. (μM)	DIC_{50}[d] ± S.D. (μM)	RIC_{50}[e] ± S.D. (μM)	SI[f] GIC_{20}/RIC_{50}
TFV[g]	>2048	114.94 ± 25.37	0.35 ± 0.0.18	0.35 ± 0.07	328.4
AZT	1196.52 ± 168.29	32.57 ± 15.46	0.45 ± 0.009	0.41 ± 0.12	79.44

[a] Concentration of the drug able to cause cytotoxicity in 20% of the cells (CC_{20}), as assessed by trypan blue exclusion test after 7 days in culture with IL-2 in PBMCs from healthy donors.
[b] The S.D. has been calculated using the values obtained from experiments performed with PBMCs from three different donors.
[c] Concentration of the drug able to cause growth inhibition by 20% (GIC_{20}) after 7 days in culture with IL-2 in PBMCs from healthy donors.
[d] Antiviral activity expressed as inhibitory concentration by 50% of proviral DNA (DIC_{50}).
[e] Antiviral activity expressed as inhibitory concentration by 50% of viral RNA (RIC_{50}).
[f] Selectivity index (SI) derived from the GIC_{20}/RIC_{50} ratio.
[g] TFV, Tenofovir.

possessed a similar efficacy in inhibiting proviral DNA and viral RNA. Values of DIC_{50} and RIC_{50}, calculated from DNA- and RT-PCR assays, respectively, showed a slightly higher activity of tenofovir, as reported in Table 1. The cytotoxic potential versus the antiviral potency of tenofovir in comparison with AZT, as a reference compound, is expressed by the SI values, calculated as the GIC_{20} to RIC_{50} ratio. Due to the higher antiviral activity and the lower cytotoxicity of tenofovir in comparison with AZT, the SI value of tenofovir for HTLV-1 was about four times higher than that of AZT, as shown in Table 1 ($p = 0.05$).

4. Discussion

The use of antiretrovirals in HTLV-1 infection could be reasonably debated. In fact, it is generally assumed that HTLV-1 infection should be transcriptional silent in vivo. This should render any effort to control the infection by means of antiviral drugs targeting viral replication, in the absence of evidence for viral particles or viral RNA and proteins in the serum of patients, very difficult, or even futile. In spite of this, recent results have shown that there is persistent, detectable expression of HTLV-1 genes. Especially Tax has been found to be frequently expressed

in vivo in infected patients and this finding has been related not only to the proliferation of infected cells, but also to the balance between viral replication and immune response (Bangham, 2003). Relevant is the recent demonstration that HTLV-1 can spread directly via cell-to-cell transmission of infectious virions, in the absence of an extracellular phase of viral replication (Igakura et al., 2003). These observations highlight the point that HTLV-1 replication and viral load maintenance in vivo is presumably sustained not only by the mitotic, vertical route, but also by horizontal transmission by means of cell-to-cell contacts, even in the absence of appreciable levels of infectious viral particles in the serum. Thus, although the exact contribution of the infectious route to the pathogenesis of HTLV-1 infection is still unknown, we can assume that a possible aim of antiretroviral therapy in HTLV-1 infection is to keep intracellular viral load as low as possible by blocking cell-to-cell transmission. As a consequence, treatment with antiretrovirals could be hypothetically useful both in asymptomatic HTLV-1 positive individuals and, in combination with antiblastic- and immuno-therapy, in patients suffering overt, HTLV-1- related diseases. Currently, very little information exists on the efficacy of antiretroviral drugs against HTLV-1. Particularly, only AZT has effectively been shown to prevent the transmission of infectious virions to human PBMCs. Recently, by means of an experimental model consisting of single-cycle infection with recombinant vectors of a rhesus lung fibroblast cell line, the potential efficacy of tenofovir to prevent HTLV-1 infection has been suggested (Hill et al., 2003). Thus, it was worthwhile investigating the effect of tenofovir directly on HTLV-1 transmission to human PBMCs in vitro. In fact, a possible therapeutic application of antivirals in humans should greatly benefit from in vitro studies using the actual target cells of HTLV-1 in vivo and from an experimental model of cell-to-cell-mediated virus transmission which accurately reproduces the modalities of virus maintenance and spread in infected patients by the horizontal route. Moreover, PBMCs, i.e. the actual cellular target of HTLV-1, are suitable for assaying both the antiviral efficacy of the drug as well as investigating its toxicity. The present study shows that pre-treatment with tenofovir is able to efficiently control de novo HTLV-1 infection to human PBMCs from healthy donors, in vitro. Conversely, no effect was observed when addition of tenofovir occurred at the same time as viral infection. This is not unexpected, considering that it was demonstrated that the uptake of ANPs does not occur via the nucleoside transport system but through a slow, energy-dependent mechanism, compatible with an endocytic process, with increasing amounts of cell-associated drug up to 1 h post-incubation (Palù et al., 1991). Tenofovir exerted a remarkably lower toxic effect toward PBMCs, when compared to AZT. Particularly, we found that the inhibitory action of tenofovir on cell growth was undoubtedly lower than that of AZT, while its CC_{20} value clearly exceeded the highest concentration assayed. Moreover, based on the virological parameters assayed in our experiments involving cell-to-cell transmission, the anti-HTLV-1 antiviral activity of tenofovir was slightly higher than that of AZT. As a result of its lower toxicity and of its slightly higher antiviral activity in comparison with AZT, tenofovir showed a selectivity index about four times higher than that of AZT, indicating a more favourable ratio between the cytotoxic versus the antiviral activity. The novel HTLV-1 RT-inhibition assay, that we describe in the present study, cannot be considered as a true quantitative one. Moreover, in the present study we have not determined the actual concentrations in the reaction mixture of the phosphorylated, active forms of tenofovir and AZT, following pre-incubation with the crude extract. Thus, we cannot compare the efficiency of the two compounds in inhibiting HTLV-1 RT activity using this assay. Nevertheless, our results of the cell-free assay are in agreement with what we observed in cell-to-cell transmission assays, and unequivocally demonstrate that tenofovir exerts a strong, direct inhibitory activity towards cDNA elongation by HTLV-1 RT. Finally, the fact that the efficacy of tenofovir against HTLV-1 has been consistently suggested by such different experimental models, as the above-mentioned (Hill et al., 2003) and those utilized by us in the present study, highlights the potential usefulness of tenofovir in counteracting HTLV-1 infection.

Taken all together, our results show that tenofovir is able to control HTLV-1 replication cycle and this encourages further investigation to establish the real impact of this drug, as well as of other acyclic nucleoside phosphonates, in limiting viral load in HTLV-1-associated leukemia or inflammatory diseases.

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