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A new antiviral: Chimeric 3TC–AZT phosphonate efficiently inhibits HIV-1 in human tissues *ex vivo*

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ABSTRACT

Although more-recently developed antivirals target different molecules in the HIV-1 replication cycle, nucleoside reverse transcriptase inhibitors (NRTIs) remain central for HIV-1 therapy. Here, we test the anti-HIV activity of a phosphonate chimera of two well-known NRTIs, namely AZT and 3TC. We show that this newly synthesized compound suppressed HIV-1 infection in lymphoid tissue *ex vivo* more efficiently than did other phosphonates of NRTIs. Moreover, the new compound was not toxic for tissue cells, thus making the chimeric phosphonate strategy a valid approach for the development of anti HIV-1 compound heterodimers.

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1. Introduction

Despite intensive research to find new drugs to fight HIV-1/AIDS, nucleoside reverse transcriptase inhibitors (NRTIs) remain at the core of HIV-1 treatment and are an important component of HAART. Among eight NRTIs that have been used before now or are currently being used for treatment of HIV-infected patients, the most extensively studied is 3′-azido-3′-deoxythymidine (AZT, zidovudine, Retrovir®) (De Clercq, 2002). Although AZT has been widely used since the beginning of the antiretroviral era, this drug has significant side effects. In particular, zidovudine induces mitochondrial disorder with massive liver steatosis, myopathy, lactic acidosis, and mitochondrial DNA depletion (Chariot et al., 1999). Also, upon AZT monotherapy, resistant virions are quickly selected. In particular, five mutations in HIV reverse transcriptase (RT) contributing to the development of high-level resistance to zidovudine have been described (Kellam et al., 1992; Ren et al., 1998).

Phosphonate derivatives of AZT have shown a significant decrease in cellular toxicity and improvement of AZT therapeutic properties (Khandazhinskaya et al., 2010; Wainberg and Cameron, 1998). In particular, the introduction of an H-phosphonate group into the AZT 5′ position resulted into a new anti-HIV drug, Nikavir® (AZT 5′-H-phosphonate, **1**). The main advantages of Nikavir® over AZT include its lower toxicity, a longer half-life in organism, and much slower selection for virus-drug resistance (Wainberg and Cameron, 1998). We recently showed that AZT 5′-aminocarbonylphosphonate (**2**) shares similar pharmacokinetic parameters with AZT 5′-H-phosphonate (**1**), i.e., a slow release of the drug following oral administration, efficient penetration into cells, and decreased toxicity (Khandazhinskaya et al., 2009). Similar studies have been done on 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine, Epivir®), a cytidine analog originally developed against HBV that was approved for HIV-1 treatment by the FDA in 1995. Like AZT phosphonate derivatives, 3TC 5′-H-phosphonate (**3**) and 3TC 5′-aminocarbonylphosphonate (**4**) were found to be much less toxic than the parent 3TC in cell cultures. Also, in laboratory animals, prodrug transformation into the active nucleoside 3TC was slower (Khandazhinskaya et al., 2011), thus making phosphonate derivatives of NRTIs promising candidates for extended-release forms of the parent NRTI.

Here, using a similar strategy, we report on the development and the antiviral activity in *ex vivo* human tissues of a phosphonate heterodimer of 3TC and AZT, (3TC–AZT heterodimer), O-(L-2′,3′–dideoxy-3′-thiacytidine-5′-yl)-O′-(3′-azido-3′deoxythymidine-5′-yl) aminocarbonyl phosphonate (**5**). At this initial stage of development of this new antiviral, two major questions that need to be addressed are whether this new compound significantly inhibits

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HIV-1, and if the answer to this question is "yes" is this inhibition due to potential cell toxicity? We answer these two questions below.

The heterodimer strategy has been successfully used before: For example, Imbach and his associates synthesized the first bis-nucleozide phosphates in 1990 (Puech et al., 1990). However, that heterodimer was relatively resistant to enzymatic hydrolysis by phosphodiesterases and thus too stable to release the active compounds. Other described compounds (Laduree et al., 2003; Pontikis et al., 2000; Velazquez et al., 1995) were designed not as depot forms but rather as non-hydrolysable molecules that supposedly have double activity. In contrast, our strategy was intended to design a molecule that is quickly hydrolyzed into active fragments, two of which (AZT and 3TC) might act synergistically.

Since *in vivo* the critical pathogenic events of HIV infection occur in tissues which are not faithfully reflected by single-cell cultures, we utilized a system of human lymphoid tissue *ex vivo* developed in our laboratory. Human tissues *ex vivo* retain tissue cytoarchitecture, support HIV-1 replication, and have been shown to complement pre-clinical drug testing against different pathogens (Grivel and Margolis, 2009; Rohan et al., 2010; Vanpouille et al., 2012).

We found that the 3TC–AZT heterodimer (**5**) significantly suppressed HIV-1 replication at a level that surpasses some of the clinically used antivirals and exhibited low toxicity towards MT-4 cell cultures and various tissue lymphocytes.

2. Materials and methods

For details of the synthesis of (**5**) and the evaluation of its stability see the Supplement data file.

2.1. Human tissue culture ex vivo

Tonsillar tissues from routine surgery were obtained from the Children's National Medical Center (Washington, DC). Tissues were obtained according to an IRB-approved protocol. Tissues were dissected into 2-mm^3 blocks and cultured as described earlier (Grivel and Margolis, 2009). Human tissue blocks were inoculated with HIV-1 $X4_{LAI.04}$ as described elsewhere (Grivel and Margolis, 2009). Human tonsillar tissues (27 blocks of tissue from each of *n* donors for each experimental condition) were treated with phosphonate derivatives overnight and then infected with a prototypical X4 variant of HIV-1 (HIV-1 $X4_{LAI.04}$) (Rush University Virology Quality Assurance Laboratory, Chicago, IL).

2.2. MT-4 cell cultures

The MT-4 T-cell line was obtained from ATCC and maintained in RPMI with 10% FCS. MT-4 cells (10^5 cells) were inoculated with 10 μL of viral stock of $X4_{LAI.04}$ containing 0.7-ng/mL p24gag antigen. Cells and HIV-1 were incubated in a vial for 1h 30 min at 37 °C. Infected cell suspensions were then transferred to 12-well plates, mixed with 1 mL of medium containing the test compound at an appropriate dilution, and further incubated at 37 °C and cultured for 3 days. MT-4 was obtained through the NIH/AIDS reagent Program, Division of AIDS, NIAID, NIH: MT-4 from Dr. Douglas Richman.

2.3. Antiviral assays

We evaluated the antiviral activity of each compound by measuring inhibition of human HIV-1 replication in MT-4 cell cultures and in human lymphoid tissues. For each compound, in MT-4 cell culture or in lymphoid tissue *ex vivo*, HIV-1 inhibition, at each single concentration, was defined by the following formula: inhibition = $(1-R_{compounds}/R_{Ctl})^*100$, where $R_{compounds}$ and R_{Ctl} are the amounts of p24 accumulated in the medium in compound-treated cultures and in untreated cultures, respectively. We calculated the EC_{50} values (with 95% confidence interval (CI)) by fitting the data points to a sigmoidal dose–response curve, using Prism software, (version 4.0; GraphPad). The EC_{50} (50% effective concentration) is defined as the compound concentration required for inhibition of viral replication by 50%.

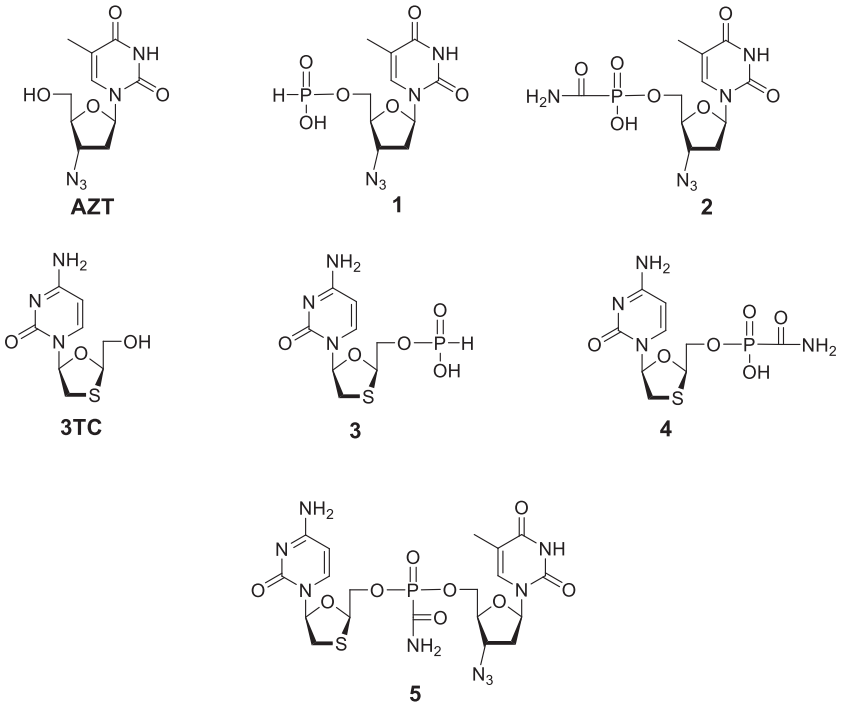

Fig. 1. Structural formulae of AZT, 3TC, and their phosphonate derivatives. Shown are structural formulae of AZT and its H-phosphonate (Nikavir®) (**1**) and 5′-aminocarbonyl phosphonate derivative (**2**), and structural formulae of 3TC and its H-phosphonate (**3**) and 5′-aminocarbonyl phosphonate derivative (**4**). Compound (**5**) is an O-(L-2′,3′-dydeoxy-3′-thiacytidine-5′-yl)-O′-(3′-azido-3′deoxythymidine-5′-yl)aminocarbonyl phosphonate, or 3TC–AZT heterodimer (**5**).

2.4. Flow cytometry

To assess the cytotoxicity of (**1**), (**2**), and (**5**) in human tonsillar tissues after 12 days of culture, cells isolated from untreated tissue blocks and from those treated with compounds were stained with combinations of the following fluorescence-labeled monoclonal antibodies: anti-CD3-QD605, anti-CD4-QD655, anti-CD8-QD705, anti-CD25-APC, anti-CD38-PE, anti-HLA-DR-APC-Cy7, anti-CXCR4-Brilliant violet 421, anti-CCR5-PR-Cy5 anti-CD45RA-FITC, and anti-CCR7-PE-Cy7 (Caltag Laboratories; Biolegend). Detection and enumeration of HIV-1–infected cells were performed with intracellular staining by means of anti-p24-PE (Beckman Coulter). Data were acquired and analyzed as described elsewhere (Grivel and Margolis, 2009). We quantified cell depletion using Trucount beads (Becton Dickinson) for volumetric control and normalized cell numbers by tissue-block weights.

3. Results

3.1. 3TC–AZT heterodimer synthesis

First, we compared two potential synthetic schemes for the 3TC–AZT heterodimer (**5**) by assessing the yields of the final product and the separation conditions of the reaction mixtures. The alternative was the coupling of (**2**) with 3TC or (**4**) with AZT (see structures of the compounds in Fig. 1). The TLC on silica gel 60 F_{254} plates permitted the conclusion that the yields of the heterodimer (**5**) under each of these couplings were close to one another and achieved 40–50%. However, in the reaction of (**2**) with 3TC the isolation and purification of (**5**) was complicated because of the remaining 3TC, which was taken in excess and whose mobility was similar to that of the target product. In contrast, after the coupling of (**4**) with AZT, AZT was easily removed because of a large difference in the chromatographic mobility. Therefore, the following synthesis of the heterodimer phosphonate was proposed (Fig. 2): We prepared ethoxycarbonylphosphonic acid by treating ethyl diethoxyphosphonoformate with trimethylbromosilane at room temperature. The interaction of the resulting acid with 3TC in the presence of dicyclohexylcarbodiimide in pyridine led to 3TC 5′-ethoxycarbonyl phosphonate. The latter compound was then treated with 25% aqueous ammonia at room temperature for 18 h to result in the formation of (**4**). We obtained 3TC–AZT heterodimer (**5**) in a yield of 45% from the reaction of (**4**) with AZT in the presence of triisopropylbenzenesulfonyl chloride in pyridine for 72 h. We confirmed its structure using ^{1}H, ^{13}C and ^{31}P-NMR spectroscopy and HRMS.

The stock solution of (**5**) was prepared in DMSO, in which the compound is stable with a half-life greater than 72 h (Fig. 3A).

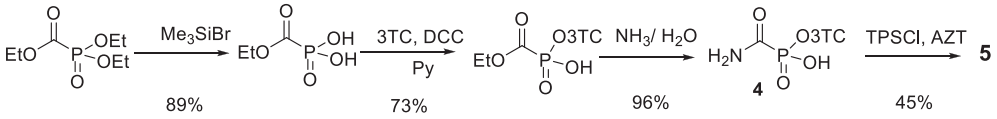

Fig. 2. 3TC–AZT heterodimer (**5**) synthesis. Presented are the synthesis of compound **5** and the yields of each chemical reaction.

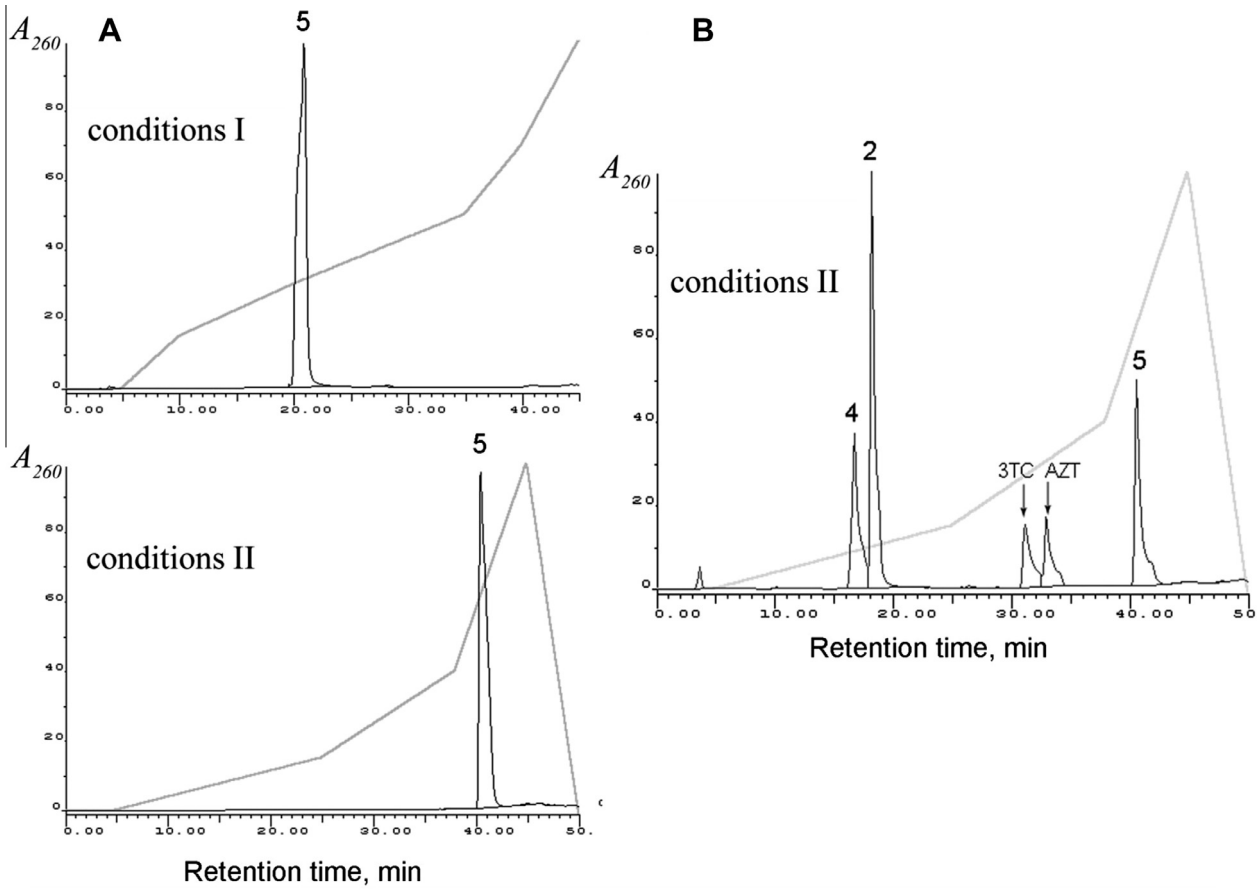

Fig. 3. The assessment of chemical stability of 3TC–AZT heterodimer (**5**) was tested at different pH levels and time points. (A) Elution profiles of heterodimer (**5**) (solution in DMSO) under different HPLC conditions (I, reverse phase mode) and (II, pseudo-ion-pair mode). (B) Elution profile after incubation at pH 5.15 for 24 h under conditions (II, pseudo-ion-pair mode) is shown in panel B. For details, see Section 2.

The half-life of the heterodimer at pH 2.2 exceeded 24 h; at pH 5.15 it was about 12 h; at pH 6.6, 1 h; and at pH 8.6, less than 5 min. In all cases the hydrolysis products were the active compounds AZT, 3TC, (**2**), and (**4**) at a ratio of 2:2:3:3 (Fig. 3B). Thus, the heterodimer under physiological conditions is quickly hydrolyzed into active RT inhibitors.

3.2. Phosphonate derivatives inhibit HIV-1 replication

We compared the anti-HIV activity of AZT 5′-H-phosphonate (**1**), AZT 5′-aminocarbonylphosphonate (**2**), and 3TC–AZT heterodimer (**5**). (**1**) (Nikavir®), (**2**), and (**5**) efficiently inhibited HIV-1 $X4_{Lai.04}$ in human lymphoid tissues *ex vivo* (Fig. 4). The absolute HIV replication level in tissues from different donors varied from 8 to 70 ng/mL with a median value of 16 ng/mL. The suppression of HIV-1 replication by (**5**), measured as p24gag accumulation in culture medium, was dose dependent (Fig. 4) with an EC_{50} in the low nanomolar range (Table 1). In the same system the EC_{50} of AZT and 3TC *ex vivo* were respectively 3 nM (95% CI: 1–16; $n = 2–13$ for each tested concentration) and 17 nM (95% CI: 3–102; $n = 2–13$ for each tested concentration) (data not shown) (see Fig. 5).

The ability of the heterodimer to suppress HIV-1 replication was confirmed in single-cell culture (MT-4 cells) in which we compared the antiviral activities of the newly developed heterodimer (**5**) with those of Nikavir® (**1**) and of (**2**) (Table 1). All compounds efficiently and dose-dependently inhibited HIV-1 replication in MT-4 cells, although the EC_{50} were different from those found in tissues (Table 1). Nevertheless, in this culture system made of iden-

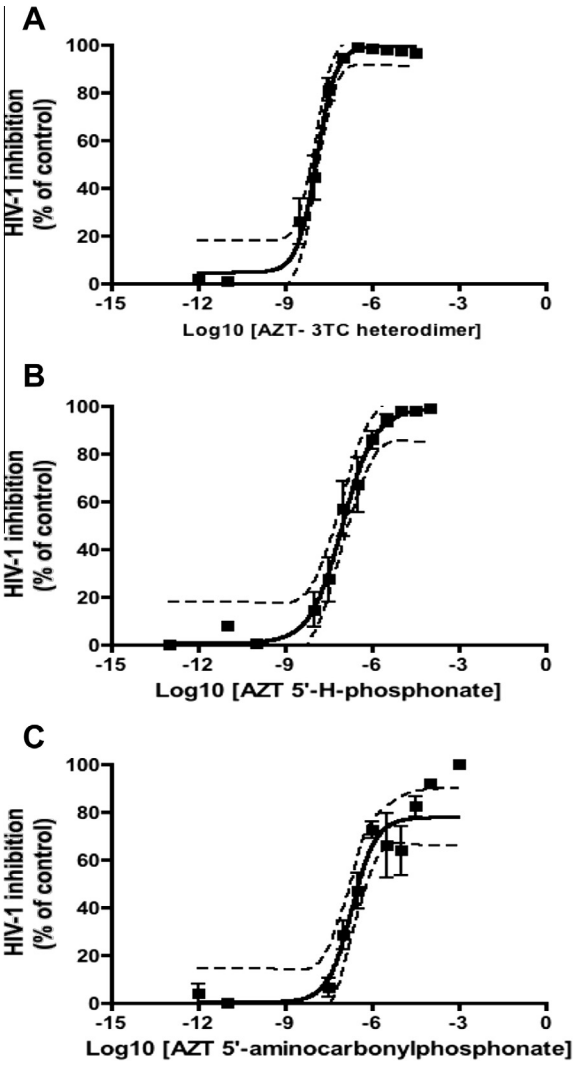

Fig. 4. Antiviral activity of NRTI phosphonate derivatives in human lymphoid tissues. Blocks of human tonsillar tissue were treated with increasing concentrations of AZT H-phosphonate (**1**) (A), AZT 5′-aminocarbonylphosphonate (**2**) (B), and heterodimer (**5**) (C) and then inoculated *ex vivo* with $X4_{LAI.04}$. The medium was changed and the drugs were replenished every 3 days. We monitored HIV-1 replication by measuring $p24_{gag}$ accumulated in culture media over 3-day periods. For each donor, each datum represents pooled viral release from 27 tissue blocks. Drugs were added at concentrations ranging from 10 pM to 3 μM, and their anti-HIV activity was evaluated from the suppression of viral replication compared with donor-matched HIV-infected tissues not treated with phosphonate derivatives. We estimated the 50% effective concentration (EC_{50}) and 95% confidence interval by fitting the data to four-parameter logistic regression. EC_{50} were estimated from independent experiments performed with tonsillar tissues from seven different donors.

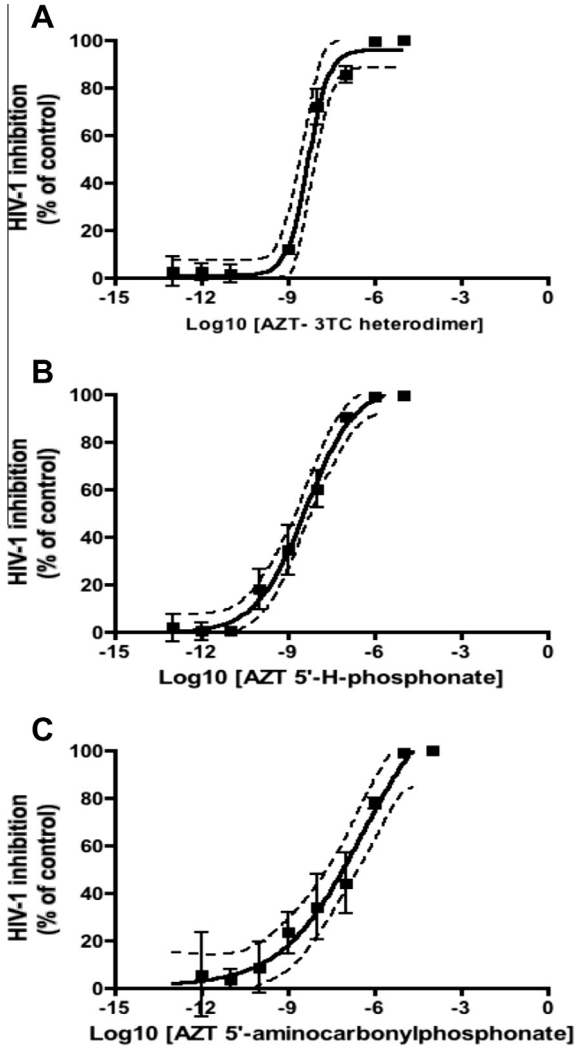

Fig. 5. Antiviral activity of NRTI phosphonate derivatives in MT-4 cell cultures. MT-4 cells (10^5 cells) were inoculated with 10 μL of viral stock of $X4_{LAI.04}$ containing 0.7-ng/mL p24gag antigen. Infected cell suspensions were then transferred to 12-well plates, mixed with 1 mL of medium containing the test compound at an appropriate dilution, further incubated at 37 °C, and cultured for 3 days. Cells were treated with increasing concentrations of AZT H-phosphonate (**1**) (A), AZT 5′-aminocarbonylphosphonate (**2**) (B), or heterodimer (**5**) (C). Drugs were added at concentrations ranging from 0.1 pM to 10 μM, and their anti-HIV activities were evaluated from the suppression of viral replication compared to untreated MT-4 cell cultures. Depending on the concentration and the compound, HIV-1 suppression was calculated from 2 to 9 independent experiments. We estimated the EC_{50} and 95% confidence interval by fitting the data to four-parameter logistic regression.

Table 1
Antiviral activity in MT-4 cell cultures and in human lymphoid tissue *ex vivo* infected with HIV-1 $X4_{LAI.04}$. MT-4 cells (10^5 cells per condition) or human tonsillar tissue cultured *ex vivo* (27 blocks per condition) were treated with increasing concentrations of AZT H-phosphonate (**1**) (A), AZT 5′-aminocarbonylphosphonate (**2**) (B), heterodimer (**5**) (C), and then inoculated *ex vivo* with $X4_{LAI.04}$. In MT-4 or human tissues, the anti-HIV activity was evaluated from the suppression of viral replication compared to untreated control cultures. In MT-4, depending on the compound concentration, HIV-1 inhibition was calculated from 2 to 9 independents experiments. In human lymphoid tissues, HIV-1 inhibition was calculated from 7 independents experiments. We estimated the 50% effective concentration (EC_{50}) and 95% confidence interval by fitting the data to four-parameter logistic regression.

Compound	MT-4 cell cultures		Human tissue *ex vivo*	
	EC_{50} (nM)	95% confidence interval (nM)	EC_{50} (nM)	95% confidence interval (nM)
Heterodimer (**5**)	3	1–11	11	8–17
AZT 5′-H-phosphonate (**1**) (Nikavir®)	9	3–26	91	39–212
AZT 5′-aminocarbonyl phosphonate (**2**)	339	4–28,000	191	85–431

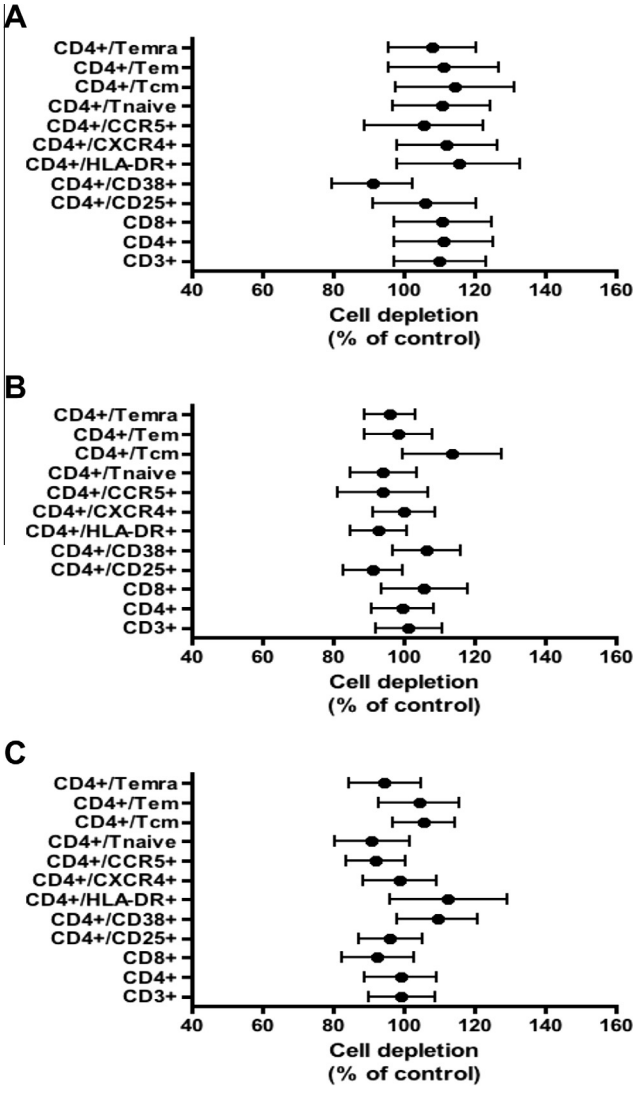

Fig. 6. Cell depletion of phosphonate derivative-inoculated *ex vivo*-cultured human lymphoid tissues. Tissue blocks (27 from each individual human tonsil donor) were treated with 10 μM heterodimer (**5**). At day 12, cells were stained for anti-CD3-QD605, anti-CD4-QD655, anti-CD8-QD705, anti-CD25-APC, anti-CD38-PE, anti-HLA-DR-APC-Cy7, anti-CXCR4-Brilliant violet 421, anti-CCR5-PR-Cy5, anti-CD45RA-FITC, and anti-CCR7-PE-Cy7. We evaluated the cytopathicity of the heterodimer for different cell subsets by comparing the number of cells in infected tissues with that in matched uninfected tissues. To account for size differences in tissue blocks, we normalized the data by the weight of the tissues. Presented are means ± SEM (error bars) of 10 independent experiments performed with tissues from 10 different donors.

tical cells, (**5**) also proved to be the most active of all the phosphonate compounds (Table 1).

3.3. Phosphonate derivatives are not toxic to tonsillar tissues

We used human tissues *ex vivo* that contain various cells to evaluate the effects of the phosphonate compounds on the viability of various lymphocyte subsets. After 12 days in culture, we isolated cells from tissue blocks treated with 10-μM heterodimer (**5**) (a concentration that completely suppresses HIV replication in this system) and from donor-matched untreated tissue and stained them for CD3, CD4, CD8, HIV-1 coreceptors (CXCR4, CCR5), activation markers (CD25, CD38, HLADR), and memory naïve markers (CD45RA, and CCR7). We evaluated cellular depletion in all three main subsets of memory CD4+ T cells: central (TCM; CD45RA−CCR7+), effector (TEM; CD45RA−CCR7−), and terminally differentiated effector (TEMRA; CD45RA+CCR7−), as well as in naïve (CD45RA + CCR7+) CD4+ T cells and activated cells. We evaluated the cytopathicity of the three compounds by comparing the numbers of cells in infected tissues with those in matched uninfected tissues. To account for size differences in tissue blocks, we normalized the data by the weight of the tissue (Fig. 6).

We compared the numbers of various subsets of lymphocytes. On day 12, the numbers of lymphocytes in corresponding subsets of CD3+, CD3+CD8+, CD3+CD4+, CD3+CD4+CXCR4+, CD3+CD4+CCR5+, as well as in activated (CD25+, CD38+ or HLA−DR+), Tnaïve, T_{CM}, T_{EM}, T_{EMRA}, and T cells were statistically not different ($p > 0.05$) in untreated tissues and in tissues treated with (**1**), (**2**), and (**5**) (Fig. 6). Thus, none of the compounds seems to deplete tissues of T lymphocytes of any of the subsets tested. The low toxicity of the compounds for tissue cells was confirmed in MT-4 cell cultures, since the compound concentrations that kill 50% of MT-4 cells (CC_{50}) were 500 μM, 180 μM, and 2.7 mM respectively for compounds (**5**), (**1**), and (**2**) (data not shown).

4. Discussion

Although more than 30 drugs, including entry inhibitors as well as inhibitors of crucial viral enzymes, have been approved for the treatment of HIV/AIDS, nucleoside analogs targeting HIV-1 RT remain important components in drug development (De Clercq, 2009). Inside the cells, NRTIs must be phosphorylated into NRTI triphosphates. As the efficacy of the triphosphorylation is low, drug doses must therefore be high, generally leading to significant toxicity and clearance of the compound. An alternative is to design the corresponding depot form, i.e., a prodrug-like derivative capable of delivering the active compound in the organism at a controlled rate (Stanczak and Ferra, 2006). Many depot forms of anti-HIV drugs have been developed (Beaumont et al., 2003; Calogeropoulou et al., 2003). For example, the two depot forms of AZT that we developed, despite being inferior to AZT in their antiviral efficacy in cell cultures, have the advantage of a slow release of AZT in a variety of laboratory animals, thus exhibiting low toxicity (Khandazhinskaya et al., 2010). A similar phenomenon has recently been described for two phosphonate forms of 3TC, 3TC

5′-H-phosphonate (**3**) and 3TC 5′-aminocarbonylphosphonate (**4**) (Khandazhinskaya et al., 2011).

Here, we describe a new phosphonate derivative, namely 3TC–AZT heterodimer: (O-L-2′,3′-dydeoxy-3′-thiacytidine-5′-yl)-O′-(3′-azido-3′deoxythymidine-5′-yl)aminocarbonylphosphonate, or 3TC–AZT heterodimer (**5**). This compound is a chimera of AZT-5′- and 3TC-5′-aminocarbonylphosphonate. In earlier studies, both of the latter compounds showed anti-HIV-1 activity *in vitro* and lower toxicity in experimental animals than did their respective parental forms (Khandazhinskaya et al., 2010, 2009). We developed the aminocarbonylphosphonate derivative and not the *H*-phosphonate derivative, as bis-substituted aminocarbonylphosphonates have been shown to be more stable than *H*-phosphonates (Khandazhinskaya et al., 2000). In this work, we compared the antiviral activity of the newly developed chimera (**5**) with that of Nikavir®, another AZT phosphate derivative (**1**).

In contrast to the testing of the majority of the newly developed antivirals, we tested the anti-HIV activity of our heterodimer in human lymphoid tissue *ex vivo*, an experimental system that faithfully reflects important aspects of lymphoid tissue *in vivo,* where critical events of HIV pathogenesis occur in HIV-infected individuals. Human lymphoid tissue supports productive HIV infection without exogenous activation and stimulation, and it retains tissue cytoarchitecture as well as the pattern of expression of key cell surface molecules relevant to HIV infection (Grivel and Margolis, 2009). Also, such a system reflects *in vivo* donor-to-donor variability and allows testing of various drugs as a preliminary step before engaging in costly and lengthy clinical trials. Importantly, *ex vivo* tissue cultures may reveal anti-HIV-1 activities of compounds, such as acyclovir, that were not revealed through use of conventional cell line cultures (Lisco et al., 2008).

We found that 3TC–AZT heterodimer (**5**) efficiently suppressed HIV-1 replication in tonsil histocultures with an EC_{50} in the range of clinically approved drugs. For example, we found that it has an EC_{50} lower than that of 3TC or Nikavir®, but higher than that of AZT. We also evaluated this activity for some of these compounds in single-cell cultures of the MT-4 cell line, where HIV-1 replication is typically much greater than in histocultures. In MT-4 cell cultures, the 3TC–AZT heterodimer (**5**) was also superior in suppressing HIV-1 compared with other tested phosphonate derivatives. However, the EC_{50}s of all the tested phosphonate compounds were closer to each other in MT-4 than in human tissues *ex vivo*, probably because all the cells in MT-4 cell cultures are equally accessible to the compounds and they do not reflect the diversity of cells present in tissues. In human tissues *ex vivo*, the EC_{50} of (**5**) was in the range of two FDA-approved NRTIs, namely AZT and 3TC (in the nM range). However, the depot form technology allows slow and continuous release of 3TC and AZT as active compounds, thus allowing slower general clearance of the two NRTIs. In tissues *ex vivo* and in MT-4 cell cultures, (**5**) was more efficient in inhibiting HIV-1 than was Nikavir®, a depot form of AZT.

It is very important for any compound that suppresses viral replication to test its toxicity, since what appears to be an antiviral effect can be the result of killing viral cell targets. To test whether it may be the case for the heterodimer, we enumerated cells in tissues treated with 3TC–AZT heterodimer (**5**) and in donor matched untreated control. There was no significant cell loss in total lymphocytes or in CD4+ or CD8+ lymphocyte subsets. However, we further investigated whether the compound might be toxic for some specific subpopulations of T cells essential for HIV-1 infection, in particular for memory and/or activated CD4+ T cells, which preferentially support HIV replication (Douek et al., 2002). We focused on the possible toxicity of the heterodimer for naïve, TCM, TEM, and TEMRA CD4+ T cells and for CD4+ T cells expressing any one of the three following activation markers: CD25, CD38, and HLA-DR. The compound did not trigger significant loss of any of the studied subsets in human tissue *ex vivo*.

The 3TC–AZT heterodimer (**5**) tissue metabolism is relatively complex: (**5**) is eventually broken down into AZT, AZT-5′ aminocarbonyl phosphonate (**2**), 3TC, and 3TC-5′ aminocarbonyl phosphonate (**4**). For the first hydrolysis, which releases two active compounds, to occur quickly the heterodimer should not be too stable in the cells. It seems that this hydrolysis is indeed quick and spontaneous and does not seem to be mediated by any enzymes. After an 18-h incubation, phosphonate derivatives (**2**) and (**4**) are in significant excess in blood plasma, as revealed by the HPLC analysis. These two released phosphonate derivatives are further slowly transformed into their unmodified NRTI form, i.e., AZT and 3TC, with a kinetic constant of reaction difficult to measure. The advantage of using the 3TC-AZT heterodimer is not that it is significantly more active than 3TC and AZT, but rather that the heterodimer will release 3TC and AZT immediately upon hydrolysis while the other two products of the heterodimer hydrolysis, namely the phosphonates of 3TC and AZT, will continue to be further hydrolyzed into 3TC and AZT, therefore smoothing the pharmacokinetic profile by prolonging their presence, thus creating a depot effect.

Detailed study of the heterodimer metabolism, together with evaluation of the relative presence of 3TC and AZT in *ex vivo* and *in vivo* systems at each time-point, is to be addressed in *in vivo* experiments that are beyond the scope of the current paper, in which we have demonstrated that the development of bis phosphonate derivatives is feasible and that such a bis phosphonate of 3TC and AZT inhibits HIV-1 in human lymphoid tissue *ex vivo* more efficiently than does Nikavir®. Its low toxicity and its complex metabolism, which is associated with a slow release of active compounds, make it a candidate for future development and demonstrate that the phosphonate strategy may be useful for the development of heterodimers of various anti-HIV-1 compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.06.019.

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