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Dual-targeted anti-CMV/anti-HIV-1 heterodimers

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Abstract

Despite the development of efficient anti–human immunodeficiency virus-1 (HIV-1) therapy, HIV-1 associated pathogens remain a major clinical problem. Human cytomegalovirus (CMV) is among the most common HIV-1 copathogens and one of the main causes of persistent immune activation associated with dysregulation of the immune system, cerebrovascular and cardiovascular pathologies, and premature aging. Here, we report on the development of dual-targeted drugs with activity against both HIV-1 and CMV. We synthesized seven compounds that constitute conjugates of molecules that suppress both pathogens. We showed that all seven compounds exhibit low cytotoxicity and efficiently inhibited both viruses in cell lines. Furthermore, we chose a representative compound and demonstrated that it efficiently suppressed replication of HIV-1

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AUTHOR CONTRIBUTIONS

Anastasia Khandazhinskaya: conceptualization, chemical synthesis of compounds, analysis of data, original draft writing, review & editing; Vincenzo Mercurio, Rogers Alberto Ñahui Palomino: testing anti-HIV activity, collecting of data; Anna A. Maslova, Mikhail S. Novikov, Elena S. Matyugina, Maria P. Paramonova: chemical synthesis of compounds, collecting of data; Marina K. Kukhanova: hydrolysis of compounds by esterase, collecting of data; Natalya E. Fedorova, Kirill I. Yurlov, Alla A. Kushch: testing anti-CMV activity, collecting of data; Olga Tarasova: PASS prediction; Leonid Margolis, Sergey N. Kochetkov: analysis of data, original draft writing, review & editing; Christophe Vanpouille: testing anti-HIV and anti-CMV activity, collecting of data, analysis of data, original draft writing, review & editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

and CMV in human lymphoid tissue *ex vivo* coinfected with both viruses. Further development of such compounds may lead to the development of dual-targeted anti CMV/HIV-1 drugs.

Keywords

HIV-1; CMV; heterodimers; human tissues; viral diseases

1. INTRODUCTION

Since the start of the AIDS epidemic, coinfections with HIV-associated pathogens have become a major clinical problem [1]. In particular, human cytomegalovirus (CMV) is among the most common opportunistic infections in people living with HIV-1. Although CMV infects a wide range of the population, it is not overly pathogenic in immunocompetent people. However, in people living with HIV-1, CMV is associated with higher risk of severe non-AIDS-defining events, such as cerebrovascular and cardiovascular events [2], and non-AIDS related death. Moreover, CMV contributes significantly to immune activation, which causes dysregulation of the immune system and premature aging [3-5]. It was reported that CMV, whose seroprevalence has reached 90–100% in people living with HIV-1, has been associated with increased inflammation and inflammation-related morbidities (reviewed in [4, 6]). In coinfected individuals, HIV-1 infection leads to reactivation of CMV in blood and semen [7-10]. In turn, CMV worsens HIV-1 disease by increasing immune activation and inflammation even in ART-treated patients [3, 11-14]. Seminal shedding of CMV has been linked to higher HIV-1 viral load in semen [10, 15-17] and to increased risk of HIV-1 transmission [18-20] whether HIV-infected individuals are under treatment or not. These observations suggest that interventions aiming at reducing CMV shedding might be a meaningful HIV-1 prevention strategy in populations with high prevalence of CMV coinfection.

In this context, the use of dual-targeted antivirals against HIV-1 and CMV may give good results both for patients already infected with HIV-1 and for prevention of HIV-1 transmission. One of the ways to construct dual activity drugs is to make heterodimers of molecules that suppress corresponding pathogens. These heterodimers constitute a prodrug of parental drugs that provide certain advantages, for example a slow release of active compounds, which typically results in less toxicity than the combination of the parent drugs [21, 22].

Here, we report on the synthesis and the biological activity of heterodimers of AZT or 3TC, as typical HIV-1 reverse transcriptase inhibitors, and 1-[ω-(phenoxy)alkyl]uracil derivatives [23]. These derivatives were synthesized by our team few years ago and showed specific inhibitory properties against CMV replication in cell culture (EC_{50} 5.5–12.0 μM). Unfortunately preclinical studies of the compounds were limited by their low solubility. So 1-[ω-(phenoxy)alkyl]uracil derivatives look perfect candidates for making prodrugs - heterodimers with NRTIs (AZT or 3TC).

2. MATERIAL AND METHODS

2.1. Chemical Synthesis

2.1.1. General—Commercial reagents Acros (Geel, Belgium), Sigma Aldrich (St. Louis, MO, USA) and Fluka (Bucharest, Romania) were used for the reactions; anhydrous solvents of high quality were used without additional purification. Silica Gel 60 0.040–0.063 mm (Merck, Darmstadt, Germany) was used for column chromatography. Silica Gel 60 F_{254} aluminum-backed plates (Merck, Darmstadt, Germany) were used for thin layer chromatography (TLC), and Silica Gel 60 F_{254} glass-backed plates (Merck, Darmstadt, Germany) for preparative layer chromatography (PLC). NMR spectra were registered on an AMX III-400 spectrometer (Bruker, Newark, Germany) with the working frequency of 400 MHz for ^{1}H NMR (Me_4Si as an internal standard for organic solvents) and 100.6 MHz for ^{13}C NMR (with carbon-proton interaction decoupling).

High resolution mass spectra were measured on Bruker micrOTOF II instruments using electrospray ionization (ESI HRMS) using standart published conditions [24].

2.1.2. Compound Synthesis and Characterization.

2.1.2.1. Synthesis of compounds 1a-1f.: The synthesis of compounds **1a-d** has been previously described (9). Compounds **1e** and **1f** were obtained in a similar way.

1-[10-(4-Bromophenoxy)decyl]pyrimidine-2,4(1H,3H)-dione (1e).: White solid; yield 82% (3.1 g, 7.32 mmol); mp 84-86 °C. R_f 0.50 (eluting with ethylacetate). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.24-1.28 (10H, m, CH_2 × 5), 1.36 (2H, q, J = 7.7 Hz, CH_2), 1.54 (2H, q, J = 6.9 Hz, CH_2), 1.67 (2H, q, J = 7.5 Hz, CH_2), 3.62 (2H, t, J = 7.3 Hz, CH_2), 3.91 (2H, t, J = 6.5 Hz, CH_2), 5.53 (1H, dd, J = 7.8 and 2.3 Hz, H-5), 6.88 (2H, d, J = 9.0 Hz, H-3', H-5'), 7.41 (2H, d, J = 9.1 Hz, H-2', H-6'), 7.63 (1H, d, J = 7.8 Hz, H-6), 11.21 (1H, s, NH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 28.8, 29.2, 31.8, 31.9, 32.0, 32.1, 32.2, 32.3, 50.8, 71.1, 104.1, 115.1, 120.1, 135.4, 149.1, 154.3, 161.3, 167.1.

1-[12-(4-Bromophenoxy)dodecyl]pyrimidine-2,4(1H,3H)-dione (1f).: White solid; yield 80% (3.2 g, 7.09 mmol); mp 95-96.5 °C. R_f 0.54 (eluting with ethylacetate). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.21-1.27 (14H, m, CH_2 × 7), 1.37 (2H, q, J = 7.6 Hz, CH_2), 1.54 (2H, q, J = 6.8 Hz, CH_2), 1.67 (2H, q, J = 7.0 Hz, CH_2), 3.62 (2H, t, J = 7.3 Hz, CH_2), 3.92 (2H, t, J = 6.6 Hz, CH_2), 5.52 (1H, dd, J = 7.9 and 2.2 Hz, H-5), 6.88 (2H, d, J = 9.0 Hz, H-3', H-5'), 7.41 (2H, d, J = 9.0 Hz, H-2', H-6'), 7.63 (1H, d, J = 7.8 Hz, H-6), 11.21 (1H, s, NH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 28.6, 29.2, 31.8, 31.9, 32.0, 32.1, 32.3, 50.8, 71.7, 104.1, 115.1, 120.1, 135.4, 149.1, 154.3, 161.3, 167.1.

2.1.2.2. General procedure for the synthesis of [3-[ω-(4-bromophenoxy)alkyl]-2,6-dioxo-3,6- dihydropyrimidin-1(2H)-yl]acetic acids 2a-f.: A mixture of 4.01 mmol of 1-[ω-(4- bromophenoxy)alkyl]uracil 1, 0.7 g (5.06 mmol) K_2CO_3 in 20 mL DMF was stirred at 80 °C for 1 hour, cooled to room temperature, and 0.5 ml (4.51 mmol) of ethyl bromoacetate was added. The resulting mixture was stirred at the same temperature for 20 hours. The reaction mass was evaporated *in vacuo*, the residue was treated with 50

mL of cold water and extracted with 1,2- dichloroethane (5 × 20 mL). The extract was evaporated under reduced pressure to give clear viscous oily liquid, which was dissolved in 15 mL of ethanol. LiOH (0.58 g, 24.22 mmol) and 10 ml of water were added to the resultant solution at room temperature under stirring. The resulting mixture was stirred at the same temperature for 24 hours. Ethanol was evaporated under reduced pressure, the residue was acidified with 6% aqueous hydrochloric acid, and the precipitate was filtered, washed on the filter with water and air dried. Yields of target acids were within 61-79%, after crystallization from a mixture ethyl acetate-hexane.

[3-[3-(4-bromophenoxy)propyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2a).: White solid; yield 61% (0.94 g, 2.45 mmol); mp 153-154 °C. R_f 0.57 (eluting with ethylacetate- iPrOH-40% aqv.NH_4OH (6:9:5)). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 2.04 (2H, q, J = 6.3 Hz, CH_2), 3.90 (2H, t, J = 6.8 Hz, NCH_2), 3.98 (2H, t, J = 6.0 Hz, OCH_2), 4.42 (2H, s, CH_2CO), 5.72 (1H, d, J = 7.9 Hz, H-5), 6.86 (2H, d, J = 8.9 Hz, H-3', H-5'), 7.42 (2H, d, J = 8.9 Hz, H-2', H-6'), 7.73 (1H, d, J = 7.9 Hz, H-6), 13.10 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 31.1, 44.9, 49.9, 68.6, 103.3, 115.4, 120.1, 135.5, 148.2, 154.3, 161.0, 165.4, 172.5. HRMS: m/z[M + H]$^+$ calcd for $C_{15}H_{15}BrN_2O_5$: 383.0237, found: 383.0237.

[3-[5-(4-bromophenoxy)pentyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2b).: White solid; yield 65% (1.07 g, 2.60 mmol); mp 144-146 °C. R_f 0.56 (eluting with ethylacetate- iPrOH-40% aqv.NH_4OH (6:9:5)). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.39 (2H, q, J = 6.6 Hz, CH_2), 1.65 (2H, q, J = 7.4 Hz, CH_2), 1.72 (2H, q, J = 7.3 Hz, CH_2), 3.75 (2H, t, J = 7.1 Hz, NCH_2), 3.93 (2H, t, J = 6.3 Hz, OCH_2), 4.44 (2H, s, CH_2CO), 5.74 (1H, d, J = 7.8 Hz, H-5), 6.89 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.42 (2H, d, J = 8.8 Hz, H-2', H-6'), 7.77 (1H, d, J = 7.8 Hz, H-6), 12.90 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 22.6, 28.37, 28.41, 41.9, 48.8, 67.8, 100.2, 112.1, 117.1, 132.4, 145.0, 151.2, 158.2, 162.3, 169.5. HRMS: m/z[M + H]$^+$ calcd for $C_{17}H_{19}BrN_2O_5$: 411.0541, found: 411.0550.

[3-[6-(4-bromophenoxy)hexyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2c).: White solid; yield 79% (1.35 g, 3.17 mmol); mp 127-129 °C. R_f 0.45 (eluting with ethylacetate- iPrOH-40% aqv.NH_4OH (6:9:5)). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.30 (2H, q, J = 6.8Hz, CH_2), 1.39 (2H, q, J = 6.8 Hz, CH_2), 1.41 (2H, q, J = 6.6 Hz, CH_2), 1.61 (2H, q, J = 7.1 Hz, CH_2), 3.73 (2H, t, J = 7.1 Hz, NCH_2), 3.93 (2H, t, J = 6.6 Hz, OCH_2), 4.44 (2H, s, CH_2CO), 5.73 (1H, d, J = 7.8 Hz, H-5), 6.89 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.42 (2H, d, J = 9.1 Hz, H-2', H-6'), 7.76 (1H, d, J = 7.8 Hz, H-6), 12.89 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 25.4, 25.8, 28.6, 28.7, 41.9, 48.9, 67.9, 100.2, 112.0, 117.1, 132.4, 145.0, 151.2, 158.3, 162.3, 169.5. HRMS: m/z[M + H]$^+$ calcd for $C_{18}H_{21}BrN_2O_5$: 425.0707, found: 425.0707.

[3-[8-(4-bromophenoxy)octyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2d).: White solid; yield 68% (1.24 g, 2.74 mmol); mp 114-115.5 °C. R_f 0.47 (eluting with ethylacetate-iPrOH-40% aqv.NH_4OH (6:9:5)). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.25-1.31 (6H, m, 3×CH_2), 1.37 (2H, q, J = 6.4 Hz, CH_2), 1.58 (2H, q, J = 7.1 Hz, CH_2),

1.68 (2H, q, J = 6.8 Hz, CH_2), 3.71 (2H, t, J = 7.4 Hz, NCH_2), 3.93 (2H, t, J = 6.6 Hz, OCH_2), 4.44 (2H, s, CH_2CO), 5.73 (1H, d, J = 7.8 Hz, H-5), 6.88 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.42 (2H, d, J = 9.1 Hz, H-2', H-6'), 7.75 (1H, d, J = 7.8 Hz, H-6), 12.90 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 25.7, 26.0, 28.6, 28.8, 28.9, 41.9, 48.9, 68.1, 100.2, 112.0, 117.0, 132.4, 145.0, 151.2, 158.3, 162.3, 169.5. HRMS: m/z[M + H]$^+$ calcd for $C_{20}H_{25}BrN_2O_5$: 453.1020, found: 453.1027.

[3-[10-(4-bromophenoxy)decyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2e).: White solid; yield 61% (1.18 g, 2.45 mmol); mp 106-107.5 °C. R_f 0.49 (eluting with ethylacetate-iPrOH-40% aqv.NH_4OH (6:9:5)). ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.23-1.29 (10H, m, 5×CH_2), 1.37 (2H, q, J = 7.3 Hz, CH_2), 1.57 (2H, q, J = 6.6 Hz, CH_2), 1.67 (2H, q, J = 6.8 Hz, CH_2), 3.71 (2H, t, J = 7.3 Hz, NCH_2), 3.92 (2H, t, J = 6.6 Hz, OCH_2), 4.44 (2H, s, CH_2CO), 5.73 (1H, d, J = 7.8 Hz, H-5), 6.88 (2H, d, J = 9.0 Hz, H-3', H-5'), 7.41 (2H, d, J = 9.0 Hz, H-2', H-6'), 7.75 (1H, d, J = 7.8 Hz, H-6), 12.91 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 25.8, 26.0, 28.7, 28.9 (2), 29.0, 29.1, 29.2, 41.8, 49.0, 68.1, 100.2, 112.0, 117.0, 132.4, 145.0, 151.1, 158.3, 162.3, 169.4. HRMS: m/z[M + H]$^+$ calcd for $C_{22}H_{29}BrN_2O_5$: 481.1320, found: 481.1327.

[3-[12-(4-bromophenoxy)dodecyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]acetic acid (2f).: White solid; yield 73% (1.49 g, 2.92 mmol); mp 102-103°C. R_f 0.47 (eluting with ethylacetate- iPrOH-40% aqv.NH_4OH (6:9:5)) ^{1}H NMR (400 MHz, DMSO-d6), δ, ppm: 1.22-1.28 (14H, m, 7×CH_2), 1.37 (2H, q, J = 7.8 Hz, CH_2), 1.57 (2H, q, J = 6.4 Hz, CH_2), 1.67 (2H, q, J = 6.8 Hz, CH_2), 3.70 (2H, t, J = 7.3 Hz, NCH_2), 3.92 (2H, t, J = 6.6 Hz, OCH_2), 4.44 (2H, s, CH_2CO), 5.73 (1H, d, J = 7.8 Hz, H-5), 6.88 (2H, d, J = 9.1 Hz, H-3', H-5'), 7.41 (2H, d, J = 9.0 Hz, H-2', H-6'), 7.74 (1H, d, J = 7.9 Hz, H-6), 12.90 (1H, br.s, COOH). ^{13}C NMR (100.6 MHz, DMSO-d6), δ, ppm: 25.8, 26.1, 28.7, 28.88 (2), 28.92, 29.1, 29.25 (2), 29.30, 41.8, 49.0, 68.1, 100.2, 112.0, 117.0, 132.4, 145.0, 151.1, 158.3, 162.3, 169.4. HRMS: m/z[M + H]$^+$ calcd for $C_{24}H_{33}BrN_2O_5$: 411.0541, found: 411.0550.

2.1.2.3. General procedure for the synthesis of (3'-azido-3'-deoxythymidine)-2-[3-[ω-(4-bromo- phenoxy)alkyl]-2,6-dioxo-3,6-dihydropyrimidin-1(6H)-yl]acetates 3a-f.: The mixture of the corresponding acid **2a-f** (0.4 mmol) and 3'-azido-2',3'-dideoxythymidine (0.4 mmol, 107 mg) was coevaporated twice with pyridine (5 mL) and then dissolved in dry pyridine (5 mL). 1,3-Dicyclohexylcarbodiimide (0.48 mmol, 99 mg) was added to the resulting solution and left for 24 hours with stirring. The solvents were evaporated and the residue was purified by PLS in a chloroform: methanol (96: 4). Yields of target products were within 57-74%, after final repurification by PLC in ethyl acetate: chloroform (3: 2) with 1% methanol added.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)propyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (3a).: Light yellow oil; yield 61% (154 mg, 0.24 mmol). R_f 0.30 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.92 (s, 3H, CH_3, Thy), 2.16-2.19 (2H, m, CH_2), 2.39-2.45 (m, 2H, 2'CH_2, Azt), 3.95-3.98 (m, 4H, NCH_2, OCH_2), 4.01-4.03 (m, 1H, 4'CH, Azt), 4.24-4.26 (m, 1H, 3'CH, Azt), 4.39-4.52 (m, 2H, 5'H-2, Azt), 4.59-4.84

(m, 2H, CH_2CO), 5.73-5.75 (1H, d, J = 7.9 Hz, H-5, Ura), 6.08-6.11 (1H, t, J=8 Hz, 1'CH, Azt), 6.73-6.76 (2H, m, H-3', H-5', Ph), 7.17-7.19 (1H, d, J = 8 Hz, H-6,Ura),7.24 (s, 1H, H-6, Thy), 7.36-7.38 (2H, m, H-2', H-6', Ph), 8.48 (s, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ ppm: 12.63, 28.23, 37.54, 41.96, 47.61, 60.20, 63.65, 64.58, 81.90, 85.37, 101.43, 111.49, 113.60, 116.27×2, 132.56×2, 135.56, 143.57, 150.04, 151.25, 157.47, 162.36, 163.43, 167.65. HRMS: m/z[M + H]$^+$ calcd for $C_{25}H_{26}BrN_7O_8$: 632.1099, found: 632.1093.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)pentyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (3b).: Light yellow oil; yield 74% (195 mg, 0.30 mmol). R_f0.31 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.48-1.52 (2H, m, CH_2), 1.76-1.80 (m, 4H, $2CH_2$), 1.92 (s, 3H, CH_3, Thy), 2.35-2.44 (m, 2H, 2'CH2, Azt), 3.74 −3.78 (t, J= 8 Hz, 2H, NCH_2), 3.89-3.92 (m, 2H, OCH_2), 4.01-4.02 (m, 1H, 4'CH, Azt), 4.23-4.25 (m, 1H, 3'CH, Azt), 4.41-4.49 (m, 2H, 5'CH_2, Azt), 4.61-4.84 (m, 2H, CH_2CO), 5.76-5.78 (d, 1H, J=6Hz, H-5, Ura), 6.08-6.12 (t, 1H, 1'CH, Azt), 6.73-6.75 (m, 2H, 2CH, H-3', H-5', Ph), 7.16-7.18 (d, 1H, H-6, J=6 Hz, Ura), 7.25 (s, 1H, H-6, Thy), 7.32-7.35 (m, 2H, $2CH_2$, H-2', H-6', Ph), 8.86 (s, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 12.57, 23.05, 28.68, 28.74, 37.47, 41.96, 49.85, 60.18, 63.63, 67.67, 81.82, 85.24, 101.42, 111.44, 112.88, 116.32×2, 132.30×2, 135.49, 143.05, 150.11, 151.17, 158.05, 162.34, 163.59, 167.62. HRMS: m/z[M + H]$^+$ calcd found for $C_{27}H_{30}BrN_7O_8$: 660.1413, found: 660.1412.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)hexyl)-2,6-dioxo-2,3-dihydropyrimidin- 1(6H)-yl)acetate (3c).: Light yellow oil; yield 60% (162 mg, 0.24 mmol). R_f0.31 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.37-1.41 (m, 2H, CH_2), 1.47-1.52 (m, 2H, CH_2) 1.70-1.78 (m, 4H, $2CH_2$), 1.93 (s, 3H, CH_3, Thy), 2.35-2.44 (m, 2H, 2'CH_2, Azt), 3.73 – 3.76 (t, J=8 Hz, 2H, NCH_2), 3.89-3.39 (m, 2H, OCH_2), 4.00-4.04 (m, 1H, 4'CH, Azt), 4.22-4.26 (m, 1H, 3'CH, Azt), 4.38-4.52 (m, 2H, 5'CH_2, Azt), 4.61-4.85 (m, 2H, CH_2CO), 5.75-5.77 (d, H, J=6 Hz, H-5, Ura), 6.08-6.12 (t, 1H, J= 8 Hz, 1'CH, Azt), 6.73-6.76 (m, 2H, H-3',H-5', Ph), 7.15-7.17 (d, 1H, H-6, J=8 Hz, Ura), 7.26 (s, 1H, 6CH, Thy), 7.32-7.35 (m, 2H, $2CH_2$, H-2', H-6', Ph), 8.40 (s, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 12.62, 25.69, 26.20, 29.02×2, 37.54, 42.01, 49.95, 60.22, 63.64, 67.94, 81.90, 85.25, 101.45, 111.50, 112.84, 116.38×2, 132.34×2, 135.48, 143.07, 150.02, 151.22, 158.19, 162.38, 163.38, 167.67. HRMS: m/z[M + H]$^+$ calcd for $C_{28}H_{32}BrN_7O_8$: 674.1568, found: 674.1563.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)octyl)-2,6-dioxo-2,3-dihydropyrimidin- 1(6H)-yl)acetate (3d).: Light yellow oil; yield 66% (185 mg, 0.26 mmol). R_f0.32 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.34-1.43 (m, 8H, $4CH_2$), 1.67-1.76 (m, 4H, $2CH_2$), 1.93 (s, 3H, CH_3, Thy), 2.33-2.44 (m, 2H, 2'CH_2, Azt), 3.73 (t, 2H, J = 7.4 Hz, NCH_2), 3.88-3.91 (m, 2H, OCH_2), 4.01-4.02 (m, 1H, 4'CH, Azt), 4.24-4.25 (m, 1H, 3'CH, Azt), 4.38-4.42 (m, 2H, 5'CH_2, Azt), 4.49-4.85 (m, 2H, CH_2CO), 5.76-5.78 (d, H, H-5, J=6 Hz, Ura), 6.10-6.12 (t, 1H, 1'CH, Azt), 6.73-6.77 (m, 2H, H-3', H-5', Ph), 7.16-7.17 (d, 1H, H-6, J=6 Hz, Ura), 7.27 (s, 1H, 6CH, Thy), 7.32-7.36 (m, 2H, H-2', H-6', Ph), 8.56 (c, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 12.56, 25.89, 26.35,

28.99, 29.05, 29.11, 29.13, 37.46, 41.97, 50.01, 60.20, 63.66, 68.18, 81.80, 85.14, 101.29, 111.45, 112.64, 116.37×2, 132.23×2, 135.46, 143.14, 150.23, 151.15, 158.25, 162.40, 163.76, 167.64. HRMS: m/z[M + H]$^+$ calcd for $C_{30}H_{36}BrN_7O_8$: 702.1881, found: 702.1872.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)decyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (3e).: Light yellow oil; yield 57% (166 mg, 0.23 mmol). R_f 0.32 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.25-1.41 (m, 12H, 6CH_2), 1.67-1.75 (m, 4H, 2CH_2), 1.92 (s, 3H, CH_3, Thy), 2.35-2.44 (m, 2H, 2'CH_2, Azt), 3.72 - 3.74 (m, 2H, NCH_2), 3.87-3.90 (m, 2H, OCH_2), 4.02 (m, 1H, 4'CH, Azt), 4.22-4.24 (m, 1H, 3'CH, Azt), 4.38-4.52 (m, 2H, 5'CH_2, Azt), 4.61-4.84 (m, 2H, CH_2CO), 5.76-5.78 (d, H, H-5, J=6 Hz, Ura), 6.12 (t, 1H, 1'CH, Azt), 6.73-6.75 (m, 2H, H-3', H-5', Ph), 7.16-7.18 (d, 1H, H-6, J=6 Hz, Ura), 7.27 (s, 1H, 6CH, Thy), 7.32-7.34 (m, 2H, H-2', H-6', Ph), 9.05 (s, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 12.73, 26.02, 26.50, 29.10, 29.19, 29.21, 29.33, 29.40, 29.45, 37.62, 42.08, 50.14, 60.28, 63.72, 68.33, 81.87, 85.18, 101.42, 111.55, 112.65, 116.42×2, 132.27×2, 135.53, 143.29, 150.26, 151.20, 158.33, 162.46, 163.75, 167.69. HRMS: m/z[M + H]$^+$ calcd for $C_{32}H_{40}BrN_7O_8$: 730.2194, found: 730.2190.

(3'-azido-3'-deoxytimidine)-2-(3-((4-bromophenoxy)dodecyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (3f).: Light yellow oil; yield 58% (176 mg, 0.23 mmol). R_f 0.33 (eluting with $CHCl_3$-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.24-1.41 (m, 16H, 8CH_2), 1.72-1.75 (m, 4H, 2CH_2) 1.92 (s, 3H, CH_3, Thy), 2.34-2.43 (m, 2H, 2'CH_2, Azt), 3.71 (t, J = 7.5 Hz, 2H, NCH_2), 3.89 (t, J=6 Hz, 2H, OCH_2), 4.01-4.02 (m, 1H, 4'CH, Azt), 4.23-4.25 (m, 1H, 3'CH, Azt), 4.37-4.53 (m, 2H, 5'CH_2, Azt), 4.61-4.84 (m, 2H, CH_2CO), 5.76 (d,1H, H-5, J=6 Hz, Ura), 6.12 (t, J=6 Hz, 1H, 1'CH, Azt), 6.73-6.76 (m, 2H, H-3', H-5', Ph), 7.16 (d, 1H, H-6, J=6Hz, Ura), 7.26 (s, 1H, 6CH, Thy), 7.31-7.35 (m, 2H, H-2', H-6', Ph), 8.97 (s, 1H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 12.60, 26.04, 26.49, 29.07, 29.22×2, 29.39, 29.47, 29.54×3, 37.51, 42.01, 50.01, 60.23, 63.67, 68.35, 81.86, 85.13, 101.30, 111.52, 112.63, 116.42×2, 132.26×2, 135.44, 143.17, 150.22, 151.19, 158.34, 162.44, 163.68, 167.68. HRMS: m/z[M + H]$^+$ calcd for $C_{34}H_{44}BrN_7O_8$: 758.2507, found:758.2507.

2.1.2.4. Synthesis of compound 4c.

((−)-L-2′,3′-dideoxy-3′-thiacytidine)-2-(3-((4-bromophenoxy)hexyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (4c).: Light yellow oil; yield 30% (27 mg, 0.04 mmol). Rf 0.5 (eluting with CHCl3-MeOH (95:5)). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.36-1.43 (m, 2H, CH_2), 1.47-1.55 (m, 2H, CH_2) 1.72-1.79 (m, 4H, 2CH_2), 3.01-3.07 (m, 1H, 2'CH_2, 3TC), 3.49-3.55 (m, 1H, 2'CH_2, 3TC), 3.73-3.78 (t, J=8 Hz, 2H, NCH_2), 3.89-3.93 (t, 2H, OCH_2), 4.44-4.56 (m, 2H, 5'CH_2, 3TC), 4.69-4.82 (m, 2H, CH_2CO), 5.33-5.36 (m, 1H, 4'CH_2, 3TC), 5.76-5.79 (d, H, J=6 Hz, H-5, 3TC), 5.93-5.96 (d, 1H, J=6 Hz, H-5,Ura), 6.30-6.34 (t, 1H, J=8 Hz, 1'CH, 3TC), 6.73-6.79 (m, 2H, H-3',H-5', Ph), 7.20-7.22 (d, 1H, H-6, J=8 Hz, Ura), 7.33-7.38 (m, 2H, 2CH_2, H-2', H-6', Ph), 7.63-7.65 (d, 1H, H-6, J=8 Hz, 3TC). ^{13}C NMR (100.6 MHz, $CDCl_3$), δ, ppm: 25.6, 26.1, 28.9×2, 37.6, 41.8, 49.8, 65.3, 67.9, 82.1, 87.7, 95.2, 101.3, 112.7, 116.3×2, 132.2×2, 140.6, 143.1, 151.1, 155.4, 158.1,162.4, 165.7, 167.5. HRMS: m/z[M + H]$^+$ calcd for $C_{26}H_{30}BrN_5O_7S$: 636.1122, found: 636.1127.

2.2. Hydrolysis of the synthesized compounds by esterase from porcine liver

Hydrolysis of the compounds was assayed in 30 μl 50 mM Tris-HCl buffer pH 8.2 containing NaCl 250 mM, $CaCl_2$ 6 mM, esterase 5.2 units/test, different concentrations of compounds **3b- 3d** and **4c** (2 - 10 mM) in methanol. The reactions were proceeded at 37 °C for 0-18 h. Reaction products were separated by TLC in chloroform-ethanol 32:1 for compounds **3b** and **3d**, or chloroform-methanol 95:5 for compounds **3c** and **4c**. The compounds **1b-d**, **2-2d,** AZT and 3TC were used as references. R_f of hydrolysis products: **3b** – 0.72, **2b** – 0.31, **1b** – 0.72, **3c** – 0.69, **2c** – 0.03, **1c** – 0.44, **4c** – 0.50, 3TC – 0.30, **3d** – 0.80, **2d** – 0.38, **1d** – 0.60, AZT – 0.51. The products were identified by mass-spectrometry. Retention time: 3.4 min for 2',3'-dideoxy-3'-azidothymidine **2b** - 4.1 min, **2c** - 4.3 min, **2d** - 4.7 min, **3b** - 6.3 min, **3c** - 6.5 min, **3d** - 7.1 min. Retention time for **4c** was 7.5 min, methanol gradient elution.

2.3. Cells and reagents

Human T lymphocyte MT-4 cell line was obtained from Dr. D. Richman (NIH/AIDS reagent program, division of AIDS, NIAID, NIH). The fibroblastic WS1 cell line was obtained from ATCC (Manassas, VA). MT-4 was cultured in RPMI 1640 FBS 10% while WS1 was cultured in DMEM FBS 10% (FBS, Gemini Products, Sacramento, CA) respectively. Human lymphoid tissues were surgically removed during routine tonsillectomies at Children's National Medical Center (Washington, DC) and obtained within 4-5h of excision according to an NIH Institutional review Board-approved protocol. Tonsils were cut in 2-3-mm tissue-blocks. Nine blocks were placed on a collagen sponge at the air-liquid interface and cultured for 12 to 15 days in 6-well plates with a change of medium every 3 days. Tissue blocks were cultured in RPMI 1640 supplemented with 15% FBS, L-Glutamine, essential amino acids, pyruvate, 50 ng/mL gentamycin and 50 ng/mL Fungizone. Typically 3 wells of 9 tissue blocks were used per experimental condition.

The prototypical X4 HIV-1 variant $X4_{LAI.04}$ (stock at 50 ng of $p24_{gag}$ /mL) was obtained from VQA Laboratory (Chicago, IL).

CMV AD169 (TCID50 of $10^{4,3}$/0,2 mL) used to infect WS1 cell lines was obtained from The State Virus Collection of the Gamaleya National Research Center for Epidemiology and Microbiology of the Ministry of Healthcare of the Russian Federation, Moscow, Russia (NRCEM, Moscow, Russia).

2.4. Antiviral activity

2.4.1. Anti HIV-1 activity in MT-4 cell cultures.—The anti HIV-1 activity of all seven newly synthesized compounds was evaluated in MT-4 cell cultures. MT-4 cells (100 μL at 10^7 cells/mL) were incubated with 100 μL of $HIV\text{-}1_{Lai.04}$ for 90 min at 37°C. The excess of virus was washed off twice with 25 mL of PBS and resuspended. After resuspending cell pellet with 1 mL of complete medium 100 μL of infected cells were added per well followed by the addition of compound diluted in 900 uL of complete medium. After 3 days of culture, $p24_{gag}$ concentration was assessed.

2.4.2. ***Anti HIV-1 activity in Human tissue culture* ex vivo.—**27 tonsillar tissue blocks were pretreated with compound **3c** at different concentrations overnight and then inoculated by application of 7 μL of $X4_{LAI.04}$ viral stock (50 ng/mL) on top of each block. Compound **3c** was re-added every 3 days when medium was changed and the tissue blocks were cultured for 12 days. HIV-1 replication was assessed by measuring $p24_{gag}$ concentration in tissue culture. HIV inhibition was calculated as percent of HIV replication in tissue blocks cultured in presence of drugs compared to HIV replication in tissue blocks cultured with DMSO control.

2.4.3. Anti-CMV activity in WS1 cell cultures.—We evaluated the anti-CMV activity of heterodimers **3a-3f** and their parental molecules **1d, 1f,** and **2a-2f** using a standard plaque reduction assay. The human embryo fibroblasts WS1 cell monolayers were infected with CMV AD169 (TCID50 $10^{4.3}$/0,2 mL) and incubated for 1 h at 37°C under 5% CO_2. Then, the infected cells were washed twice with PBS and cultured in cell culture media containing different concentrations of compounds. Cells were cultured at 37°C for 5 days. We calculated the plaque inhibition rate using the standard method as described previously [25]. The EC_{50} (50% effective concentration) of tested compounds was calculated by regression analysis.

2.4.4. ***Anti-CMV/HIV-1 activity of heterodimer 3c and 4c in coinfected human tissues* ex vivo.—**27 tonsillar tissue blocks were simultaneously infected with 7 μL of HIV-1 ($X4_{LAI.04}$) and 5 μL of CMV (AD169) viral stocks. Tissue cultures were pre-treated 12 h prior to viral infection with compound **3c** or **4c**. Medium was changed every 3 days. Tissue blocks were cultured for 15 days. The anti-HIV-1 and anti CMV activities of **3c** or **4c** was evaluated by comparing HIV or CMV replication in tissues cultured in presence of drugs versus HIV or CMV replication in tissue-control.

2.4.5. Antiviral Activity Evaluation.—Productive HIV-1 infection was evaluated from measurements of p24gag antigen in culture supernatant by Luminex, as previously described [26]. Productive CMV replication was evaluated by measurement of CMV DNA copies present in culture supernatant using real-time polymerase chain reaction as previously described [27]. Antiviral activity was either expressed as (i) EC_{50}s (heterodimer concentration that inhibits viral replication by 50%) or (ii) viral inhibition expressed as percent of control (untreated infected condition). Means ± SD of EC_{50} for each compound are presented in Table 1 or giving in the text (compound **4c**). Each experiment was performed between 2 to 5 times.

2.5. Cell Viability

2.5.1. Viability assay in MT-4 cell cultures.—Cell viability was performed using a NucleoCounter® NC-100™ automated cell counting system (ChemoMetec, Lillerød, Denmark) using nucleocassette pre-loaded with the propidium iodide (PI) fluorescent dye for dead cells. Viability of cells was calculated by comparing the number of total cells (obtained by lysing cells before measurement) and the number of dead cells (no pre-treatment). Each condition was run in duplicate. Cell viability for each concentration of each compound was expressed as percentage of cells in untreated control.

2.5.2. Viability assay in WS1 cell cultures.—Compound cytotoxicity was also tested by MTT method. WS1 cells were cultured in 96 well plates containing different concentrations of compounds in the cell culture medium at 37 °C in 5% CO_2 atmosphere. Three days later, the cell culture medium was replaced with 50 μL of fresh medium containing 1 mg/mL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Cells were incubated at 37 °C for 4 h, and 100 μL of acid-isopropanol (HCl 0.1N in isopropanol) was added to each well. After ensuring that all crystals were dissolved, the absorbance in the plates were measured using an automatic plate reader (TECAN, Switzerland) at 570 nm test wavelength and a 690 nm reference wavelength. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration that caused a 50% reduction in the number of viable cells.

2.5.3. Viability assay in human lymphoid tissue.—To assay viability of cells in tissues *ex vivo*, tissue blocks were digested with collagenase IV at day 12. Cells from **3c**-(10 μM) treated tissue blocks and untreated-control tissues were stained with anti-CD3QD655, anti-CD4QD605, anti-CD8QD800, anti-CD45RA-FITC, anti-CCR7-PECy7, anti-CXCR4-PE, and anti-CCR5-APC antibodies (stain 1) or stained with anti–CD3-QD655, anti–CD4-QD605, anti–CD8-QD800, anti-CD25-APC, anti-CD38-PE, anti-CD95-PE-Cy7, and anti-HLA-DR-BV570 antibodies (stain 2) (B&D Biosciences or Caltag laboratories, CA). Data were processed on a NovoCyte cytometer (AceaBiosciences, Inc). The depletion of each $CD3^+$ lymphocytic cell subsets was assessed by using Trucount beads (Becton-Dickinson, NJ).

3. RESULTS

3.1. Chemical synthesis

The reaction of 1-[ω-(4-bromophenoxy)alkyl]uracils **1a-f** with ethyl bromoacetate in the presence of K_2CO_3 in DMF, followed by alkaline hydrolysis at room temperature, gave the corresponding [2,6-dioxo-3-[ω-(4-bromophenoxy)alkyl]-3,6-dihydropyrimidine-1(2H)-yl] acetic acids **2a-f** in one-pot with 60–79% yields (Scheme 1). The target conjugates **3a-f** were prepared by condensation of 2',3'-dideoxy-3'-azidothymidine and acids **2a-f** in the presence of dicyclohexylcarbodiimide (DCC) in pyridine. The yields of 5'-[2,6-dioxo-3-(ω-phenoxyalkyl)-3,6-dihydropyrimidine-1(2H)-yl]acetates of 2',3'-dideoxy-3'-azidothymidine (**3a-f**) were 52-74% after isolation and purification by column chromatography on silica gel.

((−)-L-2′,3′-dideoxy-3′-thiacytidine)-2-(3-((4-bromophenoxy)hexyl)-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)acetate (**4c**) was synthesized in a similar manner starting from 3TC and **2c**.

3.2. Hydrolysis of the synthesized compounds by esterase from porcine liver

We assumed that the compounds **3a-f** could be hydrolyzed enzymatically, for instance by esterases, and generate biologically active components. Towards this goal, we investigated the hydrolysis of compounds by porcine esterase. We showed that reaction with esterase generated two main products identified by TLC and high-resolution mass spectrometry as 2',3'-dideoxy-3'-azidothymidine and the corresponding 1-[ω-(4- bromophenoxy)alkyl]uracil

acetic acids **2b-2d**, as well as 5-10% of unidentified products. It should be noted that $T_{1/2}$ for **3b** and **3c** was about 7-8 hrs, but $T_{1/2}$ for with longer linker was 10-12 hrs although hydrolysable bonds were the same. $T_{1/2}$ for **4c** was about 24 hrs. Thymidine diacetate was used as a positive control; its hydrolysis was totally completed after 5-6 hours under the same conditions.

3.3. Heterodimers suppressed HIV-1 replication in MT-4 cell cultures

HIV inhibition of all six AZT-based newly-developed anti-CMV/HIV-1 heterodimers was evaluated in MT-4 cells inoculatyed with HIV-$1_{LAI.04}$. In this system, HIV-1 replication, evaluated as p24gag accumulation in culture medium over 3 days, reached on average (195.8 ± 26.5) x 10^3 ng/mL p24gag (*n*=3). All six tested compounds efficiently and dose-dependently suppressed HIV-1 replication with EC_{50} ranging from 0.21 μM to 1.96 μM (Table 1; Fig. S1).

3.4. Heterodimers suppressed CMV replication in human embryo fibroblast cultures

The anti-CMV activity of the heterodimers **3a-f** as well as their hydrolysis products **2a-f** were tested for inhibition of CMV replication in human embryo fibroblast (WS1) cultures infected with CMV (AD169) (Table 2). EC_{50} of heterodimers **3a-3f** ranged from 3.2 to 12.2 μM and showed similar activity to that of control compound ganciclovir (EC_{50} 2 μM), **1d** and **1f** (EC_{50} 13.1 and 3.1 μM). Compounds **2c** and **2b** were less active (EC_{50} 78.1 and 104 μM, respectively); compounds **2a**, **2e**, and **2f** were inactive. The only active compound among acids **2a-2f** was **2d**, with EC_{50} 11.9 μM.

3.5. AZT-based heterodimers exhibit low cytotoxicity

In principle, viral inhibition could be the result of cell toxicity. To exclude the possibility that the HIV-1 inhibition reported here was due to cell death, we cultured MT-4 cells in the presence of increasing concentrations of each heterodimer and assessed cell viability after 3 days of culture. Five out of six compounds did not show any significant cytotoxicity, even at 100 μM (Table 1; Fig. S2). Compound **3a** showed some cell toxicity, killing around 50% of cells at 100 μM (500 times its EC_{50}). Compounds **3c** and **3f**, however, were two of the best, with very low cytotoxicity, even at 100 μM (respectively 9.0 ± 3.9% and 10.3 ± 4.2% at 100 μM) (Table 1; Fig. S2).

3.6. *Heterodimer 3c suppressed the replication of HIV-1 in human tonsillar tissue* ex vivo

All our newly developed anti CMV/anti-HIV-1 compounds efficiently inhibited HIV-1 in cell cultures (MT-4) Fig. S1. We chose one of them, compound **3c,** to investigate its anti-HIV-1 activity in tonsillar tissue *ex-vivo*. This system of culture was shown to retain tissue cytoarchitecture and to support HIV-1 replication without exogenous activation [29]. Tonsillar tissue blocks were pre-treated with **3c** overnight and inoculated with HIV-1. In control untreated HIV-1-infected cultures, the cumulative HIV-1 replication ([$p24_{gag}$]) levels in tissues from different donors varied from 2.5 to 97.9 ng/mL, as typically observed in human tissues *ex vivo*, where cell activation and coreceptor expression are highly variable from donor to donor. Compound **3c** at concentrations ranging from 1 nM to 10 μM inhibited HIV-1 replication in a dose-dependent manner by 0 ± 0.5%, 17.9 ± 8.2%, 31.4 ± 7.7%,

76.5 ± 3.3%, and 96.2 ± 1.0%, respectively (n=9 (except for 1 nM (n=2)), p>0.05; p>0.05; p<0.05; p<0.05; p<0.05) (Fig. 2). The EC_{50} of AZT alone in human tissue *ex vivo* was previously reported (3.1 nM ((95% CI (0.63–15.7)) [30].

3.7. *Heterodimer 3c was not cytotoxic in human lymphoid tissues* ex vivo

As an adequate model to assess the efficiency of newly-synthesized drugs or microbicides [30-32], the system of human tissue explants is also a trademark system to evaluate potential cytotoxicity of various drugs for different cell subsets. Since HIV-1 mainly replicates in lymphocytes, here we used human tonsillar tissues *ex vivo* to assess the effect of **3c** on cell a variety of lymphocytic cell subsets. At the end of the culture (day 12), cells were isolated from control tissue blocks not treated or from tissue blocks treated with **3c** at 10 μM, a concentration that completely abrogated HIV-1 replication in this system. Cells were stained with different cell markers, and we evaluated the effect of **3c** on cell viability by comparing the numbers of cells in tonsillar tissue blocks treated with **3c** and with those in untreated tonsillar tissue control. We evaluated the depletion of T cells (CD3+), CD3+CD4+ cells, CD3+CD8+ cells, and various subsets of CD4+ T cells: CD4+ expressing HIV-1 coreceptor CXCR4 or CCR5, CD45+CCR7+ ($T_{naïve}$), CD45RA-CCR7+, CD45RA-CCR7−, CD45RA+CCR7−, CD4+ expressing different markers of activation (CD25+, CD38+, CD95+, HLA-DR+). Figure 3 shows that **3c**, even at a concentration of 10 μM, didn't significantly deplete any cells (n=7, p<0.05).

3.8. *Heterodimer 3c suppressed CMV and HIV-1 replication in coinfected lymphoid tissues* ex vivo

CMV reactivation is a major problem in immunocompromised people, including people living with HIV-1. To mimic more closely the *in vivo* situation where HIV-1 and CMV both replicate in coinfected individuals, we coinfected human tonsillar tissues *ex vivo* with both viruses and investigated the effect of compound **3c**, as a representative of our newly synthesized heterodimers. Both HIV-1 and CMV replication in coinfected tissues were typical of replication of these viruses in singly infected human tissues. The cumulative HIV-1 replication levels in coinfected tonsillar tissues varied from 10.3 to 83.3 ng/mL $p24_{gag}$, while the cumulative CMV replication levels varied from 3,226 to 32,667 CMV DNA copies/μL (n=6). In HIV-1/CMV-coinfected human lymphoid tissue, **3c** retained its inhibitory activity against both viruses: 10 μM **3c** inhibited the cumulative production of $p24_{gag}$ by 97.2 ± 2.1% (n=6; p<0.05) (Fig. 4A) and the number of CMV DNA copies/uL by 86.9 ± 3.3% (n=6; p<0.05) (Fig. 4B). In coinfected tissues, AZT and 3TC controls inhibited HIV-1 replication respectively by 97.0 ± 1.6% and 99.7 ± 0.2% (n=3; p<0.05). Control compounds **1c** and **2c** inhibited CMV respectively by 92.8 ± 2.1% and 68.6 ± 11.3% (n=3; p<0.05). AZT and 3TC have no effect on CMV replication [28]. Compound **1c** has been reported to be inactive against HIV-1 [23], while **2c** was tested and did not inhibit HIV-1 either (Table 1).

3.9. Heterodimer 4c suppressed CMV and HIV-1 replication

As a proof-of-concept, next we synthesized heterodimer **4c**, the equivalent of **3c** using 3TC as the anti-HIV-1 molecule instead of AZT (Fig. 5A). EC_{50} of **4c** in MT-4 cell cultures was

2.0 μM (95% CI (1.4–2.8) (Fig. 5B). CC_{50} of **4c** was greater than 100 μM. The EC_{50} and CC_{50} of the 3TC control were 17 nM (n=3) and >100 μM, respectively [30]. In human tissue *ex vivo* infected with HIV-1, compound **4c** at concentrations ranging from 1 nM to 10 μM inhibited HIV-1 replication in a dose-dependent manner by 0.6 ± 0.2%, 14.6 ± 6.5%, 45.7 ± 3.7%, 88.6 ± 2.9%, and 99.6 ± 0.1%, respectively (n=4; p>0.05; p>0.05; p<0.05; p<0.05; p<0.05) (Fig. 5C). The EC_{50} of 3TC alone in human tissue *ex vivo* was previously reported (16.6 nM (95% CI (2.7–101.6)) [30].

In HIV-1/CMV-coinfected human lymphoid tissue, **4c** retained its inhibitory activity against both viruses: 10 μM **4c** inhibited the cumulative production of $p24_{gag}$ by 99.4 ± 0.4% (n=3; p<0.05) (Fig. 5D) and the number of CMV DNA copies/μL by 78.7 ± 1.7% (n=3; p<0.05) (Fig. 5E). In these coinfected tissues, 3TC control inhibited HIV-1 replication by 99.7 ± 0.2% (n=3; p<0.05).

4. DISCUSSION

Here, we present newly-designed anti CMV/anti-HIV-1 heterodimers which are prodrugs of corresponding antiviral agents. A prodrug strategy is a well-known and effective way to overcome shortcomings of active compounds such as poor solubility and low bioavailability. Also, it can improve the pharmacokinetic profile and make drug delivery to the target more efficient [30, 33, 34].

AZT, an HIV-1 nucleoside reverse transcriptase inhibitor, was chosen as anti-HIV-1 agent because of our previous experience in synthesizing its effective prodrugs [30, 34]. AZT had been approved by the FDA and has been in clinical use for many years but its pharmacokinetic profile with jumps in blood levels results in toxicity and in emergence of HIV-1 resistant strains. On the other hand, nonnucleoside inhibitors of CMV are hydrophobic, have poor water solubility and some of them were cytotoxic. Therefore, we suggested that synthesizing mutual prodrugs may solve the problems of both antiviral agents. We decided to connect two active parts of prodrugs with acetic acid as a short linker. Six nonnucleoside inhibitors of CMV with linkers of different lengths, connecting uracil and p-bromophenoxy fragment, were used, and thus six corresponding target prodrugs were synthesized with good yields in two step-synthesis. The cytotoxicity and the antiviral activity of the compounds **1a-1f**, which were chosen as anti-CMV molecules in the design of our heterodimers, were all described in our previous publication [23].

We tested the efficiency of these six compounds in inhibiting HIV-1 replication in cell cultures of MT-4 cells, a lymphocytic cell line known to support a high level of HIV-1 replication, and in cultures of WS1 cells, a fibroblastic cell line known to support CMV replication. All six compounds were active with similar inhibitory capabilities against both HIV-1 and CMV and were generally not cytotoxic. To further characterize the biological activity of the heterodimers, we chose one of them, **3c**, for analysis in human tonsillar tissues *ex vivo*.

Specifically, we tested the antiviral activity and tissue cytotoxicity in a system of human lymphoid tissue *ex vivo*. This system of culture is an adequate model for preclinical drug

testing, as it does not require cell activation or stimulation. Moreover, this system retains the original cytoarchitecture of the tissue and the expression of key cell-surface molecules that are important for viral infections [30]. Although the range of viral production can be large in human tissue *ex vivo*, as observed in patients *in vivo*, the kinetics of replication are very similar from tissue-donor to tissue-donor [29].

Ex vivo human lymphoid tissues have previously been used for microbicide pretesting [29, 32]. By specifically investigating the effect of the representative compound **3c** on different T-cell subpopulations, we confirmed the low cytotoxicity of our chimeric compound in human lymphoid tissue.

The dual inhibitory activity of our compounds is an important feature, as the goal of developing such drugs is to interrupt the vicious circle of mutual facilitation between HIV-1 and CMV. Indeed, although both viruses have evolved complex strategies that manipulate our immune system to their own advantage, CMV infection alone is often asymptomatic. However, when present together, CMV infections can be particularly devastating, leading to severe organ disease and death. We found that in experimental control condition (no compound **3c**), the replication of both viruses was typical of viral replication observed in human tissue *ex vivo* [29]. In tissues treated with **3c**, both viruses were fully suppressed, thus confirming the hydrolysis of **3c** in two active compounds inside cells.

Similar results were also obtained with compound **4c**, for which AZT was replaced by 3TC, thus validating our strategy.

5. CONCLUSION

We designed, synthesized, and tested biological activities of new compounds active against HIV-1 and CMV, which is one of the major HIV-1 copathogens. The heterodimers described here are the first dual-targeted compounds supressing both HIV-1 and CMV. Further development and testing of the biological activity of such compounds and optimization of their pharmacokinetic profiles may lead to the development of dual-targeted anti CMV/HIV-1 antivirals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- We synthetized seven dual-targeted anti-CMV/HIV heterodimers.
- AZT or 3TC were used as anti HIV molecules while 1-[ω-(phenoxy)alkyl]uracil derivatives were used as anti CMV molecules.
- These compounds inhibited CMV and HIV respectively in WS1 and MT-4 cell cultures.
- Both AZT and 3TC-based heterodimers inhibited CMV and HIV replication in coinfected human lymphoid tissues.

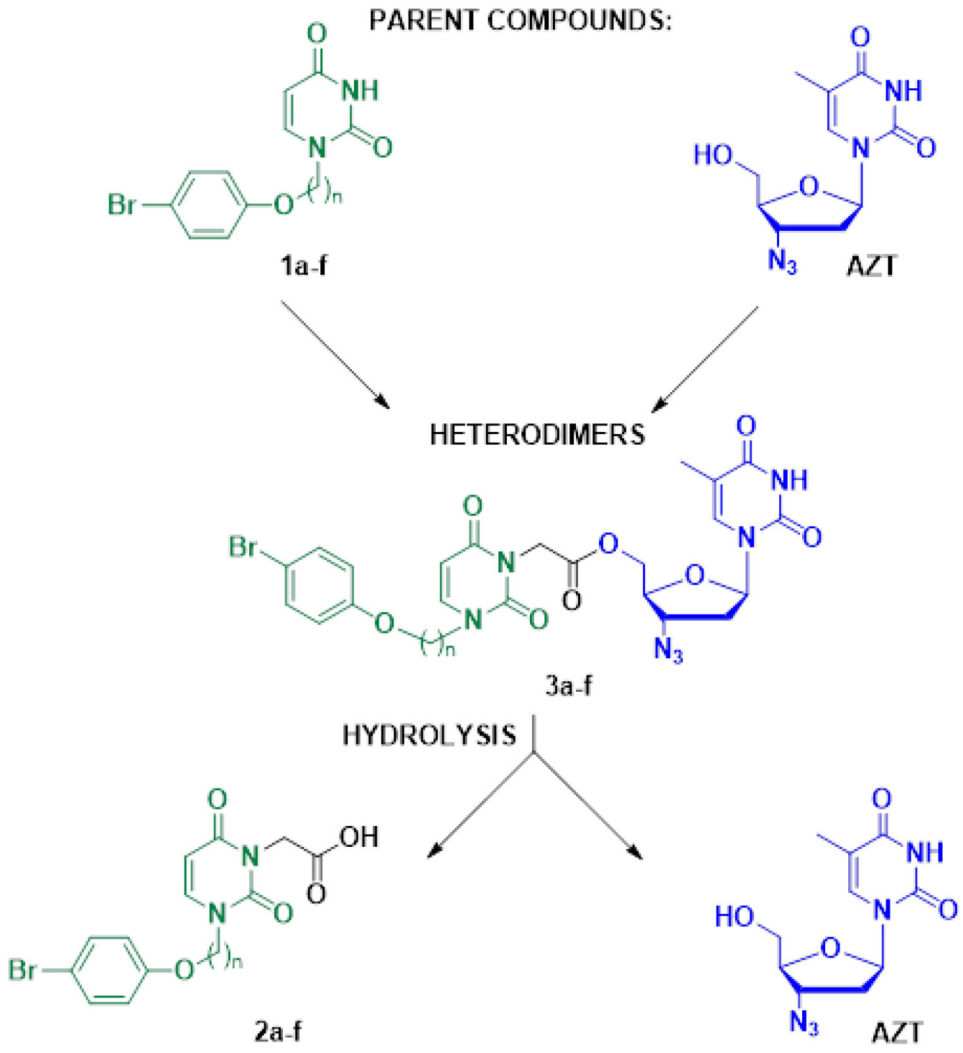


Figure 1. Structure of the compounds used for the synthesis of heterodimer 3c or resulting from its hydrolysis

The anti-CMV compound **1a-1f** were combined with the anti HIV-1 compound **AZT** to synthesize six AZT-based heterodimers **3a-3f**. Upon hydrolysis, heterodimers **3a-3f** generate **AZT** and the anti CMV compounds **2a-2f**. The anti HIV-1 activity of all heterodimers and **AZT** are presented in Table 1. The anti CMV compounds **1a-1f** had no anti HIV-1 activity, as previously reported [23]. The anti CMV compound **2c** (chosen as a representative of the group of compounds **2a-2f**) also did not have any anti HIV-1 activity (see Table 1). The anti CMV activities of the compounds **1a-1f** are in the range 4–20 μM [23]. The anti-CMV activity of the heterodimers **3a-3f** and compounds **2a-2f** are presented in Table 2. AZT does not have any anti CMV activity [28].

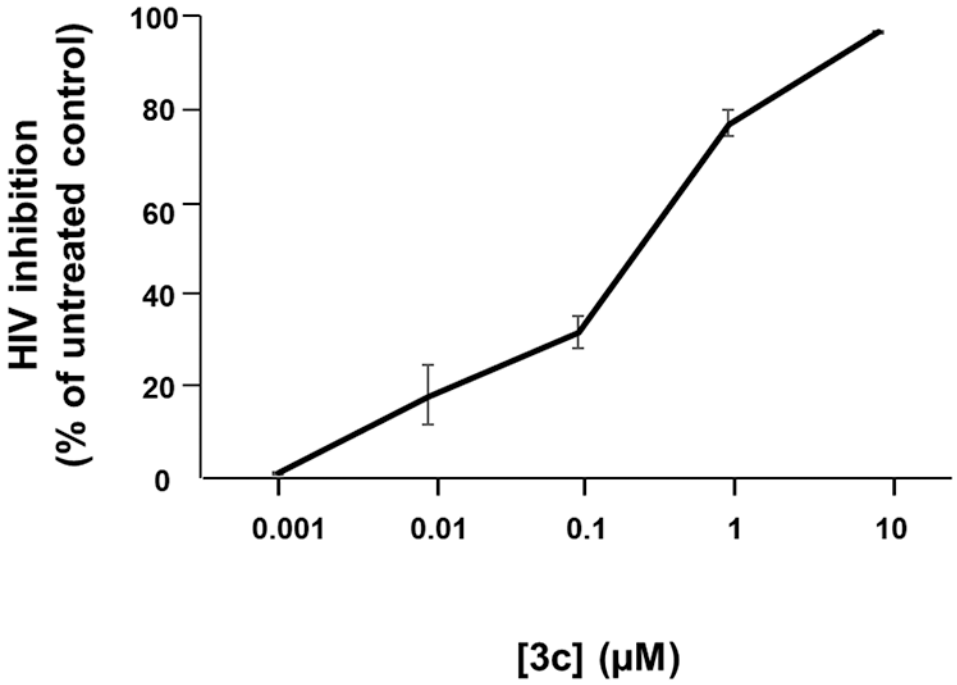


Figure 2. Inhibition by heterodimer 3c of HIV-1 replication in human tonsillar tissues

27 tonsillar blocks from each of 6 donors were infected with $X4_{LAI.04}$ and incubated with **3c** at five concentrations ranging from 1 nM to 10 μM for 12 days or used as untreated controls. We evaluated HIV-1 replication from $p24_{gag}$ core antigen present in tissue culture medium using a Luminex bead-based assay. The inhibition of HIV-1 was expressed as percentage of donor-matched untreated control for each concentration of **3c**. Presented are means ± SEM of HIV-1 inhibition.

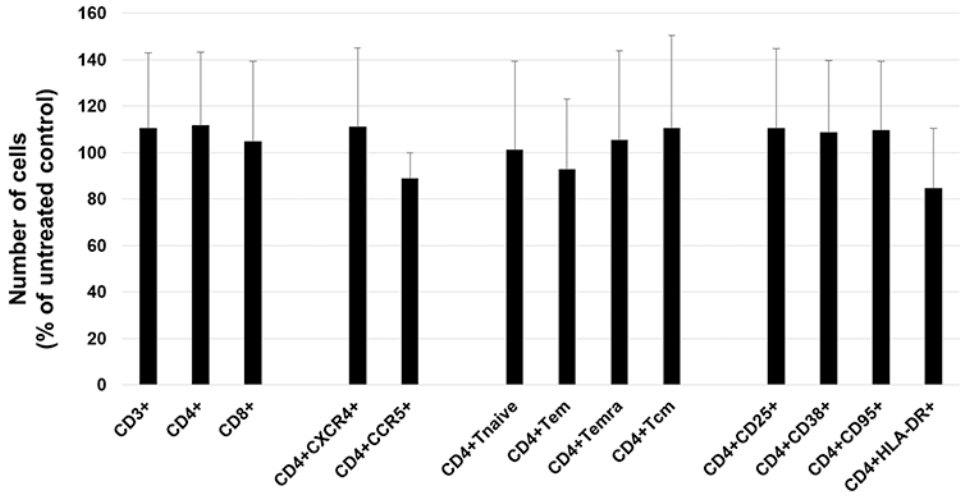


Figure 3. Cell toxicity of heterodimer-inoculated *ex vivo* tissue cultures

At day 12 of culture, 27 human lymphoid tissue blocks treated or not with **3c** were stained with anti-CD3QD655, anti-CD4QD605, anti-CD8QD800, anti-CD45RA-FITC, anti-CCR7-PECy7, anti-CXCR4-PE, and anti-CCR5-APC antibodies (stain 1) or stained with anti-CD3-QD655, anti-CD4-QD605, anti-CD8-QD800, anti-CD25-APC, anti-CD38-PE, anti-CD95-PE-Cy7, and anti-HLA-DR-BV570 antibodies (stain 2). Means ± SEM (error bars) of various cell subsets compared to matched untreated tissue performed with tissues from seven different donors are presented. Cell depletion for each cell subset was normalized by tissue-block weights.

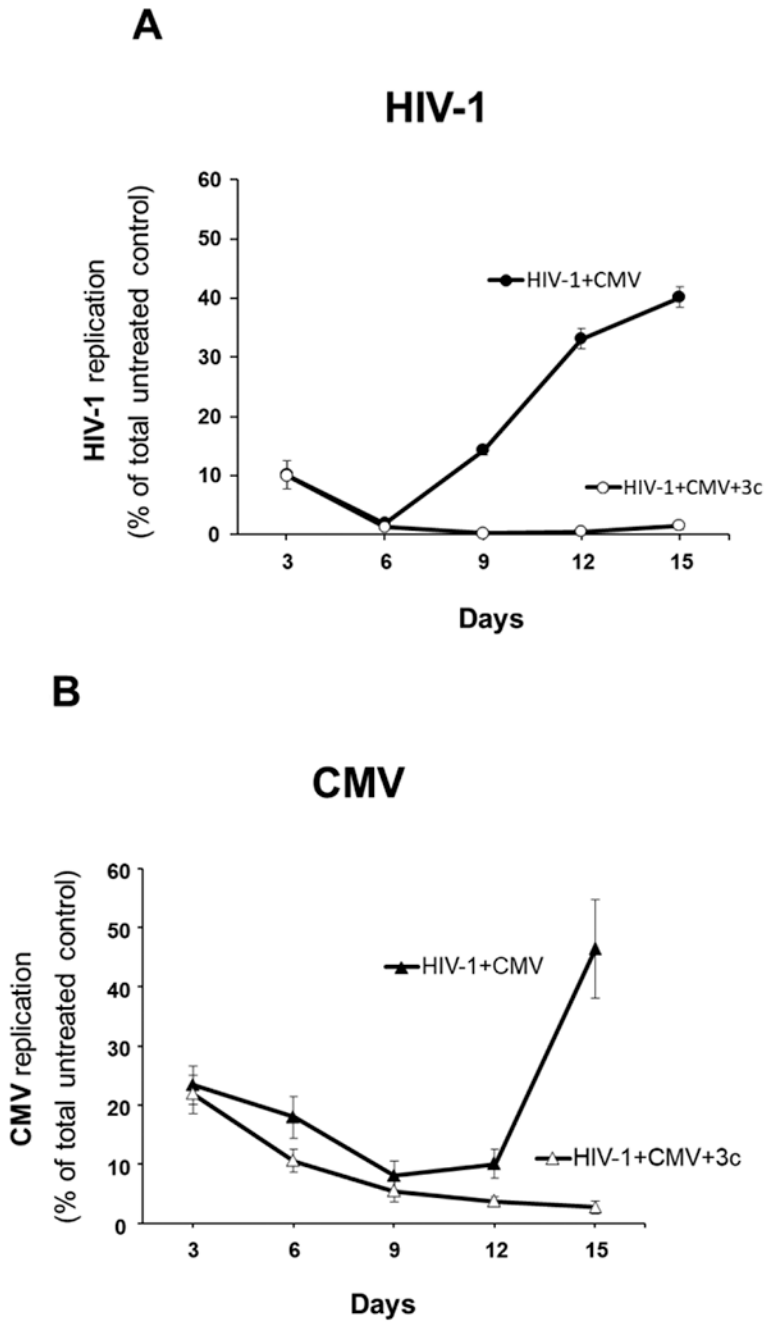
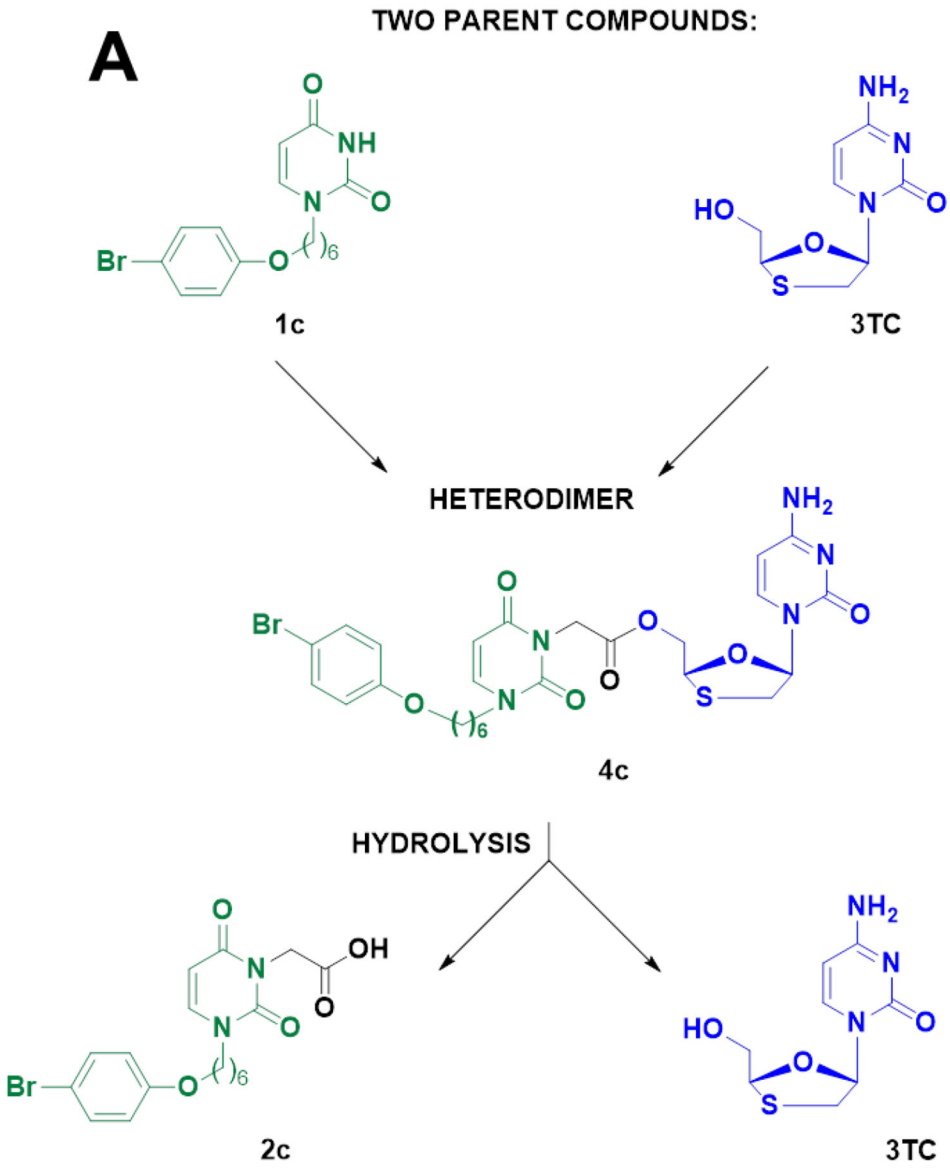


Figure 4. Suppression of HIV-1 and CMV replication in coinfected tonsillar tissues

(A) Blocks of human tissue were co-inoculated with HIV-1 ($X4_{LAI.04}$) and CMV (AD169) and cultured for 15 days at 37°C. Tissues were treated with **3c** at 10 μM or left untreated (control). We evaluated HIV-1 replication by measuring p24gag in culture medium, using a Luminex bead-based assay. (B) The replication of CMV was measured with real-time PCR for viral DNA accumulated in culture medium. For each day, presented are means ± SEM of viral replication as percent of cumulative virus produced in untreated control condition in 6 tonsillar tissues from 6 different donors.



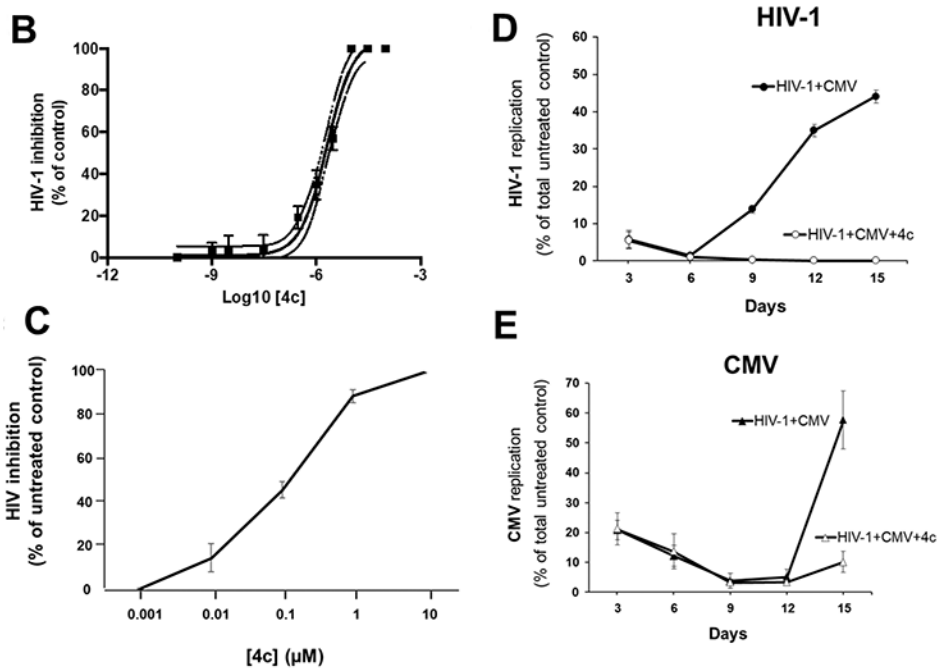
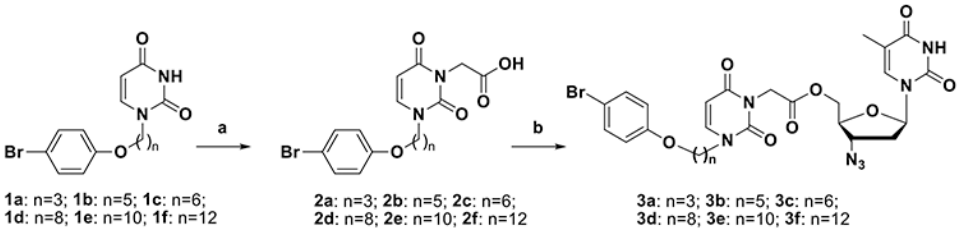


Figure 5. Inhibition of HIV-1 and CMV replication by heterodimer 4c

(A) Structure of the compounds used in the synthesis of the 3TC-based heterodimer **4c** or resulting from its hydrolysis. (B) Anti HIV-1 activity of heterodimer **4c** in MT-4 cell cultures. Graph was obtained by fitting the data points to a sigmoidal dose-response curve using Prism software. EC_{50} of **4c** in MT-4 cell cultures was 2.0 μM (95% CI (1.4–2.8). (C) Inhibition of HIV replication in human tonsillar tissues. Human tonsillar tissues *ex vivo* were inoculated with $X4_{LAI.04}$ and incubated with **4c** at five concentrations ranging from 1 nM to 10 μM for 12 days. HIV-1 inhibition was expressed as percentage of donor-matched untreated control for each concentration of **4c**. Means ± SEM of HIV-1 inhibition in tonsillar tissues blocks from 3 donors, relative to results for matched untreated tissues are presented. (D & E) Suppression of HIV-1 and CMV replication in coinfected tonsillar tissues. As for heterodimer **3c**, tonsils were coinfected with HIV-1 ($X4_{LAI.04}$) and CMV (AD169) and treated with **4c** 10 μM or left untreated (control). (D) The replication of HIV-1 was assessed by measuring p24gag using a Luminex bead-based assay. (E) Replication of CMV was evaluated by real-time PCR by measuring viral DNA in culture medium. For each day, means ± SEM of viral replication as percent of total virus produced in untreated control condition in tonsillar tissues from 3 different donors are presented.



Scheme 1.

Synthesis of conjugates **3a-f.** Reagents and conditions: (a) 1. $BrCH_2COOEt$, K_2CO_3, DMF; 2. LiOH, H_2O/EtOH; (b) AZT, DCC, Py.

Table 1.

Anti-HIV-1 activity and cytotoxicity of synthesized heterodimers and the products of their hydrolysis in the MT-4 cell line.

Compounds	Antiviral activity[a] EC_{50} ± SD (μM)	Cytotoxicity[b] CC_{50} (μM)
3a	1.96 ± 0.78	82.1
3b	0.61 ± 0.31	>100
3c	0.53 ± 0.29	>100
3d	0.23 ± 0.11	>100
3e	0.44 ± 0.29	>100
3f	0.21 ± 0.1	>100
AZT	3 nM (95%CI: 1-16)[1]	>100[1]
2c	>100	>100

[a] EC_{50}: 50% Effective concentration or compound concentration required to inhibit HIV-1 replication by 50%. (2 < n < 5)

[b] CC_{50}: 50% Cytotoxic concentration or compound concentration that decreases cell viability by 50%.

[1] See Vanpouille et al. (2014); Antiviral Res **109**, 125-131

Table 2.

Anti-CMV activity and cytotoxicity of synthesized heterodimers and the products of their hydrolysis in the human fibroblast WS1 cell line infected with CMV AD169.

Compounds	Antiviral activity[a] EC_{50} ± SD (μM)	Cytotoxicity[b] CC_{50} (μM)	Controls	Antiviral activity[a] EC_{50} ± SD (μM)	Cytotoxicity[b] CC_{50} (μM)
3a	3.2 ± 0.8	347	**2a**	NA	965
3b	4.4 ±2.1	173	**2b**	103.6±12.2	749.4
3c	7.9 ± 3.0	393	**2c**	78.1±18.3	724
3d	12.2 ± 1.7	600	**2d**	11.9±2.1	90.5
3e	3.2 ± 0.5	182	**2e**	NA	164
3f	5.1 ± 1.2	253	**2f**	NA	22
			GCV[c]	2.0 ± 0.5	500

[a] EC_{50}: 50% Effective concentration or compound concentration that inhibits CMV replication by 50% (n=3)

[b] CC_{50}: 50% Cytotoxic concentration or compound concentration required to decrease cell viability by 50%.

[c] The common anti-CMV drug ganciclovir (GCV) was used as control of the experimental system.