Adenosine Dioxolane Nucleoside Phosphoramidates as Antiviral Agents for Human Immunodeficiency and Hepatitis B Viruses

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BIOLOGICAL ASSAYS:

HIV Assay: human PBM cells were isolated by Ficoll-Hypaque discontinuous-gradient centrifugation from healthy seronegative donors. Cells were stimulated with phytohemagglutinin A (PHA) for 2 to 3 days prior to infection. AZT was included as positive control. The antiviral EC_{50} and EC_{90} were obtained from the concentration-response curve using the median effective method described previously.[1]

HBV Assay: using HepAD38 system: The HepAD38 cell line replicates HBV under conditions that can be regulated with tetracycline. In the presence of tetracycline, the supernatant is virtually free of viral DNA, but in the absence of tetracycline, these cells secrete HBV-like particles into the supernatant. The cells were incubated with no compound, test compound, or control drugs at concentrations up to 10 μM for 5 days. After a 5-day incubation, the cell supernatant was collected and stored at -70 °C until the HBV DNA was quantified. HBV DNA was extracted from the supernatant with DNeasy kit (Qiagen). The TaqMan probe and primers were designed by using Primer Express software (Applied Biosystems); their sequences cover highly conserved sequences complementary to the DNA sequences present in

HBsAg. A total of 5 µl of DNA was amplified by using the reagents and conditions described by the manufacturer (Applied Biosystems). All samples were performed in duplicates or triplicates.

Cellular toxicity assays: log-phase cells were seeded at 5 x 10^3 to 5 x 10^4 cells/well in 96-well plates containing 10-fold serial dilutions of test compound. The cultures were incubated for 2 to 4 days in a humidified 5% CO_2 at 37 °C, following which viability was determined by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Promega, Madison, WI). Cytotoxicity was also evaluated in HepG2 (human hepatoma) cells by the uptake of neutral red dye. The median 50% cytotoxic concentration was derived from the concentration-response curve using the median effect method.

Cellular Pharmacology:

Cell Treatment Procedure: Human PBM cells were exposed to media containing 50 µM nucleoside for 4 h at 37 °C. The drug containing media was then removed and cells were washed with 3 × PBS to remove extracellular drugs. The intracellular drugs and metabolites were extracted from 5 × 10(6) PBM cells using 1 mL 70% ice cold methanol (containing 20 nM of internal standard ddATP). Following extraction, samples were stored at -80 °C before drying. Prior to analysis, each sample was reconstituted in 200 µL mobile phase.

LC-MS/MS: An Ultimate 3000 HPLC system was used for separation. The processed samples were injected on a Hypersil GOLD column (100 × 1 mm) with a 3 µm particle size. Ion-pair method (Mobile phase A, consisted of 2 mM ammonium phosphate and 3 mM hexylamine, and mobile phase B, acetonitrile) was used for a gradient elution. Mobile phase B was increased from 5 to 80% in 13 min, kept at 80% for 4 min, and returned to initial conditions without ramp. The flow rate was maintained at 50 µL/min. Mass spectrometric detection was performed on a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with electrospray ionization source. Positive SRM detection mode was used with a spray voltage of 3.2 KV, sheath gas at 55 (arbitrary units), ion sweep gas at 0.3 (arbitrary

units), auxiliary gas at 5 (arbitrary units), and a capillary temperature of 380°C. The collision cell pressure was maintained at 1.5 mTorr.

Data Processing: Xcalibur 2.0 was used to perform the data analysis. The calibration curves were generated from standards of parent nucleosides, ProTides, and DOA-TP. The limits of quantification for these three analytes were 1, 1, and 5 nM, respectively.

EXPERIMENTAL SECTION:

Unless otherwise stated, all reactions were carried out under an atmosphere of dry argon or nitrogen in oven-dried glassware. Anhydrous solvents were purchased from EMD Chemicals Inc. (E. Merck, Darmstadt, Germany). Reagents and materials used were obtained from commercial suppliers unless noted otherwise. Thin layer chromatography (TLC) was carried out on silica gel GHLF 250 mm plates (No. 21521 from Analtech, Inc., Newark, DE, USA). Preparative layer chromatography (PLC) was employed for purification of some products (No. 02013 from Analtech, Inc., Newark, DE, USA). ^{1}H NMR spectra were taken on a Varian Unity Plus 400 spectrometer (Varian Inc., Palo Alto, CA, USA) at room temperature and reported in parts per million downfield from internal tetramethylsilane. Signal multiplicities are represented by s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quadruplet), br (broad), bs (broad singlet), and m (multiplet). All *J* values are in hertz. Mass spectral analyses were performed on a Micromass TOF instrument (Hewlett-Packard, HPLC driven electrospray MS instrument). Purity of final compounds was determined to be > 95%, using an analytical HPLC analyses performed on a Hewlett-Packard 1100 HPLC with a Phenomenex Gemini-NX column (2 mm x 50 mm, 3 μm, C18, 110 Å), elemental analyses and/or clean NMR spectra. Mobile phase flow was 0.5 mL/min with a 3.5 min initial hold, a 6.5 min gradient from 96% aqueous media (0.05% formic acid) to 96% CH_3CN (0.05% formic acid), and a 5.5 min total acquisition time. Photo diode array detection ranged from 190 to 360 nm.

General Procedure for Preparation of dichlorophosphoramidates (10) and (11). A solution of the substituted phenol (1 eq) and triethylamine (1 eq) in anhydrous ether was added drop-wise to a stirred

solution of $POCl_3$ (1 eq) at 0 °C over a period of 2 h. The reaction mixture was stirred overnight at room temperature. The triethylamine salt was filtered and the filtrate concentrated and used directly in the next step.

General Procedure for Preparation of chlorophosphoramidates (14), (15) and (16). Dichlorophosphoramidate (1 eq) and the amino acid (**12** or **13**) (1 eq) in anhydrous CH_2Cl_2, was cooled to -78 ° C. To the reaction mixture a solution of Et_3N (1 eq) in anhydrous CH_2Cl_2 was added slowly over a period approximately of 30 min to 1 h and stirred at room temperature overnight. Solvent was removed under reduced pressure and the resulting residue dissolved in ether and filtered. The filtrate was dried under reduced pressure to afford an oil.

General Procedure for the Preparation of Dioxolane Phosphoramidates

To a solution of the appropriate nucleoside analog (1 eq) in THF was added *t*-BuMgCl (3 eq) dropwise. The reaction mixture was stirred for 30 min at room temperature followed by the addition of phenyl ethoxyalaninyl phosphorochloridate (3.0 eq). The reaction mixture was stirred for 3 h then ethanol (2 mL) and ammonium chloride (5 mL) were added to the reaction. The reaction mixture was purified by column chromatography to afford product as a mixture of phosphorous diastereomers.

(-)-β-D-(((2*R*,4*R*)-4-(6-amino-2-chloro-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (3). A solution of **2** (0.20 g, 0.55 mmol) was dissolved in freshly prepared saturated methanolic ammonia (10 mL) and was stirred at room temperature for 10 h. The reaction mixture was then concentrated and column chromatography over silica gel MeOH: CH_2Cl_2 (1:10) gave the 6-amino analog **3** (0.96 g, 63 %) and the 6-methoxy analog **4** (0.02 g, 15%). The 6-amino analog **3**: ^{1}H NMR (DMSO, 400 MHz): δ 2.46 (s, 1H), 3.57 (brs, 2H), 4.25-4.29 (m, 1H), 4.15-4.18 (dd, *J* = 3.9 Hz, 1H), 4.48-4.46 (d, *J* = 7.5 Hz, 1H), 5.01 (s, 1H), 5.15 (s, 1H), 6.30 (d, *J* = 3.0 Hz, 1H), 7.7 (s, 2H), 8.26 (s, 1H). ^{13}C-NMR ($CDCl_3$): 62.5, 72.0, 80.3, 106.0, 117.9, 139.8, 151.2, 154.0, 157.8. HRMS (EI): *m/z* calcd for $C_9H_{10}ClN_5O_3$ $[M + H]^+$: 272.0550, found: 272.05449.

(-)-β-D-((2*R*,4*R*)-4-(2-chloro-6-methoxy-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (4). A

solution of **2** (0.20 g, 0.55 mmol) in methanol was added K_2CO_3 at room temperature for 1 h. The reaction goes to completion with trace formation of the 2,6-dimethoxy analog. The reaction mixture was dissolved in 10:90 (MeOH:CH_2Cl_2) and the solid was filtered through silica-gel MeOH:CH_2Cl_2 (1:20) to afford 2-chloro-6-methoxy analog **4** (0.16 g, 90%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.95 (brd, *J* = 4.8 Hz, 2H), 4.18 (s, 3H), 4.29 (q, *J* = 3.9 Hz, 1H), 4.48 (dd, *J* = 1.2 Hz, *J* = 2.7 Hz 1H), 5.20 (t, *J* = 1.8 Hz, 1H), 6.45 (dd, *J* = 1.2 Hz, *J* = 2.7 Hz, 1H), 8.35 (s, 1H). ^{13}C-NMR ($CDCl_3$): 19.6, 61.1, 71.2, 80.2, 106.4, 132.2, 144.8, 152.2, 152.9, 161.4. LC/MS (ESI): *m/z* 287.1 $[M + H]^+$.

(-)-β-D-((2*R*,4*R*)-4-(2-chloro-6-(cyclopropylamino)-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (5). A solution of **2** (0.5 g, 1.3 mmol) in cyclopropylamine (15 mL) was refluxed at 45 °C for 2 h. The reaction mixture was then concentrated and treated with saturated methanolic ammonia (10 mL) and stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure and then purified by column chromatography over silica gel MeOH: CH_2Cl_2 (1:20) to afford **5** (0.34 g, 79%). ^{1}H NMR (CD_3OD, 400 MHz): δ 0.60-0.63 (m, 2H), 0.84-0.86 (m, 2H), 1.09 (d, *J* = 5.1 Hz, 1H), 3.76-3.78 (m, 2H), 4.26 (q, *J* = 3.9 Hz, 1H), 4.49 (d, *J* = 4.8 Hz, 1H), 5.10 (t, *J* = 1.8 Hz, 1H), 6.39 (d, *J* = 3.9 Hz, 1H), 8.35 (s, 1H). ^{13}C-NMR ($CDCl_3$, 400 MHz): 18.7, 34.6, 42.6, 60.8, 71.5, 80.3, 105.9, 115.4, 134.2, 139.2, 149.4, 154.4; HRMS (EI): *m/z* calcd for $C_{12}H_{14}ClN_5O_3$ $[M + H]^+$: 312.0863, found 312.0857.

(-)-β-D-((2*R*,4*R*)-4-(6-amino-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (6). Compound **3** (0.30 g, 1.0 mmol) was dissolved in methanol (20 mL) and added 2N NaOH (1.5 mL) and catalytic amount of 10% Pd/C. The suspension was stirred in a Parr apparatus under an atmosphere of H_2 (50 psi) for 7 h. The catalyst was removed by filtration, the reaction mixture was then concentrated and column chromatography over silica gel afforded **6** (0.20, 82%). ^{1}H NMR (CD_3OD, 400 MHz): δ 3.78 (m, 2H), 4.29 (q, *J* = 5.6 Hz, 1H), 4.50 (dd, *J* = 1.2 Hz, *J* = 11.2 Hz 1H), 5.13 (t, *J* = 2.4 Hz, 1H), 6.49 (dd, *J* = 1.2 Hz, *J* = 2.8 Hz, 1H), 8.18 (s, 1H), 8.44 (s, 1H). HRMS (EI): *m/z* calcd for $C_9H_{11}N_5O_3$ $[M + H]^+$: 238.0940, found: 238.0938.

(-)-β-D-((2*R*,4*R*)-4-(6-methoxy-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (7). Synthesized from **4** using similar conditions to compound **6**; (0.20, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.29 (t, *J* = 4.8 Hz, 1H), 3.94 (dd, *J* = 1.6 Hz, *J* = 4.8 Hz, 2H), 4.17 (s, 3H), 4.34 (q, *J* = 6.0 Hz, 1H), 4.52 (dd, *J* = 2.0 Hz, *J* = 7.6 Hz 1H), 5.25 (t, *J* = 2.4 Hz, 1H), 6.45 (dd, *J* = 2.4 Hz, *J* = 2.8 Hz, 1H), 8.25 (s, 1H), 8.52 (s, 1H). HRMS (EI): *m/z* calcd for $C_{10}H_{12}N_4O_4$ $[M + H]^+$: 253.0937, found: 253.0936.

(-)-β-D-((2*R*,4*R*)-4-(6-(cyclopropylamino)-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methanol (8). Synthesized from **5** using similar conditions to compound **6**; (0.42, 79%). ^{1}H NMR (CD_3OD, 400 MHz): δ 0.59-0.63 (m, 2H), 0.84-0.89 (m, 2H), 2.92 (brs, 1H), 3.32 (s, 1H), 3.77-3.78 (m, 2H), 4.28 (q, *J* = 5.2 Hz, 1H), 4.51 (dd, *J* = 0.8 Hz, *J* = 10 Hz, 1H), 5.12 (t, *J* = 2.4 Hz, 1H), 6.45 (d, *J* = 4.4 Hz, 1H), 8.26 (s, 1H), 8.40 (s, 1H). HRMS (EI): *m/z* calcd for $C_{12}H_{15}N_5O_3$ $[M + H]^+$: 278.1253, found: 278.1252.

(2*S*)-ethyl 2-(((((2*R*,4*R*)-4-(6-amino-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate (17). Yield: (0.07 g, 70%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.15-1.33 (m, 6H), 2.37 (s, 1H), 3.85-4.02 (m, 1H), 4.04-4.03 (m, 4H), 4.26-4.34 (m, 2H), 4.95-4.53 (m, 1H), 5.27-5.29 (m, 1H), 6.13-6.15 (m, 2H), 6.48 (m, 1H), 7.08-7.27 (m, 5H), 8.14 (s, 1H), 8.29 (s. 1H). HRMS (EI): *m/z* calcd for $C_{20}H_{25}N_6O_7P$ $[M + H]^+$: 493.1601, found: 493.1603.

Ethyl 3-(2-(((((2*R*,4*R*)-4-(6-amino-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate (18). Yield: (0.06 g, 51%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.14-1.35 (m, 9H), 2.53-2.63 (m, 2H), 2.89-2.96 (m, 2H), 3.79-3.94 (m, 1H), 4.01-4.10 (m, 6H), 4.30-4.37(m, 2H), 4.65-4.67 (m, 1H), 5.33-5.36 (m, 1H), 6.48-6.51 (m, 1H), 6.98-7.43 (m, 4H), 8.20 (s, 1H), 8.21 (s, 1H), 8.31 (s. 1H), 8.34 (s, 1H). HRMS (EI): *m/z* calcd for $C_{25}H_{33}N_6O_9P$ $[M + H]^+$: 593.2125, found: 593.2125.

(2*S*)-ethyl 2-(((((2*R*,4*R*)-4-(6-methoxy-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate (19). Yield: (0.06 g, 68%). ^{1}H NMR (CD_3Cl_3, 400 MHz): δ 1.17-1.35 (m, 6H), 3.79-3.97 (m, 2H), 4.08-4.17 (m, 4H), 4.29-4.33 (m, 5H), 4.52-4.57 (m, 1H), 5.29-5.33(m, 1H), 6.29-6.63 (m, 1H), 7.07-7.30 (m, 5H), 8.24 (s, 1H), 8.25 (s. 1H), 8.51(s, 1H) and

8.52 (s, 1H). HRMS (EI): *m/z* calcd for $C_{21}H_{26}N_5O_8P$ $[M + H]^+$: 508.1597, found: 508.1601.

Ethyl 3-(2-(((((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)(((2*R*,4*R*)-4-(6-methoxy-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)phosphoryl)oxy)phenyl)propanoate (20). Yield: (0.06 g, 55%). ^{1}H NMR (CD_3Cl_3, 400 MHz): δ 1.15-1.38 (m, 9H), 1.84 (brs, 1H), 2.55-2.61 (q, *J* = 8.0 Hz, 1H), 2.89-2.96 (m, 1H), 3.79-3.84 (m, 1H), 4.06-4.15 (m, 8H), 4.27-4.33 (m, 3H), 4.53-4.58 (m, 1H), 5.29-5.32 (m, 1H), 6.51-6.54 (m, 1H), 7.04-7.34 (m, 4H), 8.21 (s, 1H), 8.23 (s. 1H), 8.49 (s, 1H) and 8.50 (s, 1H). HRMS (EI): *m/z* calcd for $C_{26}H_{34}N_5O_{10}P$ $[M+H]^+$: 608.2122, found: 608.2127.

Isopropyl 3-(2-(((((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)(((2*R*,4*R*)-4-(6-methoxy-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)phosphoryl)oxy)phenyl)propanoate (21). Yield: (0.07 g, 53%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.12-1.36 (m, 15H), 2.09 (brs, 1H), 2.51-2.88 (m, 2H), 2.88-2.95 (m, 2H), 3.81-3.97 (m, 2H), 4.12-4.13 (m, 3H), 4.26-4.31 (m, 3H), 4.51-4.56 (m, 1H), 4.89-4.97 (m, 2H), 5.27-5.31 (m, 1H), 6.50-6.52 (m, 1H), 6.98-7.32 (m, 4H), 8.19 (s, 1H), 8.22 (s. 1H), 8.47 (s, 1H) and 8.48 (s, 1H); ^{31}P NMR (400 MHz, $CDCl_3$): δ 4.23, 3.98; HRMS (EI): *m/z* calcd for $C_{28}H_{38}N_5O_{10}P$ $[M+H]^+$: 636.2435, found: 636.2441.

(2*S*)-ethyl 2-(((((2*R*,4*R*)-4-(6-(cyclopropylamino)-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate (22). Yield: (0.07 g, 74%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.61-0.66 (m, 2H), 0.89-0.92 (m, 2H), 1.18-1.35 (m, 5H), 1.33-1.35 (m, 1H), 3.0 (brs, 1H), 3.46-3.79 (m,1), 3.89-3.98 (m, 2H), 4.08-4.14 (m, 2H), 4.28-4.34 (m, 3H), 4.50-4.57 (m, 1H), 5.29-5.33 (m, 1H), 6.0 (s, 1H), 6.48-6.51 (m, 1H), 7.08-7.30 (m, 5H), 8.07 (s, 1H), 8.44 (s. 1H). HRMS (EI): *m/z* calcd for $C_{23}H_{29}N_6O_7P$ $[M+H]^+$: 533.1914, found: 533.1917.

Ethyl 3-(2-(((((2*R*,4*R*)-4-(6-(cyclopropylamino)-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)(((*S*)-1-ethoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate (23). Yield: (0.06 g, 60%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.61-0.65 (m, 2H), 0.88-0.92 (m, 2H), 1.17-1.25 (m, 9H), 1.35-1.37 (m, 1H), 2.56-2.64 (m, 2H), 2.90-2.98 (m, 2H), 3.72-4.03 (m, 1H), 4.08-4.14 (m, 4H), 4.25-4.33 (m, 4H), 4.52-4.54 (m, 1H), 5.28-5.30(m, 1H), 6.0 (s, 1H), 6.47-6.50 (m, 1H), 6.99-7.24 (m, 4H), 8.06 (s, 1H),

8.07 (s, 1H), 8.43 (s, 1H), 8.44 (s. 1H). LC/MS (ESI): *m/z* calcd for $C_{28}H_{37}N_6O_9P$: 633.0, $[M + H]^+$.

Isopropyl 3-(2-(((((2*R*,4*R*)-4-(6-(cyclopropylamino)-9*H*-purin-9-yl)-1,3-dioxolan-2-yl)methoxy)((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)phosphoryl)oxy)phenyl)propanoate (24). Yield: (0.08 g, 65 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.58-0.60 (m, 2H), 0.54-0.88 (m, 2H), 1.18-1.16 (m, 12H), 1.21-1.32 (m, 2H), 2.50-2.57 (m, 2H), 2.87-2.96 (m, 3H), 3.87-4.14 (m, 1H), 4.23-4.32 (m, 4H), 4.48-4.51 (m, 1H), 4.88-4.95 (m, 2H), 5.25-5.28 (m, 1H), 6.27 (brs, 1H), 6.32 (brs, 1H), 6.44-6.47 (m, 1H), 6.95-7.23 (m, 4H), 8.04 (s, 1H), 8.40 (s. 1H); ^{31}P NMR (400 MHz, $CDCl_3$): δ 4.28, 4.03. HRMS (EI): *m/z* calcd for $C_{30}H_{41}N_6O_9P$ $[M+H]^+$: 661.2751, found: 661.2758.

[1] Chou, T. C.; Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **1984**, *22*, 27-55.