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Phosphatidyl-2′,3′-dideoxy-3′-thiacytidine: synthesis and antiviral activity in hepatitis B- and HIV-1-infected cells

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Abstract

We recently found that phosphatidyl-2′,3′-dideoxycytidine (phosphatidyl-ddC) had substantial anti-hepatitis B virus (HBV) activity in vitro compared to 2′,3′-dideoxycytidine (ddC) (Hostetler et al. (1994) Antiviral Res. 24, 59–67). Upon administration of liposomal phosphatidyl-ddC to mice, a 40-fold higher drug area under curve was observed in the liver. To evaluate the possibility of using liver-targeted anti-HBV nucleosides to treat woodchuck hepatitis virus, we wanted to find the most potent and selective lipid conjugates. It has been shown that 2′,3′-dideoxy-3′-thiacytidine as a racemic mixture of the *cis*-isomer (*cis*-(±)-BCH-189) has much greater activity against HBV viruses than ddC in vitro. Recently, it was shown that the (−)-β-L-enantiomer (3TC) is more active and less toxic than the (+)-β-D-form ((+)-BCH-189). To determine whether phospholipid conjugates of 3TC retain antiviral activity in 2.2.15 cells as demonstrated previously with ddC, we

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synthesized the 1,2-dipalmitoyl-*sn*-glycerol-3-phosphate conjugates of (±)-BCH-189 and 3TC and assessed their anti-HBV and anti-HIV activities, in vitro. Phosphatidyl-3TC and phosphatidyl-BCH-189 had antiviral activity comparable to the respective free drugs in 2.2.15 cells which chronically produce HBV. In HIV-1-infected human peripheral blood mononuclear cells and HT4-6C cells, phosphatidyl-3TC and phosphatidyl-(±)-BCH-189 exhibited significantly lower activity than the corresponding free nucleosides. In view of the documented ability of phosphatidyl-ddC to target drug to the liver, it seems reasonable to expect that phosphatidyl-3TC or phosphatidyl-(±)-BCH-189 could be employed to provide greatly enhanced hepatic antiviral activity in HBV infection in vivo.

Keywords: Hepatitis B virus; Human immunodeficiency virus type 1; Phosphatidyl-2′,3′-dideoxy-3′-thiacytidine; Liver targeting

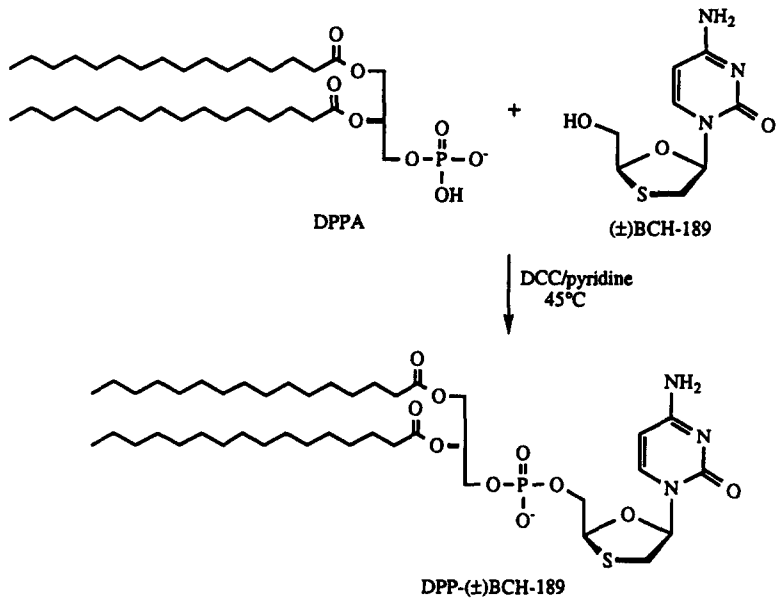
1. Introduction

2′,3′-Dideoxy-3′-thiacytidine (BCH-189) is a synthetic nucleoside analog in which the 3′-carbon has been replaced by a sulfur atom (Wainberg et al., 1990). As a racemic mixture of the *cis*-isomers, *cis*-(±)-BCH-189 was found to show activity against human hepatitis B virus (HBV) (Doong et al., 1991) as well as human immunodeficiency viruses (HIV) in culture (Greenberg et al., 1990; Wainberg et al., 1990; Soudeyns et al., 1991; Schinazi et al., 1992a). Further studies have shown that the (−)-β-L-enantiomer (3TC), the unnatural enantiomer, is responsible for the antiviral effect and that some of the cytotoxicity is caused by the corresponding (+)-enantiomer ((+)-BCH-189) (Chang et al., 1992a,b; Coates et al., 1992; Schinazi et al., 1992a,b). 3TC is currently undergoing clinical evaluation in AIDS and HBV infection (Dienstag et al., 1994). Recently, we found that lipid prodrugs of 2′,3′-dideoxycytidine (ddC) such as dioleoylphosphatidyl-2′,3′-dideoxycytidine (DOP-ddC) demonstrate increased selectivity in 2.2.15 cells which chronically produce HBV in culture, primarily due to decreased toxicity (Hostetler et al., 1994a). DOP-ddC, when administered intraperitoneally in liposomes to mice, provided a 40-fold increase drug area under curve in the liver over 24 h versus an equimolar dose of ddC (Hostetler et al., 1994a). We are seeking a highly effective and selective antiviral nucleoside to do testing in woodchuck hepatitis virus comparing free nucleoside and its liposomal lipid conjugates. Therefore, it was of interest to evaluate whether lipid prodrugs of 3TC and its racemic mixture, BCH-189 also exhibit this effect. We now report the synthesis of dipalmitoylphosphatidyl derivatives of 3TC and BCH-189 and their antiviral activity in HBV- and HIV-1-infected cells.

2. Materials and methods

2.1. Synthesis of phospholipid conjugates of (±)-BCH-189 and 3TC

The synthesis of dipalmitoylphosphatidyl-(±)-BCH-189 (DPP-(±)-BCH-189) is outlined in Scheme 1.



Scheme 1. Synthesis of dipalmitoylphosphatidyl-(±)-2′,3′-dideoxy-3′-thiacytidine (DPP-(±)-BCH-189).

Both 3TC and (±)-BCH-189 were synthesized as previously described (Choi et al., 1991; Hoong et al., 1992). Dipalmitoylphosphatidic acid monosodium salt (DPPA-Na) (400 mg, 0.59 mmol; Nippon Fine Chemicals, Japan) was converted to the free acid as previously described (Hostetler et al., 1990) followed by conversion to the pyridinium salt by co-evaporation with pyridine; (±)-BCH-189 (95 mg, 0.41 mmol) was added and the mixture was dried by co-evaporation with pyridine 3 times and dissolved in 6 ml of anhydrous pyridine at 45°C. Dicyclohexylcarbodiimide (300 mg, 1.45 mmol) was added and the mixture was stirred under nitrogen at 45°C for 48 h. Pyridine was removed by evaporation under vacuum and the residue was dried in vacuo with toluene twice. The residue was then dissolved in chloroform (10 ml) and filtered to remove dicyclohexylurea. The concentrated filtrate was loaded on a DEAE Sephadex A-25 (HCO_3^- form) column (2.9 × 5.2 cm). The column was first washed with $CHCl_3/MeOH/H_2O$ (2:3:1, 200 ml), then eluted with a linear gradient of 0–0.2 M NH_4HCO_3 in $CHCl_3/MeOH/H_2O$ (2:3:1, 500 ml). The appropriate fractions were pooled and concentrated and further purified on a silical gel column (2 × 6 cm) eluted with $CHCl_3/MeOH/28\%$ NH_4OH/H_2O (75:25:4:1) (100 ml) to give 52 mg (15%) of the desired product, DPP-(±)-BCH-189, as a single UV and phosphorus-positive spot on TLC by phosphorus spray (R_f 0.55: Analtech silica gel plate, $CHCl_3/CH_3OH/28\%$ NH_4OH/H_2O, 70:38:8:2 by volume). The structure of the product was confirmed by ^{1}H-NMR and UV spectra. ^{1}H-NMR ($CDCl_3/CD_3OD$ 10:1) δ ppm 0.88 (t, 6H, CH_3), 1.26 (brs, 48H, $24CH_2$), 1.60 (brs, 4H, β-CH_2), 2.32 (m, 4H, α-CH_2), 3.3–3.6 (m, 4H, CH_2-*sn*-1,3), 4.02 (brs, 2H, H-2′), 4.40 (dd, 2H, H-5′), 5.25 (brs, 1H, CH-*sn*-2), 5.38 (brs, 1H, H-4′), 6.10 (d, 1H, H-5), 6.28 (brs, 1H, H-1′), 8.39 (d, 1H, H-6). λ_{max} ($CHCl_3$)

280.9 nm ($\epsilon = 10.69 \times 10^3$). Elemental analysis calculated for $C_{43}H_{78}N_3O_{10}PS.2\ H_2O$: C, 57.63; H, 9.22; N, 4.69. Found: C, 57.82; H, 8.76; N, 4.67. DPP-3TC was synthesized and purified similarly and its physical and chemical characteristics such as R_f value on TLC and proton NMR were identical to that of DPP-(±)-BCH-189.

2.2. *Dipalmitoylphosphatidyl-3TC containing liposomes for in vitro experiments*

DPP-3TC and DPP (±)-BCH-189 were dried with dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG) and cholesterol (Chol) at a molar ratio of DOPC/DOPG/Chol/drug of 50:10:30:10 in a small serum vial (2 ml). The dried lipid film was hydrated with sterile isotonic sorbitol containing 5 mM sodium acetate (pH 5.4), sealed and sonicated in a cup horn of a Heat Systems sonicator at 45°C for 30 min at maximum output to provide small unilamellar vesicles. A lipid control (DOPC/DOPG/Chol, 60:10:30 mol%) matched to the 30 μM DPP-ddG sample for total lipid content was prepared in the same manner.

2.2.1. *2.2.15 cells and assessment of drug effects on HBV-specific DNA*

(±)-BCH-189 and 3TC (both as 5 mM aqueous solutions) and their lipid conjugates, DPP-(±)-BCH-189 and DPP-3TC (formulated as described above), were added to wells containing 2.2.15 cells (clonal cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) (Sells et al., 1988). After 9 days HBV-specific virion DNA in the medium and cellular replication intermediates were measured by DNA probe assays as reported previously (Korba and Gerin, 1992).

2.2.2. *HT4-6C cells and plaque assay of drug effects on HIV replication*

The effect of antiviral compounds on HIV replication was measured by a plaque-reduction assay (Chesebro and Wehrly, 1988; Larder et al., 1989). Briefly, monolayers of HT4-6C cells (Bruce Chesebro, Hamilton, MT) were infected with 100–200 PFU of virus per well in 24-well microdilution plates. Various concentrations of drug were added to the culture medium. After 3 days at 37°C, the monolayers were fixed with 10% formaldehyde solution in phosphate-buffered saline and stained with 0.25% crystal violet to visualize virus plaques. Antiviral activity in drug-treated samples was plotted as the percentage of the no-drug control plaques and the EC_{50} and EC_{90} values were obtained as previously described (Hostetler et al., 1992).

2.2.3. *Anti-HIV effect of drugs in primary human lymphocytes acutely infected with HIV-1 (strain LAI)*

Uninfected phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PBMC) were uniformly distributed in 25-cm^2 flasks to give a 5-ml suspension containing about 2×10^6 cells per ml. Suitable dilutions of HIV-1 were added to infect the cultures. The mean reverse transcriptase (RT) activity of the inoculum was 50,000 dpm/ml which is equivalent to about 100 $TCID_{50}$ (50% tissue culture infective doses) in PBMC (Groopman et al., 1987). The tested drugs at twice their final concentrations in 5 ml of RPMI 1640 medium supplemented as described above, were added to the cultures. Uninfected and untreated PBMC were grown in parallel at equivalent cell

concentrations as controls. The cultures were maintained in a humidified 5% CO_2–95% air incubator at 37°C for 6 days after infection, at which point all cultures were assayed for supernatant RT activity as described previously (Schinazi et al., 1988).

3. Results and discussion

3TC and its racemic mixture (±)-BCH-189, were potent and selective inhibitors of HBV replication in 2.2.15 cells (Table 1). The selectivity of 3TC was greater than (±)-BCH-189, 4030–6280 versus 620–1120, due to increased efficacy and reduced toxicity. Interestingly, dipalmitoylphosphatidyl-(±)-BCH-189 (DPP-(±)-BCH-189) was more effective and less toxic than free (±)-BCH-189, giving a selectivity index of 1280–1960 versus 620–1120. The increased selectivity of DPP-(±)-BCH-189 was somewhat more pronounced when considering its effects on HBV cellular replicative intermediates. DPP-3TC was similar to free 3TC in both efficacy and toxicity. Both compounds and their lipid prodrugs were substantially more active and selective than ddC (30- to 212-fold). A lipid control consisting of liposomes without DPP-3TC had no effect on measures of HBV replication to a 100 μM concentration (data not shown) and its cytotoxicity was not significant to concentrations of 1000 μM (Hostetler et al., 1994a).

Table 1
Toxicity and antiviral activity of test compounds against HBV DNA replication in 2.2.15 cells

Compound	TC_{50} (μM)	EC_{50} (nM)	EC_{90} (nM)	Selectivity index (TC_{50}/EC_{90})
2′,3′-ddC [c]	231 ± 24	1100 ± 100 [a]	6200 ± 600 [a]	37 [a]
		2300 ± 300 [b]	12200 ± 2300 [b]	19 [b]
DOP-ddC [c]	1000	n.d.	18000	56
		n.d.	23000	43
3TC	1180 ± 125	33 ± 4	188 ± 20	6280
		76 ± 8	293 ± 31	4030
DPP-3TC	1114 ± 127	30 ± 4	214 ± 23	5210
		68 ± 8	290 ± 44	3840
(±)-BCH-189	496 ± 54	76 ± 7	444 ± 38	1120
		144 ± 15	803 ± 8	620
DPP-(±)-BCH-189	784 ± 77	69 ± 8	401 ± 46	1960
		109 ± 14	612 ± 69	1280

Data are expressed as mean ± S.D. ($n = 4$). TC_{50} is the concentration of drug which reduces cell viability by 50% and EC_{50} and EC_{90} are the concentrations of drug which reduce HBV virion (extracellular DNA) (upper line) or HBV replicative intermediate (intracellular DNA) (lower line) by 50 or 90%, respectively. Viable cell number was determined using neutral red as previously described (Hostetler et al., 1994a). n.d., not determined.

[a] For HBV virion (extracellular DNA), upper line.

[b] For HBV RI (intracellular DNA), lower line.

[c] Data adapted from Hostetler, et al., 1994a.

Table 2
Effect of 3TC and its lipid prodrugs on HIV-1 replication

Compound	Antiviral efficacy		Toxicity
	PBMC EC_{50}	HT4-6C EC_{50}	CEM cells TC_{50}
DPP-(±)-BCH-189	1.09	4.0	200
DPP-3TC	0.80	0.38	100
(±)-BCH-189	0.02–0.06	0.11	52.6 [a]
3TC	0.002–0.07	0.08	> 100 [a]

Data are the average of two determinations. EC_{50}, TC_{50} data are μM and represent the concentration of compound which inhibits viral replication by 50% or reduces viable cell number by 50%. n.d., not determined. PBMC, human peripheral blood mononuclear cells; DPP-(±)-3TC, dipalmitoylphosphatidyl-(±)-BCH-189; 3TC, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine.
[a] Taken from Schinazi et al., 1992a.

The effects of 3TC, (±)-BCH-189 and their corresponding prodrugs on HIV-1 replication in HT4-6C cells and in primary human PBMC are summarized in Table 2. The results indicate that the lipid derivatives exhibited substantially less activity than the respective free nucleosides in HIV-infected human PBMC. In HT4-6C cells, the activity of DPP-3TC was 21% of that of 3TC and DPP (±)-BCH-189 was 2.8% of the (±)-BCH-189. This contrasted with our earlier findings with phosphatidyl-ddC and ddC showing roughly equivalent activity in HT4-6C cells (Hostetler et al., 1994b).

In summary, phospholipid derivatives of (±)-BCH-189 and 3TC have been synthesized by coupling the nucleoside analog with dipalmitoylphosphatidic acid. In 2.2.15 cell assays based on reductions in HBV-specific DNA, DPP-(±)-BCH-189 was more potent and less toxic than the free drug, while DPP-3TC was comparable to 3TC in antiviral activity and selectivity. DPP-3TC is the most potent and selective anti-HBV lipid prodrug synthesized and tested to date. By analogy to phosphatidyl-ddC (Hostetler et al., 1994a,b), we predict that high degrees of liver targeting can be achieved by administering DPP-3TC in a liposomal formulation and conclude that compounds of this type may be useful in treating HBV infection if oral administration of the free nucleoside is less than fully successful. Preliminary phase II results indicate that only a small percentage of patients (16%) have sustained responses to oral lamivudine therapy (Dienstag et al., 1994). It would be of interest to determine if targeted hepatic delivery of DPP-3TC would improve the duration of the response in animal models, such as woodchuck hepatitis virus. Preliminary studies in woodchucks with phosphatidyl-ddG indicate that serum WHV DNA remains low for 1–2 weeks after discontinuance of dosing, suggesting that low frequency dosing regimens might be possible using a liver-targeted agent (H. Xie, B.E. Korba, B.C. Tennant and K.Y. Hostetler, submitted for publication).

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