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Synthesis and anti-HIV activity of novel 2′,3′-dideoxy-3′-thiacytidine prodrugs

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ABSTRACT

We report here the synthesis of a novel series of 5′-*O*-carbonates of 3TC, using different aliphatic alcohols and *N,N*-carbonyldiimidazol. Its antiviral activity was determined in peripheral blood mononuclear cells (PBMCs) showing some carbonate derivatives with an activity similar to or better than 3TC, except 3TC-Metha and 3TC-2Pro with less activity. In vitro assays in PBMCs have demonstrated that cytotoxicity increases as the carbon chain length of the alcohol moiety increases, showing compounds with a normal chain length of $n = 2–5$ good selective index, compared to the parent drug. Thus, this work is an important contribution leading to the suppression of HIV replication.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a degenerative infectious disease of the immune system caused by the human immunodeficiency virus (HIV).[1,2] HIV, a retrovirus, specifically infects a type of white blood cell called CD4+ T lymphocytes or T helper cells, which plays a key role in directing the immune response against infections.[3–6]

The search for an effective chemotherapeutic treatment against HIV infection has led to the development of agents that target specific and critical events in the HIV replicative cycle. The best known and the most intensively studied active drugs against HIV are reverse transcriptase (RT) inhibitors, viral protease inhibitors, entry inhibitors and, more recently, integrase inhibitors.[7–12] In particular, the inhibition of viral RT, a key enzyme in the replicative cycle of the HIV, by nucleoside reverse transcriptase inhibitors (NRTIs) activated intracellularly by biotransformation to their active triphosphate form or to alternate substrate (chain termination), has been an important target in anti-HIV drug design.[8–10,13]

The prodrug design is one of the methods selected to improve the anti-HIV efficacy of a drug by enhancing its spectrum of chemotherapeutic properties for the effective treatment of HIV/AIDS, leading to a major distribution and retention of the parent compound in the body for a longer time.[7,8,13] Thus, prodrugs have been rationally designed to decrease the toxicity associated with nucleoside drugs, to release active species by their degradative metabolism and to allow larger amounts of drug to enter the cell.[13–17]

The bioavailability of a parent compound depends on the rate of hydrolysis of the prodrug in T-lymphocytes or macrophages, the primary target cells involved in HIV replication. A useful prodrug must also be optimized for a number of unrelated but structurally dependent properties, such as its lipophilicity, hydrolysis rate, systemic metabolism, retention and elimination.[13–17] A lipophilic prodrug has a stronger possibility of passing through the blood–brain barrier and entering the brain. For this last purpose many nucleoside analogues with interesting biological properties have been developed by substitution at the 5′-*O* position of the NRTI with lipophilic chemical moieties, through enzymatically hydrolysable functions, such as ester and carbonate bonds.[17–21]

Lamivudine (3TC, 2′,3-deoxy-3′-thiacytidine) has been shown to be somewhat less toxic than other NRTIs[22–25] and active against zidovudine-resistant HIV isolates.[13] Therefore, for the treatment of HIV infection, 3TC is currently recommended in combination with other NRTIs and with a protease inhibitor or a non-*nucleoside* reverse transcriptase inhibitor.[14,15] The structural characteristics of lamivudine exclude hydroxyl substituent at both its 2′- and 3′-positions like ddNs (2′,3′-dideoxynucleosides) analogues with two heteroatoms on the sugar moiety.[26,27]

Like AZT prodrugs, the lipophilic character of the side chains at the 5′-*O* position in the absence of an active nucleoside transport system should influence their ability to cross the cell membrane by passive diffusion.[28–30] The usefulness of the prodrug of 2′,3′-dideoxy-3′-thiacytidine should be determined not only by the stability of the prodrug for its transport across the cell membrane, but also by its reversion to the parent compound intracellular especially in the virally infected cells. The partition coefficient of 2′,3′-dideoxy-3′-thiacytidine prodrugs may have a significant effect

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on cellular transport. As stated by Parang et al.,[18] more selective compounds can be designed by using the strategy of 5′-*O*-carbonates substituted 3TC. However, although the clinical application of these approaches remains unknown, they hold the promise of becoming an important tool in the treatment of HIV infection and its related consequences.

Anastasi et al. have obtained two 5′-*O*-carbonates of 3TC, with a primary amine group in their terminal chain, showing a similar antiviral activity than 3TC, but one of them with a minor and the other with a major toxicity than 3TC.[16]

Therefore, as part of our ongoing efforts to search for novel antiviral agents,[28–31] we have found it interesting to design, synthesize and evaluate carbonate prodrugs of lamivudine with the objective of generating 3TC derivatives able to suppress HIV-replication more efficiently than its parent drug.

Thus, the rationale of this study was to obtain compounds with enhanced lipophilicity to facilitate the transport across cell membrane independently of the nucleoside transport system. Hence, we used a 5′-*O*-carbonate substitution strategy by linking different aliphatic alcohols on the side chains 5′-*O* position of lamivudine in order to improve in vitro anti-HIV activity of 3TC, as well as to evaluate the anti-HIV activity of the novel prodrugs obtained.

2. Results and discussion

2.1. Chemistry

Highly selective chemistry, capable of discriminating between two different functional groups on the same molecule with similar reactivity, has applications in different synthetic strategies to form controlled structures without lengthy protection/deprotection steps. Hence, taking into account that lamivudine (3TC, 1) has two primary reactive functional groups such as 5′-OH and 4-NH_2, an imidazole carboxylic ester, *N,N*-carbonyldiimidazole (CDI), was used in the controlled and selective formation of carbonate derivatives of lamivudine. Thus, in this study, our synthesis strategy focused on the association of 5′-OH of the 3TC with different aliphatic alcohols,[21–23,30] achieved in a two-step reaction sequence (Fig. 1).

The precursor lamivudine intermediate called 3TC-5′-CI (2) was obtained in quantitative yields by refluxing 3TC with CDI for three hours in a dry mixture of dimethylformamide (DMF)-triethylamine (TEA). Subsequent *in situ* reaction of intermediate 3TC-5′-CI with the respective aliphatic alcohols, methanol, ethanol, n-propanol, 2-propanol, butanol, pentanol, hexanol and octanol gave the corresponding carbonates in 67–76% yields named 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-methyl (3TC-Metha, 3), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-ethyl (3TC-Etha, 4), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-propyl (3TC-nPro, 5), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-2-propyl (3TC-2Pro, 6), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-butyl (3TC-Buta, 7), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-pentyl (3TC-Penta, 8), 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-hexyl (3TC-Hexa, 9) and 2′,3′-dideoxy-3′-thiacytidin-5′-yl O-octyl (3TC-Octa, 10) carbonates, respectively (Fig. 2). In all cases, unreacted 3TC and CDI were not detected from the reaction media.

The reaction is thought to proceed via an intermediate imidazole anhydride which decomposes after either intra or internucleophilic attack by an imidazole group of CDI, as proposed by Rannard et al.[32,33] It is important to point out that the advantages of this method include mild reaction conditions, relatively high yields, lack of formation of 4-NH_2 derivatives and removal of lengthy purification stages. The purity of the synthesized compounds was checked by TLC and by HPLC, and the compounds of this study were identified by spectral data.

The structures of nucleoside derivatives **3**–**10** were characterized by spectroscopic data. The ^{1}H NMR, ^{13}C NMR, DEPT 135 and COSY homo and heteronuclear spectra of **3**–**10** were performed in DMSO-d_6. ^{1}H and ^{13}C NMR, data given in Section 4, are in full agreement with the structures **3**–**10** (Fig. 1) proposed. Signals of ^{1}H and ^{13}C resonances observed were characteristic of each of the moieties constituting structures of C-5′ substituted pyrimidine nucleoside analogues (**3**–**10**).

In the ^{1}H NMR spectra, the signals of the protons of the prepared prodrugs were identified on the basis of their chemical shifts, multiplicities and coupling constants. The spectra showed

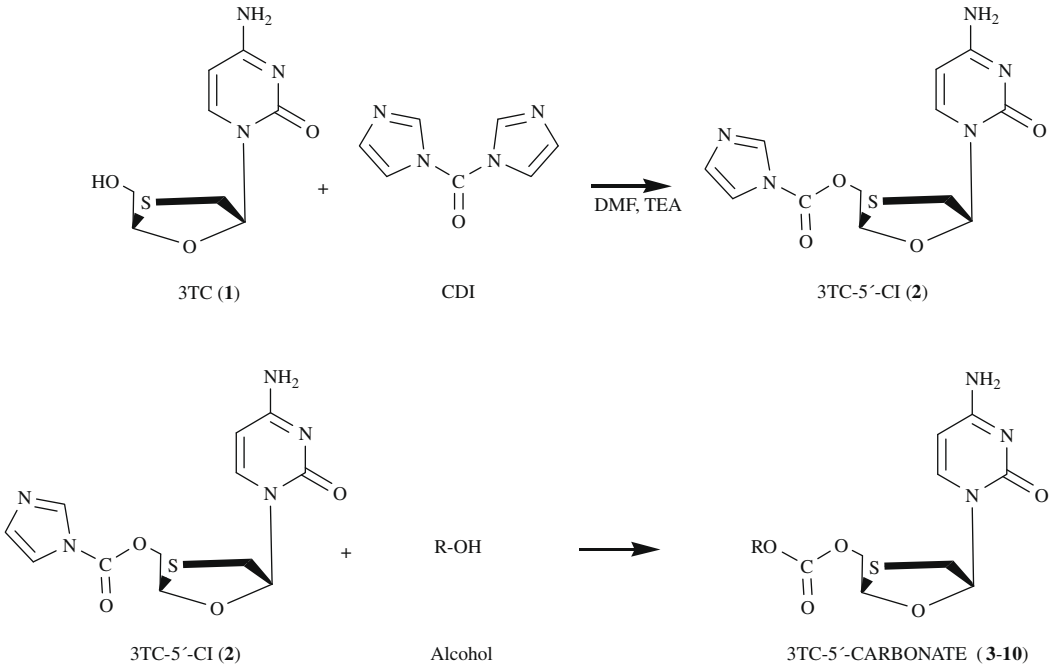

Figure 1. Protocol for the new synthetic compounds.

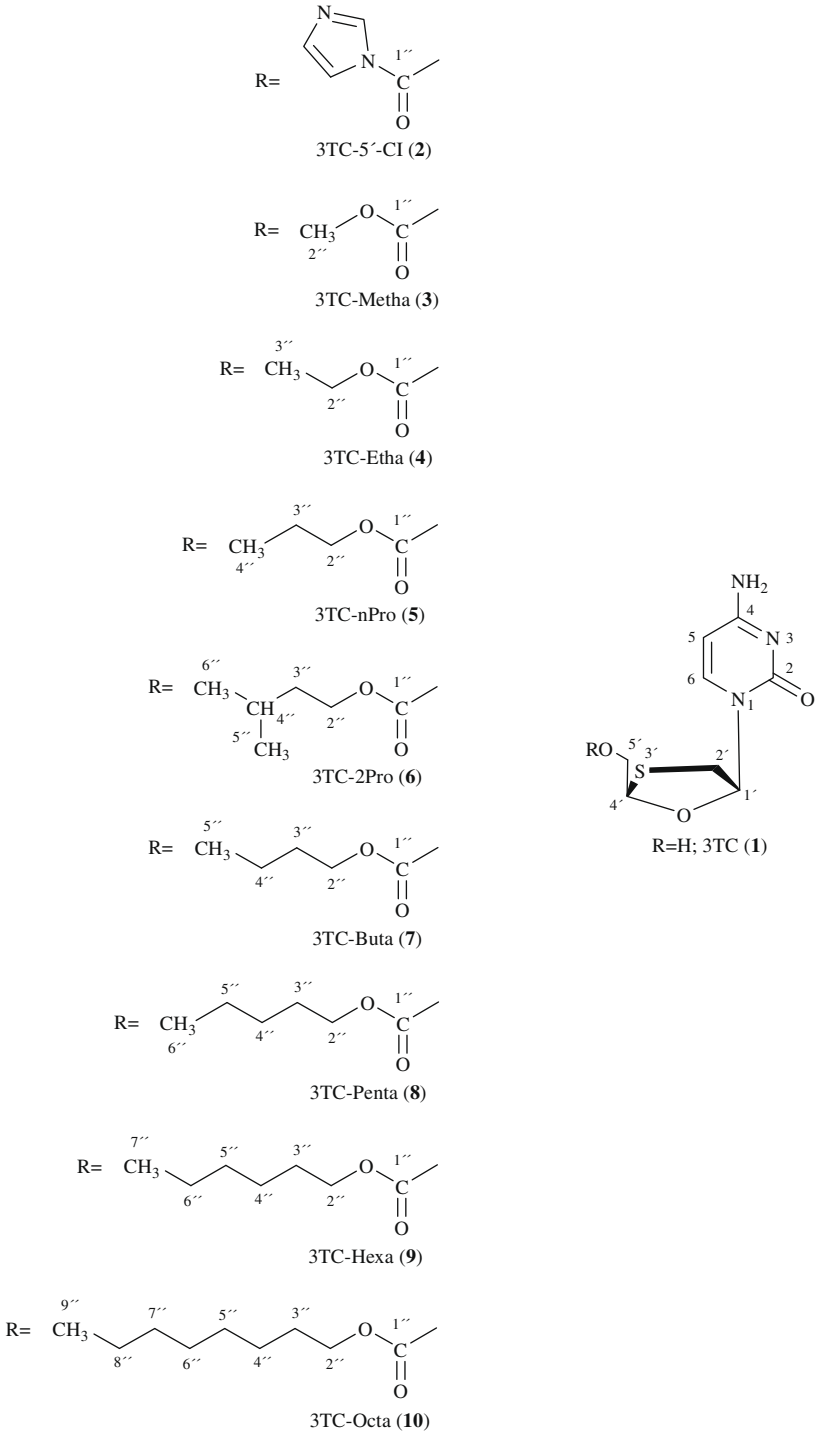

Figure 2. Chemical structures of lamivudine and their derivatives (**1**–**10**).

a D_2O exchangeable broad singlet at a $\delta \cong 7.25$ ppm corresponding to $-NH_2$-group, which remained unalterable in all carbonate derivatives, while 5′-hydroxyl group signals were not present in these compounds.

The proton signals of NH_2-base, H-5, H-6, H-1′ and H-2′ correlated well with those of 3TC, while H-4′ ($\delta \cong 5.17$ ppm) and H-5′ ($\delta \cong 3.77$ ppm) showed certain chemical shift differences of about $\delta \cong 0.28$ ppm and $\delta \cong 0.42$ ppm, respectively, with the parent compound. The assignment of all exchangeable protons was confirmed by the addition of D_2O, which indicated that the NH_2-base signals ($\delta \cong 7.25$ ppm) remained unchanged. Proton signals were successfully assigned using COSY homo (H–H) and heteronuclear (C–H) spectra.

The most significant features in the ^{13}C NMR spectra of **3–10** were the signals at $\delta \cong 153$–154 corresponding to the CO carbons from CDI. Other ^{13}C NMR signals are also in concordance with those of 3TC and the corresponding alcohols.[30]

In the IR spectra of **3–10**, characteristic vibrational regions of the structural features were found.[30] The intensive absorption at 1690 cm^{-1} and 1744 cm^{-1} indicated the presence of carbonyl groups and the particular stretching of the carbonate group respectively, while for all the compounds, those at 3370–

3410 cm^{-1} (NH_2) corresponded to the primary amine group at position 4.

2.2. Optical rotation measurements

2′,3-Deoxy-3′-thiacytidine has two chiral carbon atoms at 1′ and 4′ positions on oxathiolane ring (Fig. 1). Thus, a pair of diastereoisomers or four possible enantiomers can be present (α and β diastereoisomers have 1′ and 4′ substituents in *trans* and *cis* relationships, respectively).[24,25] Chu et al. described the anti-HIV activity of the α-D and β-D isomers of 2′,3′-dideoxy-3′-thiacytidine in human lymphocytes, suggesting that β-L-(−) isomer, known as 3TC, is the most potent one. Our results show that the optical rotation of 3TC has a $[\alpha]_D^{25} = -122.63$ (lit.$[\alpha]_D^{25} = -121.60$[24,25]) and those of all carbonate derivatives (see Section 4) respond to isomer β-L-(−)-2′*R*,5′*S* indicating that **3**–**10** as well as 3TC correspond to the (−) enantiomers.

2.3. Antiviral and cytotoxicity evaluation

Lamivudine prodrugs **3–10** were evaluated in vitro for anti-HIV activity in a primary culture of activated peripheral blood mononuclear cells (PBMCs) infected with HIV as previously described[31] and the activities were compared with those of 3TC as a standard drug. Assays on infected and uninfected PBMCs were performed in order to calculate the concentration of drug that inhibits 50% of viral replication (IC_{50}) and the concentration that inhibits 50% of normal cell growth ($CCID_{50}$), respectively. Selectivity indexes (SI = $CCID_{50}/IC_{50}$) were in turn calculated for each compound and compared to that of 3TC. Results are summarized in Table 1.

Data obtained in uninfected PBMCs indicate that 3TC-Metha, 3TC-Etha, 3TC-nPro, 3TC-2Pro and 3TC-Buta have similar $CCID_{50}$ (range 3000–4500 μM) than 3TC ($CCID_{50}$ = 4000 μM). However, 3TC-Metha and 3TC-2Pro have significantly higher IC_{50} than that of 3TC, resulting in poor SI. Compounds 3TC-Etha and 3TC-Buta were found to be equipotent to lamivudine concerning their IC_{50} giving as a result relevant SI (15,000 and 22,500, respectively). Similarly, compound 2′,3′-dideoxy-3′-thiacytidin-5′-yl *O*-pentyl carbonate (**8**, 3TC-Penta) has SI equivalent to that of 3TC. It should be noted that this compound showed a potent IC_{50} (0.065 μM, 3 times lower than parent drug) but also a higher toxicity ($CCID_{50}$ = 1286 μM). Thus, in vitro assays in PBMCs have demonstrated that cytotoxicity increases as the carbon chain length of the alcohol moiety increases, showing compounds with a normal chain length of n = 2–5 good selective index compared to the parent drug, [coma] as observed for the toxicity of the aliphatic alcohols.[34]

Table 1
Anti-HIV activity, cytotoxicity and selectivity indexes of the 3TC derivatives studied in PBMC

Compound	IC_{50} (μM)[a]	$CCID_{50}$ (μM)[a]	SI[b]	Log P[c]
3TC (1)	0.200 ± 0.060	4000 ± 707	20,000 ± 4535	−0.910
3TC-Metha (3)	0.967 ± 0.001	3667 ± 1414	3792 ± 1697	−0.810
3TC-Etha (4)	0.200 ± 0.060	3000 ± 707	15,000 ± 4535	−0.310
3TC-nPro (5)	0.300 ± 0.070	3333 ± 577	11,110 ± 4551	0.200
3TC-2Pro (6)	1.270 ± 0.060	3333 ± 707	2624 ± 2018	−0.150
3TC-Buta (7)	0.200 ± 0.001	4500 ± 707	22,500 ± 4660	0.860
3TC-Penta (8)	0.065 ± 0.006	1286 ± 707	19,784 ± 4535	1.230
3TC-Hexa (9)	0.250 ± 0.075	857 ± 151	3428 ± 1002	1.570
3TC-Octa (10)	0.200 ± 0.001	450 ± 71	2250 ± 467	1.720

PBMC = peripheral blood monocellular cell.
IC_{50} = concentration of drug that inhibits 50% of viral production.
$CCID_{50}$ = concentration of drug that inhibits 50% of cell growth.
SI = Selectivity Index, defined as $CCID_{50}/IC_{50}$.
[a] Results shown as mean ± S.D.
[b] Error calculated according to error propagation law.
[c] Ref. 35.

By contrast, 3TC-Hexa and 3TC-Octa have 10-fold lower SI than that of 3TC. Although 3TC-Hexa and 3TC-Octa have the same IC_{50}, it resulted far more toxic to the cell culture than that of 3TC (i.e., showing lower $CCID_{50}$ and thus lower SI). 3TC-Metha and 3TC-2Pro have similar $CCID_{50}$ than that of 3TC; however, it had a diminished capacity to inhibit viral replication (i.e., higher IC_{50}).

The increase observed in anti-HIV IC_{50} of 3TC-Penta compared with the parent drug may be related to their improved permeability through the cell membrane, allowing higher concentrations of the prodrug accumulated in HIV target cells. Probably, lipophilicity is not the only factor that influences the antiviral activity of these prodrugs, although, as expected, log P values of **3**–**10** (log P values between −0.810 for 3TC-Metha, 3 and 1.720 for 3TC-Octa, 10) were greater than that of the parent drug 1, 3TC (log P = −0.910), rendering them as more capable of penetrating various biomembranes,[34] and consequently improving their permeation properties through viral cell membranes. They may also be expected to cross the blood–brain barrier effectively since the brain is the sanctuary of HIV.

3. Conclusion

We have described the synthesis of eight 2′,3′-dideoxy-3′-thiacytidine prodrugs, their in vitro anti-HIV activity in PBMCs and their corresponding cytotoxicity. These studies have shown the usefulness of the carbonate linkage in creating prodrugs from 3TC, indicating how physical-chemical properties of lamivudine have been modified substantially to produce different compounds with lipid solubility and good antiviral activity. Among the compounds studied, 3TC-Buta, 3TC-Penta and 3TC-Hexa appear to be promising as candidates for further evaluations.

4. Experimental

4.1. Chemistry

All chemicals, reagents and solvents were of analytical grade. The nucleoside 2′,3′-dideoxy-3′-thiacytidine (Lamivudine, 3TC, **1**) was a generous gift from Filaxis Laboratory (Buenos Aires, Argentina), and was used without purification. *N,N*-carbonyldiimidazole (purest >97%, Sigma) was used without purification. Dimethylformamide (DMF) was dried over 4 Å molecular sieves. All solid reagents were dried for several hours under high vacuum. Thin layer chromatography (TLC) was performed on Merck Sil G/UV_{254} silica gel plates with fluorescent indicator, and the spots were visualized under 254 nm illumination. All glassware was oven-dried at 130 °C overnight, and cooled in a desiccator over anhydrous $CaSO_4$. Column chromatography was performed on Silica Gel 60 (0.063–0.200 mm, Merck) and samples were applied pre-absorbed onto silica gel. Nuclear Magnetic Resonance (NMR) spectra were recorded on a 200.13 MHz Brüker spectrometer. Chemical shift values are reported in δ units relative to tetramethylsilane (TMS) as internal standard and coupling constants (J) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = doublet of doublet, t = triplet, m = multiple and br = broad. The relative integrals of peak areas are in agreement with those expected for the assigned structures. The assignment of all exchangeable protons (OH, NH_2) was confirmed by the addition of D_2O. All ^{13}C NMR spectra were proton-decoupled, and the signals were confirmed by using 135° DEPT (Distortionless Enhancement by Polarization Transfer) technique, as well as Correlation Spectroscopy (COSY ^1H–^1H) and Heteronuclear Correlations (HETCOR ^{13}C–^1H). IR spectra were obtained from potassium bromide (KBr) disks on a Nicolet 5 SXC FT-IR. Ultraviolet spectrophotometric (UV) studies were carried out on a Shimadzu Model

UV-160A spectrophotometer, using 1 cm quartz cells. High-resolution mass spectra were recorded on the Agilent LCTOF instrument (UCR Mass Spectrometry Facility, University of California, USA). Optical rotations were obtained with a JASCO P-1010 polarimeter, using 5 cm cell path length. All measurements were carried out at 25 ± 0.1 °C, at λ 272 nm (sodium D line).

4.2. General procedure for the synthesis of 5′-*O*-carbonates of 3TC

1.2 Equiv of *N,N*-carbonyldiimidazole (170 mg; 1 mmol 1.2 equiv) under N_2 stream was added to 1 equiv (200 mg, 0.872 mmoL, 1 equiv) of **1** in dried DMF (0.5 mL). A large excess of dried triethylamine (Et_3N, 120 μL) was then added to this solution. The mixture reaction was stirred at room temperature for 2 h leading to an intermediate of 3TC (3TC-5′-CI). The progress of the reaction was monitored by TLC (CH_2Cl_2/MeOH, 8:2 v/v). Once, the aliphatic alcohol (1.5 equiv) was added, and the reaction mixture was maintained in the same conditions until total conversion of 3TC-5′-CI with the formation of the corresponding carbonate product. The solvent was removed under reduced pressure, and the residual oil was washed in CH_2Cl_2 (1 × 20 mL). The organic phase was successively washed with water (3 × 20 mL). The organic layer was dried over Na_2SO_4, filtrated and evaporated to give the crude product which was purified by column chromatography on silica gel using a gradient solvent from EtOAc to $(CH_3)_2CO$/EtOAc (8:2). The appropriate fractions were combined, and the solvent removed in vacuum. Finally, **3**–**10** were recrystallized from dichloromethane–hexane. R_f values were determined using as mobile phase CH_2Cl_2/MeOH (8:2).

4.2.1. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-methyl carbonate (3, 3TC-Metha)

According to the general procedure, by adding methanol (0.106 mL, 2.617 mmol, 3 equiv), the title compound **3** was obtained as a white solid. Yield: 188 mg, (75%); R_f: 0.23.

^1H *NMR (DMSO-d_6)*: δ 7.45 (d, J = 7.31 Hz, 1H, H-6), 7.21 (br s, 2H, NH_2, exchangeable with D_2O), 6.25 (t, J = 4.75 Hz, 1H, H-1′), 5.60 (d, J = 7.31 Hz, 1H, H-5), 5.74 (t, J = 4.38 Hz, 1H, H-4′), 3.83 (d, J = 4.38 Hz, 2H, H-5′$_{a,b}$), 3.05 (dd, J = 5.12, 10.23 Hz, 1H, H-2′$_a$), 2.99 (dd, J = 4.02, 8.41 Hz, 1H, H-2′$_b$), 2.58 (br s, 3H, H-2″). ^{13}C *NMR (DMSO-d_6)*: δ 162.97 (C, C-4), 154.72 (C(O), C-2), 154.43 (C(O), C-1″), 140.40 (CH, C-6), 94.19 (CH, C-5), 87.21 (CH, C-1′), 82.81 (CH, C-4′), 67.21 (CH_2, C-5′), 54.99 (CH_3, C-2″), 36.86 (CH_2, C-2′). *UV* (H_2O)/nm λ_{max}: 207.5, 241.0 and 272.0. *IR* (KBr disk) ν_{max}: 3496.5–3380.8 (NH_2), 3230.1 (CH_2, C=C), 1754.3 (OC(O)O), 1650.2 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{10}H_{14}N_3O_5S^+$ 288.0649; found 288.065. $[\alpha]_D^{25}$ − 110.28.

4.2.2. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-ethyl carbonate (4, 3TC-Etha)

According to the general procedure, by adding ethanol (0.149 ml, 2.617 mmol, 3 equiv), the title compound **4** was obtained as a white solid. Yield: 187 mg (71%); R_f: 0.28.

^1H *NMR* (DMSO-d_6): δ 7.70 (d, J = 7.31 Hz, 1H, H-6), 7.27 (br s, 2H, NH_2, exchangeable with D_2O), 6.27 (t, J = 5.48 Hz, 1H, H-1′), 5.79 (d, J = 7.31 Hz, 1H, H-5), 5.41 (t, J = 4.38 Hz, 1H, H-4′), 4.49 (d, J = 4.38 Hz, 2H, H-5′$_{a,b}$), 4.05 (q, J = 6.94 Hz, 2H, H-2″), 3.40 (dd, J = 5.48, 10.23 Hz, 2H, H-2′$_a$), 3.12 (dd, J = 5.48, 9.87 Hz, 2H, H-2′$_b$), 1.11 (t, J = 7.31 Hz, 3H, H-3″). ^{13}C *NMR* (DMSO-d_6): δ 165.57 (C, C-4), 154.59 (C(O), C-2), 153.85 (C(O), C-1″), 140.49 (CH, C-6), 94.39 (CH, C-5), 86.83 (CH, C-1′), 80.74 (CH, C-4′), 68.16 (CH_2, C-5′), 62.85 (CH_2, C-2″), 36.21 (CH_2, C-2′), 14.02 (CH_3, C-3″). *UV* (H_2O)/nm λ_{max}: 207.5, 241.0 and 272.0. *IR* (KBr disk) ν_{max}: 3427.0–3346.0 (NH_2), 3189.7 (CH_2, C=C), 1765.9 (OC(O)O), 1655.9 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{11}H_{16}N_3O_5S^+$ 302.0805; found 302.0809. $[\alpha]_D^{25}$ − 115.50.

4.2.3. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-propyl carbonate (5, 3TC-nPro)

According to the general procedure, by adding *n*-propanol (0.196 ml, 2.617 mmol, 3 equiv), the title compound **5** was obtained as a white solid. Yield: 184 mg (67%); R_f: 0.34.

^1H *NMR (DMSO-d_6)*: δ 7.71 (d, J = 7.67 Hz, 1H, H-6), 7.24 (br s, 2H, NH_2 exchangeable with D_2O), 6.26 (t, J = 5.48 Hz, 1H, H-1′), 5.74 (d, J = 7.67 Hz, 1H, H-5), 5.44 (t, J = 4.38 Hz, 1H, H-4′), 4.43 (d, J = 4.38 Hz, 2H, H-5′$_{a,b}$), 4.08 (t, J = 6.57 Hz, 2H, H-2″), 3.40 (dd, J = 5.48, 9.50 Hz, 2H, H-2′$_a$), 3.15 (dd, J = 5.48, 10.60 Hz, 2H, H-2′$_b$), 1.50–1.71 (m, 2H, H-3″), 0.91 (t, J = 6.57 Hz, 3H, H-4″). ^{13}C *NMR (DMSO-d_6)*: δ 165.96 (C, C-4), 154.63 (C(O), C-2), 153.40 (C(O), C-1″), 140.89 (CH, C-6), 94.61 (CH, C-5), 87.15 (CH, C-1′), 81.38 (CH, C-4′), 69.74 (CH_2, C-2″), 67.98 (CH_2, C-5′), 36.15 (CH_2, C-2′), 21.84 (CH_2, C-3″), 10.36 (CH_3, C-4″). *UV* (H_2O)/nm λ_{max}: 207, 241.5 and 272.0. *IR* (KBr disk) ν_{max}: 3421.2–3380.7 (NH_2), 3149.2 (CH_2, C=C), 1760.1 (OC(O)O), 1667.5 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{12}H_{18}N_3O_5S^+$ 316.0962; found 316.097. $[\alpha]_D^{25}$ − 86.56.

4.2.4. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-2propyl carbonate (6, 3TC-2Pro)

According to the general procedure, by adding *2*-propanol (0.201 ml, 2.617 mmol, 3 equiv), the title compound **6** was obtained as a white solid. Yield: 192 mg (70%); R_f: 0.24.

^1H *NMR (DMSO-d_6)*: δ 7.68 (d, J = 7.67 Hz, 1H, H-6), 7.26 (br s, 2H, NH_2 exchangeable with D_2O), 6.27 (t, J = 5.48 Hz, 1H, H-1′), 5.76 (d, J = 7.31 Hz, 1H, H-5), 5.41 (t, J = 4.02 Hz, 1H, H-4′), 4.49 (d, J = 4.02 Hz, 2H, H-5′$_{a,b}$), 3.65–3.80 (m, 1H, H-2″), 3.26–3.56 (m, 1H, H-2′$_a$), 3.17 (dd, J = 5.48, 12.06 Hz, 1H, H-2′$_b$), 1.24 (t, J = 6.94 Hz, 6H, H-3″$_{a,b}$). ^{13}C *NMR (DMSO-d_6)*: δ 165.57 (C, C-4), 154.59 (C(O), C-2), 153.82 (C(O), C-1″), 140.48 (CH, C-6), 94.37 (CH, C-5), 86.83 (CH, C-1′), 80.74 (CH, C-4′), 68.14 (CH_2, C-5′), 63.00 (CH, C-2″), 35.63 (CH_2, C-2′), 21.58 (CH_3, C-3″$_a$), 21.36 (CH_3, C-3″$_b$). *UV* (H_2O)/nm λ_{max}: 209.0, 241.5 and 272.0. *IR* (KBr disk) ν_{max}: 3461.2–3398.1 (NH_2), 3137.6 (CH_2, C=C), 1771.7 (OC(O)O), 1673.3 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{12}H_{18}N_3O_5S^+$ 316.0962; found 316.0963. $[\alpha]_D^{25}$ − 98.06.

4.2.5. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-butyl carbonate (7, 3TC-Buta)

According to the general procedure, by adding *n*-butanol (0.239 ml, 2.617 mmol, 3 equiv), the title compound **7** was obtained as a white solid. Yield: 207 mg (72%); R_f: 0.36.

^1H *NMR* (DMSO-d_6): δ 7.71 (d, J = 7.31 Hz, 1H, H-6), 7.32 (br s, 2H, NH_2 exchangeable with D_2O), 6.28 (t, J = 5.48 Hz, 1H, H-1′), 5.79 (d, J = 7.31 Hz, 1H, H-5), 5.41 (t, J = 4.02 Hz, 1H, H-4′), 4.47 (d, J = 4.02 Hz, 2H, H-5′), 4.05 (t, J = 6.94 Hz, 2H, H-2″), 3.46 (dd, J = 5.48, 13.89 Hz, 1H, H-2′$_a$), 3.13 (dd, J = 5.85, 13.16 Hz, 1H, H-2′$_b$), 1.24–1.13 (m, 4H, H-3″, H-4″), 0.87 (t, J = 7.31 Hz, 3H, H-5″). ^{13}C *NMR (DMSO-d_6)*: δ 165.64 (C, C-4), 154.67 (C(O), C-2), 153.88 (C(O), C-1″), 140.95 (CH, C-6), 93.97 (CH, C-5), 86.58 (CH, C-1′), 81.03 (CH, C-4′), 68.18 (CH_2, C-5′), 67.64 (CH_2, C-2″), 38.26 (CH_2, C-3″), 36.29 (CH_2, C-2′), 30.07 (CH_2, C-4″), 14.06 (CH_3, C-5″). *UV* (H_2O)/nm λ_{max}: 207.5, 241.5 and 272.5. *IR* (KBr disk) ν_{max}: 3455.9–3334.4 (NH_2), 3189.7 (CH_2, C=C), 1760.1 (OC(O)O), 1650.2 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{13}H_{20}N_3O_5S^+$ 330.1118; found 330.1119. $[\alpha]_D^{25}$ − 102.12.

4.2.6. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-pentyl carbonate (8, 3TC-Penta)

According to the general procedure, by adding *n*-pentanol (0.284 ml, 2.617 mmol, 3 equiv), the title compound **8** was obtained as a white solid. Yield: 227 mg (76%); R_f: 0.40.

^1H NMR (DMSO-d_6): δ 7.70 (d, J = 7.67 Hz, 1H, H-6), 7.27 (br s, 2H, NH_2 exchangeable with D_2O), 6.26 (t, J = 5.48 Hz, 1H, H-1′), 5.76 (d, J = 7.31 Hz, 1H, H-5), 5.40 (t, J = 4.38 Hz, 1H, H-4′), 4.45 (d, J = 4.38 Hz, 2H, H-5′), 4.12 (t, J = 6.58 Hz, 2H, H-2″), 3.49 (dd, J = 5.48, 10.96 Hz, 1H, H-2′$_a$), 3.10 (dd, J = 5.48, 11.39 Hz, 1H, H-2′$_b$), 1.62 (quin, J = 6.58 Hz, 2H, H-3″), 1.45–1.17 (m, 4H, H-4″, H-5″), 0.88 (t, J = 6.57 Hz, 3H, H-6″). *^{13}C NMR (DMSO-d_6)*: δ 165.95 (C, C-4), 154.90 (C(O), C-2), 154.63 (C(O), C-1″), 140.86 (CH, C-6), 94.64 (CH, C-5), 87.17 (CH, C-1′), 81.33 (CH, C-4′), 68.25 (CH_2, C-5′), 68.01 (CH_2, C-2″), 36.09 (CH_2, C-2′), 28.07 (CH_2, C-3″), 27.61 (CH_2, C-4″), 22.00 (CH_2, C-5″), 14.11 (CH_3, C-6″). *UV* (H_2O)/nm λ_{max}: 207.5, 241.5 and 272.5. *IR* (KBr disk) ν_{max}: 3467.5–3334.4 (NH_2), 3126.0 (CH_2, C=C), 1748.6 (OC(O)O), 1621.2 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{14}H_{22}N_3O_5S^+$ 344.1275; found 344.128. $[\alpha]_D^{25}$ − 117.72.

4.2.7. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-hexyl carbonate (9, 3TC-Hexa)

According to the general procedure, by adding *n*-hexanol (0.327 ml, 2.617 mmol, 3 equiv) afforded the title compound **9** as a white solid. Yield: 218 mg (74%); R_f: 0.45.

^1H NMR (DMSO-d_6): δ 7.65 (d, J = 6.58 Hz, 1H, H-6), 7.25 (br s, 2H, NH_2 exchangeable with D_2O), 6.26 (t, J = 5.48 Hz, 1H, H-1′), 5.75 (d, J = 4.75 Hz, 1H, H-5), 5.48 (t, J = 4.39 Hz, 1H, H-4′), 4.55 (d, J = 3.65 Hz, 2H, H-5′$_{a,b}$), 4.12 (t, J = 6.58 Hz, 2H, H-2″), 3.57 (dd, J = 5.48, 11.28 Hz, 1H, H-2′$_a$), 3.20 (dd, J = 5.85, 13.16 Hz, 1H, H-2′$_b$), 1.73–1.50 (m, 2H, H-3″), 1.46–1.15 (m, 6H, H-4″, H-5″, H-6″), 0.87 (t, J = 5.12 Hz, 3H, H-7″). *^{13}C NMR* (DMSO-d_6): δ 164.50 (C, C-4), 154.64 (C(O), C-2), 153.61 (C(O), C-1″), 140.84 (CH, C-6), 94.53 (CH, C-5), 87.50 (CH, C-1′), 83.64 (CH, C-4′), 68.42 (CH_2, C-5′), 65.56 (CH_2, C-2″), 36.09 (CH_2, C-2′), 31.14 (CH_2, C-3″), 28.50 (CH_2, C-4″), 25.16 (CH_2, C-5″), 22.30 (CH_3, C-6″), 14.14 (CH_3, C-7″). *UV* (H_2O)/nm λ_{max}: 207.5, 241.5 and 272.5. *IR* (KBr disk) ν_{max}: 3427.0–3357.6 (NH_2), 3183.9 (CH_2, C=C), 1754.3 (OC(O)O), 1650.2 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{15}H_{24}N_3O_5S^+$ 358.1431; found 358.1438. $[\alpha]_D^{25}$ − 106.00.

4.2.8. 2′,3′-Dideoxy-3′-thiacytidin-5′-yl *O*-octyl carbonate (10, 3TC-Octa)

According to the general procedure, by adding *n*-octanol (0.366 ml, 2.617 mmol, 3 equiv) afforded the title compound **10** as a white solid. Yield: 235 mg (70%); R_f: 0.57.

^1H NMR (DMSO-d_6): δ 8.35 (d, J = 7.31 Hz, 1H, H-6), 7.22 (br s, 2H, NH_2 exchangeable with D_2O), 6.23 (t, J = 5.12 Hz, 1H, H-1′), 5.40 (d, J = 7.67 Hz, 1H, H-5), 5.27 (t, J = 4.02 Hz, 1H, H-4′), 4.11 (d, J = 3.65 Hz, 2H, H-5′), 3.84 (t, J = 4.38, 2H, H-2″), 3.57 (dd, J = 5.48, 10.60 Hz, 1H, H-2′$_a$), 3.20 (dd, J = 4.02, 13.89 Hz, 1H, H-2′$_b$), 1.74–1.48 (m, 2H, H-3″), 1.46–1.11 (m, 10H, H-4″, H-5″, H-6″, H-7″, H-8″), 0.87 (t, J = 3,65 Hz, 3H, H-9″). *^{13}C NMR (DMSO-d_6)*: δ 163.34 (C, C-4), 154.34 (C(O), C-2), 153.64 (C(O), C-1″), 145.20 (CH, C-6), 94.29 (CH, C-5), 87.39 (CH, C-1′), 83.35 (CH, C-4′), 65.53 (CH_2, C-5′), 62.35 (CH_2, C-2″), 37.80 (CH_2, C-2′), 31.50 (CH_2, C-3″, C-4″), 28.90 (CH_2, C-5″, C-6″), 25.50 (CH_2, C-7″), 22.38 (CH_2, C-8″), 14.24 (CH_3, C-9″). *UV* (H_2O)/nm λ_{max}: 212.0, 241.5 and 272.5. *IR* (KBr disk) ν_{max} 3461.7–3282.3 (NH_2), 3143.4 (CH_2, C=C), 1754.3 (OC(O)O), 1679.1 (CO) cm^{-1}. HMRS (ESI+): calcd for $C_{17}H_{28}N_3O_5S^+$ 386.1744; found 386.175. $[\alpha]_D^{25}$ − 86.34.

4.3. Optical rotation measurements

The samples were prepared by weighing the appropriate amount of carbonate, using methanol as solvent. All samples were left to stand 1 day before measuring the optical rotation. Each sample was prepared, and measurements were performed at least twice. For each sample, α is the average of at least 10 distinct measurements. The values of α in methanol are also described in Section 4.2.

4.4. Antiviral activity assays

4.4.1. Cells and virus

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Sweden) gradient centrifugation from peripheral blood of HIV-1-seronegative healthy donors. PBMCs were pooled from at least 3 donors, stimulated with 0.1% phytohaemagglutinin (PHA) during 3 days and cultured at 37 °C in RPMI-1640 medium (Sigma–Aldrich, USA) supplemented with 2 mM L-glutamine (Gibco BRL, USA), 100 U/ml penicillin (Gibco BRL), 100 mg/ml streptomycin (Gibco BRL) and 10% fetal calf serum (FCS, Gibco BRL) plus 10 U/ml interleukin-2 (IL-2). A stock of HTLV-IIIB strain of HIV-1 (*Titer*: 1.58×10^6 $TCID_{50}$/ml) was derived from chronically infected H9 cells and used in all experiments.

4.4.2. Inhibition of HIV replication and cytotoxicity assays

PBMCs were infected at 6.45×10^5 $TCID_{50}/10^6$ cells for 2 h at 37 °C. After infection, cells were washed and dispensed in a 96-well plate in the presence of different drug concentrations. The experiments were performed in duplicate, and wells treated with 3TC were also monitored as controls of antiviral activity. The culture medium was changed at day 4 maintaining the original drug concentration. On the seventh day, supernatant fluids were harvested and the production of p24 antigen was subsequently evaluated by using a commercial enzyme-linked immunosorbent assay (ELISA) assay (ABBOT Laboratories, USA). Absorbances were plotted against drug concentration and the concentration required to inhibit 50% of p24 production (i.e., 50% inhibitory concentration, IC_{50}) was calculated.

Cytotoxicity studies were performed in parallel on uninfected PBMCs in order to determine the concentration of drug that inhibited 50% of cell growth ($CCID_{50}$, cell culture inhibitory dose 50). Mock-infected cells were plated as described above and cell viability was determined by the staining of cells with Sulforhodamine B (SRB) dye.[33] Briefly, cells were fixed with TCA (trichloroacetic acid) 80%, dyed with SRB, and the optical density was then measured at 492 nm. Background-subtracted absorbances were plotted against drug concentration and the $CCID_{50}$ was calculated. Once IC_{50} and $CCID_{50}$ were obtained, the selectivity index (SI = $CCID_{50}/IC_{50}$) was determined.

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