

Synthesis, physicochemical and pharmacokinetic studies of potential prodrugs of β -L-2'-deoxycytidine, a selective and specific anti-HBV agent

Claire Pierra¹, Samira Benzaria¹, David Dukhan¹, Anna Giulia Loi², Paolo La Colla³, Edward Bridges⁴, John Mao⁴, David Standing⁴, Jean-Pierre Sommadossi⁴ and Gilles Gosselin^{1*}

¹Laboratoire Coopératif Idenix-CNRS-Université Montpellier II, Montpellier, France

²Cooperative Laboratory Idenix – University of Cagliari, Cagliari, Italy

³Dipartimento di Scienze e Tecnologie Biomediche, Università degli Studi di Cagliari, Cagliari, Italy

⁴Idenix Pharmaceutical, Inc., Cambridge, Mass., USA

*Corresponding author: Tel: +33 4 671 43855; Fax: +33 4 675 49610; E-mail: gosselin@univ-montp2.fr

Dedicated to the memory of Martin Bryant

β -L-2'-Deoxycytidine (β -L-dC) is a potent, selective and specific anti-hepatitis B virus (HBV) agent. To improve its oral bioavailability, several derivatives involving sugar or base acylation, as well as *N*⁴-derivatization with an *N,N*-(dimethylamino)methylene function, were synthesized. The physicochemical characteristics (including chemical stabilities, solubilities and distribution coefficient values) and pharmacokinetics of these

compounds were determined and compared with those of the parent drug, β -L-dC.

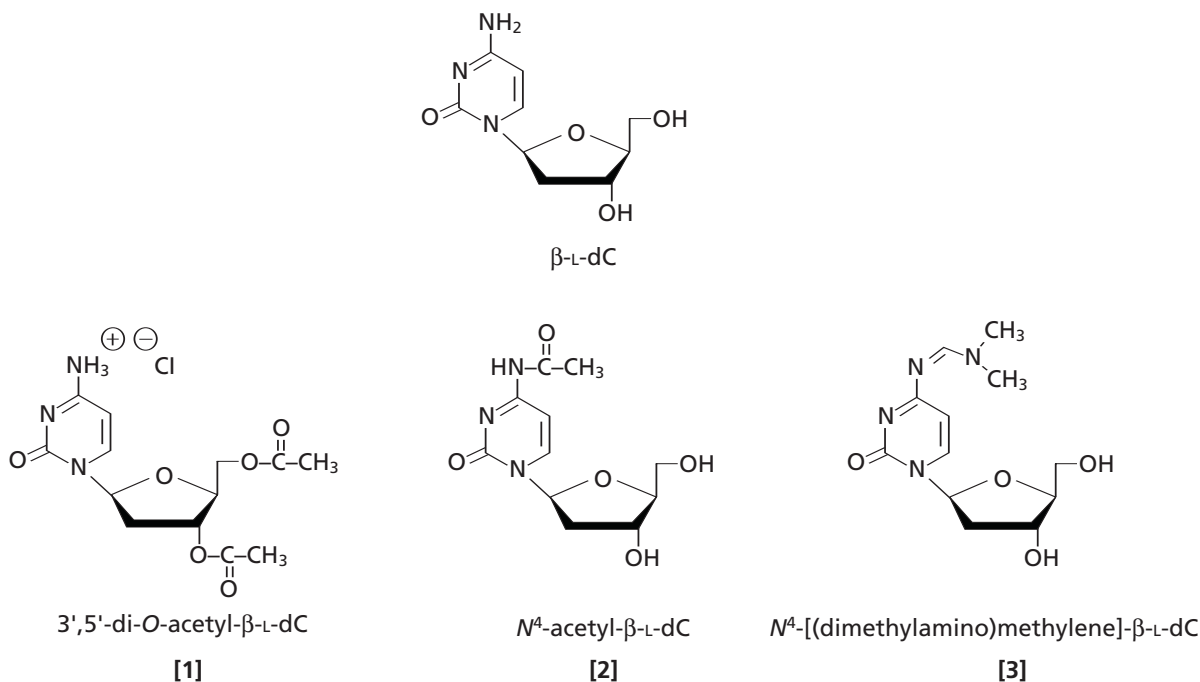
Presented in part at the *14th International Conference on Antiviral Research*, Seattle, Washington, USA, 8–13 April 2001. *Antiviral Research* 2001; 50:A79.

Keywords: hepatitis B virus, β -L-2'-deoxycytidine, prodrugs

Introduction

Morbidity and mortality of significant magnitude due to hepatitis B virus (HBV) are at the origin of intensive efforts in searching for potent and selective antiviral agents against this virus. To date, in addition to alpha interferon (IFN), only two small molecules have been approved by the US Food and Drug Administration for the treatment of hepatitis B, namely lamivudine (β -L-2',3'-dideoxy-3'-thiacytidine, 3TC) and, more recently, adefovir dipivoxyl (Yang *et al.*, 2002). Moreover, the main treatment options using IFN and lamivudine are sub-optimal due to drug-associated side effects, low response rates, unsustained viral load reduction and rapid emergence of antiviral drug resistance (Wong *et al.*, 1993; Ling *et al.*, 1996). As a consequence, more potent and specific drugs are required to efficiently suppress chronic HBV replication and to minimize or prevent hepatocellular damage and risk of liver cancer. In this regard, we have recently reported that β -L-2'-deoxycytidine (β -L-dC, Figure 1), which possesses, like lamivudine, an 'unnatural' L-configuration, is a potent,

specific and selective inhibitor of HBV in cell culture experiments (Bryant *et al.*, 2001a,b) without any cellular or mitochondrial toxicity when evaluated at high concentrations (Standing *et al.*, 2001). The potency and safety of β -L-dC has also been proven *in vivo* by using the woodchuck model of chronic HBV infection (Bryant *et al.*, 2001a,b), known to be a good model for evaluating drug candidates in the treatment of human chronic HBV infection. However, pharmacokinetic studies showed that oral bioavailabilities of β -L-dC in the woodchucks and in monkeys are only 9% and 16%, respectively (Standing *et al.*, 2001). Those results prompted us to elaborate and synthesize potential prodrugs with more favourable oral absorption profiles by derivatizing either the sugar hydroxyl functions or the cytosine exocyclic amino group. Here we report the preparation, physicochemical characteristics and pharmacokinetic studies of 3',5'-di-*O*-acetyl- β -L-dC [**1**], *N*⁴-acetyl- β -L-dC [**2**] and *N*⁴-[(dimethylamino)methylene]- β -L-dC [**3**] (Figure 1).

Figure 1. β -L-dC and its three studied derivatives

Materials and methods

Chemistry

General methods

¹H NMR spectra were recorded on a Bruker AC 250 (250 Mhz) or Bruker AC 400 (400 Mhz) spectrometer (Bruker, Mass., USA). ¹H NMR chemical shifts (δ) are quoted in parts per million (ppm) referenced to the residual solvent peak [dimethyl sulphoxide (DMSO-*d*₆) set at 2.49 ppm] relative to tetramethylsilane (TMS). The accepted abbreviations are as follows: s, singlet; dd, doublet of doublet; m, multiplet; q, quartet; pt, pseudotriplet; and br s, broad signal. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a Jeol DX 300 mass spectrometer operating with a JMA-DA 5000 mass data system (Jeol, Mass., USA) and using a mixture of glycerol and thioglycerol (1/1, v/v, G-T) as the matrix. Melting points were determined in open capillary tubes with a Büchi B-545 apparatus (Büchi Labortechnik AG, Switzerland) and are uncorrected. UV spectra were recorded on an Uvikon XS spectrophotometer (Uvikon, The Netherlands). Optical rotations were measured in a 1-cm cell on a PerkinElmer Model 241 (PerkinElmer, Calif., USA) spectropolarimeter. Elemental analyses were carried out by the Service de Microanalyses du CNRS, Division

de Vernaison (France). Thin-layer chromatography (TLC) was performed on precoated aluminium sheets of silica gel 60 F₂₅₄ (Merck, Art. 5554), visualization of products being accomplished by UV absorbance and by charring with 10% ethanolic sulphuric acid with heating. Column chromatography was carried out on silica gel 60 (Merck, Art. 9385). All moisture-sensitive reactions were carried out under an argon atmosphere using oven-dried glassware. Solvents were dried and distilled prior to use and solids were dried over P₂O₅ under reduced pressure at room temperature. High-performance liquid chromatography (HPLC) studies were carried out on a Waters Alliance unit [717 autosampler injector, 996 photodiode array detector and a Millennium data workstation (Waters Corp., Mass., USA)] using a reverse-phase analytical column [Nova-Pak® Silica 60 Å 4 μ m, C18, 150×3.9 mm (SGE, Tex., USA)]. The compound to be analysed was eluted using a linear gradient of 0–40% acetonitrile in 20 mM triethylammonium acetate buffer (TEAC, pH 7) programmed over a 15 min period with a flow rate of 1 ml/min.

Chemical synthesis

3',5'-Di-O-acetyl-2'-deoxy- β -L-cytidine ([1], hydrochloride form). A solution of β -L-dC (Pierra et al., 2000) (0.77 g, 3.37 mmol) and acetyl chloride (0.96 ml,

13.48 mmol) in glacial acetic acid (4.8 ml) was stirred at room temperature for 10 min, then dry chloroform (3.5 ml) was added and the stirring continued for 24 h (Breiner *et al.*, 1990). The solution was evaporated under reduced pressure and coevaporated with ethanol. The desired compound was recrystallized from ethanol (0.91 g, 78%); mp 192–193°C [lit. 187–189°C for the D-enantiomer (Breiner *et al.*, 1990)]; $^1\text{H NMR}$ (DMSO- d_6) δ ppm 9.8 and 8.7 (2 br s, <3 H, NH_3^+ , D_2O exchangeable), 8.0 (d, 1H, $H-6$, $J_{6-5}=7.8$ Hz), 6.18 (d, 1H, $H-5$, $J_{5-6}=7.8$ Hz), 6.08 (t, 1H, $H-1'$, $J=6.7$ Hz), 5.2 (m, 1H, $H-3'$), 4.3–4.1 (m, 3H, $H-4'$, $H-5'$, $H-5''$), 2.4–2.5 (m, 2H, $H-2'$, $H-2''$), 2.06 and 2.03 (2 s, 6H, 2 CH_3); mass spectra FAB >0 (GT): m/e 623 ($2\text{M}+\text{H}$) $^+$, 312 ($\text{M}+\text{H}$) $^+$, 201 (S) $^+$, 112 ($\text{B}+2\text{H}$) $^+$, 43 (CH_3CO) $^+$; FAB <0 (GT): 968 ($3\text{M}+\text{Cl}$) $^-$, 657 ($2\text{M}+\text{Cl}$) $^-$, 438 ($\text{M}+\text{G}+\text{Cl}$) $^-$, 346 ($\text{M}+\text{Cl}$) $^-$, 310 ($\text{M}-\text{H}$) $^-$, 110 (B) $^-$; 59 (CH_3COO) $^-$; UV (MeOH) $\lambda_{\text{max}}=277$ nm (ϵ 9900); $\lambda_{\text{min}}=246$ nm (ϵ 5000); $[\alpha]_{\text{D}}^{20}=-36.3$ ($c=1.0$, DMSO); Anal. Calcd. for $\text{C}_{13}\text{H}_{18}\text{ClN}_3\text{O}_6$: C=44.90, H=5.22, Cl=10.20, N=12.08; found: C=44.77, H=5.22, Cl=10.12, N=12.13.

***N*⁴-Acetyl-2'-deoxy- β -L-cytidine [2].** Acetic anhydride (0.20 ml, 2.20 mmol) was added to a suspension of β -L-dC (Pierra *et al.*, 2000) (0.41 g, 1.83 mmol) in dry *N,N*-dimethylformamide (DMF, 9.2 ml) and the mixture was stirred at room temperature for 24 h (Bhat *et al.*, 1989). After removal of DMF under reduced pressure, the resulting residue was purified by silica gel column chromatography (eluant: 15% MeOH in CH_2Cl_2) to afford the desired compound (0.31 g, 63%), which was recrystallized from ethanol; mp 130–170°C [lit. (Bhat *et al.*, 1989) for the D-enantiomer 150–175°C]; $^1\text{H NMR}$ (DMSO- d_6) δ ppm 10.86 (s, 1H, NH , D_2O exchangeable), 8.31 (d, 1H, $H-6$, $J_{6-5}=7.5$ Hz), 7.18 (d, 1H, $H-5$, $J_{5-6}=7.5$ Hz), 6.09 (t, 1H, $H-1'$, $J=6.3$ Hz), 5.25 (d, 1H, $\text{OH}-3'$, D_2O exchangeable, $J=4.2$ Hz), 5.03 (t, 1H, $\text{OH}-5'$, D_2O exchangeable, $J_{\text{OH}-5'}=5.0$ Hz), 4.1–4.2 (m, 1H, $H-3'$), 3.8 (m, 1H, $H-4'$), 3.4–3.6 (m, 2H, $H-5'$, $H-5''$), 2.2–2.3 (m, 1H, $H-2'$), 2.08 (s, 3H, CH_3), 2.0–1.9 (m, 1H, $H-2''$); mass spectra FAB >0 (GT): 808 ($3\text{M}+\text{H}$) $^+$, 539 ($2\text{M}+\text{H}$) $^+$, 362 ($\text{M}+\text{G}+\text{H}$) $^+$, 270 ($\text{M}+\text{H}$) $^+$, 154 ($\text{B}+2\text{H}$) $^+$, 117 (S) $^+$; FAB <0 (GT): m/e 806 ($3\text{M}-\text{H}$) $^-$, 537 ($2\text{M}-\text{H}$) $^-$, 360 ($\text{M}+\text{G}-\text{H}$) $^-$, 268 ($\text{M}-\text{H}$) $^-$, 152 (B) $^-$; UV (H_2O) $\lambda_{\text{max}}=297$ nm (ϵ 8300); $\lambda_{\text{min}}=270$ nm (ϵ 3500), $\lambda_{\text{max}}=245$ nm (ϵ 14400); $\lambda_{\text{min}}=226$ nm (ϵ 5800); $[\alpha]_{\text{D}}^{20}=-81.3$ ($c=1.1$, DMSO); Anal. Calcd. for $\text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_5 \cdot 0.4\text{H}_2\text{O}$: C=47.79, H=5.76, N=15.20; found: C=47.44, H=5.55, N=15.16.

***N*⁴-[[Dimethylamino)methylene]-2'-deoxy- β -L-cytidine [3].** The title compound was prepared according to a published procedure developed for the preparation of the corresponding D-enantiomer (Kerr & Kalman, 1994). A solution of β -L-dC (Pierra *et al.*, 2000) (0.50 g, 2.20 mmol) in DMF

(4.8 ml) was treated with dimethylformamide dimethylacetal (2.8 ml, 21.08 mmol), and stirred at room temperature overnight. The solution was evaporated under reduced pressure and coevaporated with ethanol. Recrystallization from ethanol/diethyl ether yielded the title compound (0.50 g, 81%) as light yellow crystals; mp 174–176°C [lit. 188–190°C for the D-enantiomer (Kerr & Kalman, 1994)]; $^1\text{H NMR}$ (DMSO- d_6) δ ppm 8.60 (s, 1H, $\text{N}=\text{CH}$), 8.00 (d, 1H, $H-6$, $J_{6-5}=7.2$ Hz), 6.15 (t, 1H, $H-1'$, $J=6.6$ Hz), 5.96 (d, 1H, $H-5$, $J_{5-6}=7.2$ Hz), 5.22 (d, 1H, $\text{OH}-3'$, $J_{\text{OH}-3'}=4.2$ Hz), 5.01 (t, 1H, $\text{OH}-5'$, $J_{\text{OH}-5'}=5.2$ Hz), 4.20 (m, 1H, $H-4'$), 3.80 (m, 1H, $H-3'$), 3.56 (m, 2H, $H-5'$ and $H-5''$), 3.15 and 3.02 [2s, 3H and 3H, $\text{N}(\text{CH}_3)_2$], 2.22–1.90 (2 m, 1H and 1H, $H-2'$ and $H-2''$); mass spectra FAB >0 (GT): m/z 847 ($3\text{M}+\text{H}$) $^+$, 565 ($2\text{M}+\text{H}$) $^+$, 283 ($\text{M}+\text{H}$); FAB <0 (GT): 599 ($2\text{M}+\text{Cl}$) $^-$, 317 ($\text{M}+\text{Cl}$) $^-$, 165 (B) $^-$; UV (EtOH) $\lambda_{\text{max}}=315$ nm (ϵ 32400); $\lambda_{\text{min}}=243$ nm (ϵ 1800); $[\alpha]_{\text{D}}^{20}=-12.6$ ($c=1.0$, DMSO); Anal. Calcd. for $\text{C}_{12}\text{H}_{18}\text{N}_4\text{O}_4 \cdot 0.3\text{H}_2\text{O}$: C=50.10, H=6.52, N=19.47; found: C=50.34, H=6.45, N=19.58.

Anti-HBV activity

Inhibition of HBV replication *in vitro* was assessed using the 2.2.15 cell line as previously described (Korba & Gerin, 1992). Briefly, cells were cultured for 11 days at a density of 2×10^5 cells/ml in 24-well plates in Dulbecco minimal essential medium, supplemented with 4% dialysed fetal bovine serum (FBS), and 0.5 mM L-glutamine, in the presence or absence of drug, with medium changes every 3 days. At the end of the subsequent 3-day period, an aliquot of the culture medium was harvested and processed to obtain extracellular HBV DNA by Slot blot analysis.

For intracellular HBV DNA analysis, cells were lysed (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% SDS). Total intracellular DNA was extracted; HBV DNA was digested with *Hind*III restriction endonuclease, separated by electrophoresis and transferred to a nylon membrane. Filters from Slot and Southern blot were hybridized with a ^{32}P -labelled HBV-specific probe, prepared from a full-length HBV-DNA genome template excised from plasmids. Quantification was performed on a Personal Molecular Imager FX (Bio-Rad Laboratories, Inc., Calif., USA). Inhibition of viral DNA replication was assessed by comparison of the amount of HBV DNA between untreated and drug-treated cultures.

Cytotoxicity

Cytotoxicity of compounds was based on the viability of HepG2 hepatoma cells, as monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Mosmann, 1983). Cells were seeded at a rate of 5×10^4 /well into 96-well plates in Eagle's minimum essential medium (MEM) with 10% FBS and 1 mM sodium pyruvate, in the presence or absence of drug, with

medium changes every 3 days. After an 11-day incubation at 37°C, cell viability was determined by the MTT method.

Water solubility

Aqueous solubilities of compounds [1–3] were determined in distilled water at room temperature. Excess of studied compound was added to aqueous solution and the suspension was shaken for 15 min and then centrifugated. Samples of supernatant were analysed by HPLC and concentrations of saturated solutions were determined according to calibration curves (Valette, 1998). The experiments were repeated three times.

Chemical stability

Diluted solutions of each compound (10^{-4} M) were incubated at 37°C in a KCl-HCl 20 mM buffer (pH 1.2). Aliquot samples were collected at different intervals of time and analysed by the HPLC method. Rates of decomposition of each compound were easily determined using a method developed in our laboratory and based on pseudo-first-order kinetic models (Lefebvre *et al.*, 1995).

Distribution coefficient

Distribution coefficients between 1-octanol and an aqueous phase (phosphate buffer solution 0.02 M, pH 7.2) were determined at room temperature using a shake-flask procedure (Valette, 1998). An aliquot of a 10^{-2} M aqueous solution of the studied compound was diluted to 1 ml, with the aqueous phase previously saturated with octanol. An equal volume of octanol, previously saturated with the aqueous phase, was added to give a total volume of 2 ml, and the mixture was shaken vigorously. The two phases were centrifugated and separated; samples of each phase were collected and analysed by HPLC injections. UV absorbencies for both phases were measured at the respective maximum wavelengths. The distribution coefficient was calculated from the ratio of the area of the signal detected in the octanol and aqueous phases. Each experiment was repeated twice.

Pharmacokinetics and oral bioavailability

To evaluate the pharmacokinetics and oral bioavailability of β -L-dC in monkeys, three male non-naive cynomolgus monkeys (*Macaca fascicularis*) received 10 mg/kg of β -L-dC intravenously with a tracer amount of [3 H]-labelled drug (250 μ Ci, [5,6- 3 H]-; Moravek Biochemicals, Calif., USA) dissolved in sterile 0.9% saline. Following a 6-week washout period, the same three animals received an identical dose of β -L-dC orally via gavage. Blood samples for pharmacokinetic analysis were collected in heparinized tubes at pre-dose, 0.25, 0.50, 1, 2, 3, 4, 6, 8 and 24 h after dosing. Urine was collected by pan catch over periods from 0–2, 2–4, 4–8, 8–12 and every 12 h until 336 h post-dose. Urine volumes

collected were recorded and a 5-ml aliquot frozen (-20°C) until analysis. Plasma samples (200 μ l aliquots) were treated with 10 μ l of 20% trichloroacetic acid (TCA) and centrifuged (14 000 $\times g$) to remove precipitated proteins. The supernatant solution was analysed by HPLC using a Columbus 5 micron C18 110A 250 \times 4.60 mm column (Phenomenex, Calif., USA) at a flow rate of 1 ml/min. HPLC elution was carried out with a 50 mM phosphate buffer, pH 7 and a step gradient of 2% methanol from 0–15 min and 10% methanol for the remainder of the run, with UV detection at 280 nm. Radioactivity was measured by an online 500TR Radiomatic Flo-One detector (Radiomatic Corp., Fla., USA) and by liquid scintillation counting following fraction collection. Pharmacokinetic (PK) data analysis was conducted using noncompartmental modelling. Urine samples were centrifuged, supernatants filtered through a 0.2 μ m Acrodisc and 10–200 μ l used for liquid–solid chromatography (LSC) and HPLC analysis.

Similarly, [3 H]-labelled drug 3',5'-di-*O*-acetyl- β -L-dC ([1], [5,6- 3 H]) provided by Moravek Biochemicals) was dosed orally at 10 mg/kg to three male cynomolgus monkeys. Blood samples were collected at pre-dose, 0.25, 0.50, 1, 2, 3, 4, 6, 8 and 24 h after dosing. Plasma samples (200 μ l aliquots) were treated with 20% TCA and centrifuged to remove precipitated proteins. The supernatant solution was analysed by HPLC using the same column as above and a gradient elution. HPLC mobile phases were (A) 50 mM phosphate buffer, pH 7, and (B) 50% methanol and 50% phosphate buffer (50 mM, pH 7). The gradient elution was 0 min, 100%A; 10 min, 95%A/5%B; 20 min, 70%A/30%B and 30 min, 100%B. The flow rate was 1 ml/min. Radioactivity was measured by liquid scintillation counting following fraction collection. PK data analysis was conducted using non-compartmental modelling.

Results

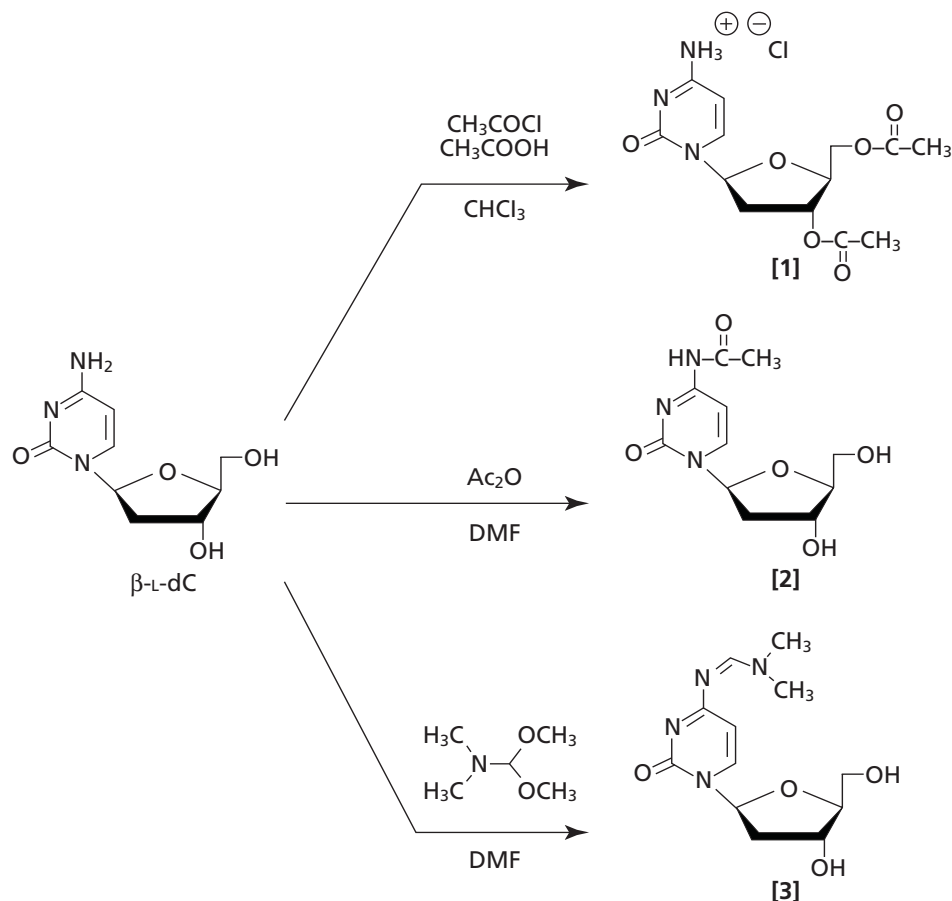
Synthesis

Selective *N*- or *O*-acylations of β -L-dC and preparation of the *N*⁴-substituted imine derivative were accomplished following similar procedures as those previously developed for the preparation of the corresponding D-enantiomer counterparts (Figure 2) (Bhat *et al.*, 1989; Breiner *et al.*, 1990; Kerr & Kalman, 1994).

Anti-HBV activity

The effects of β -L-dC, 3TC and the compounds [1–3] against HBV replication are reported in Table 1. All the compounds inhibited HBV replication in a dose-dependent manner. The three compounds [1–3] showed EC₅₀ values comparable with that of the parent nucleoside in 2.2.15 cells, as assessed by inhibition of extracellular HBV virions and intracellular replicative intermediate HBV DNA. All

Figure 2. Chemical syntheses of 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [**1**], *N*⁴-acetyl-2'-deoxy- β -L-cytidine [**2**] and *N*⁴-[(dimethylamino)methylene]-2'-deoxy- β -L-cytidine [**3**]



tested compounds were found to be non-cytotoxic in Hep-G2 cells at concentrations higher than 200 μ m.

When evaluated against human immunodeficiency virus (HIV), yellow fever virus (YFV) and bovine viral diarrhoea virus (BVDV) replication in cell-based assays (La Colla, manuscript in preparation), none of them showed cytotoxicity or antiviral activity at the highest concentration tested (100 μ M, data not shown).

Solubility

The concentration of a saturated solution of 3',5'-di-*O*-acetyl- β -L-dC [**1**] in water is 3.3 mol/l (1148 g/l). In the case of *N*⁴-acetyl and *N*⁴-formamidine derivatives ([**2**] and [**3**]), the concentrations of the saturated solutions in water are higher than 0.19 mol/l (51 g/l) and 1.87 mol/l (528 g/l), respectively. All the three compounds [**1–3**] have

an excellent water solubility which is as good as commercial β -D-dC.HCl [saturated solution=1.03 mol/l (272 g/l)] and even better in the case of compounds [**1**] and [**3**].

Chemical stability

An important factor for derivatives aimed at improving the oral bioavailability of the parent molecule is a sufficient stability in acidic medium. In order to mimic the behaviour of the prodrugs in the gastrointestinal tract, their stability at pH 1.2 has been studied.

The half-life ($T_{1/2}$ value) of 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [**1**] at pH 1.2 is 11.2 h. At this acidic pH, compound [**1**] is partially converted into a monoacetyl derivative (3' or 5'), which is later transformed into 2'-deoxy- β -L-cytidine (Figures 3 and 4). No glycosidic bond breakage has been observed in up to 2 days (no cytosine base detected).

Table 1. Anti-HBV activity and cytotoxicity of β -L-dC and its three studied derivatives

Compound	EC ₅₀ *		CC ₅₀ [†]
	HBV virion	HBV RI	
3TC	0.05	0.05	>100
L-dC	0.22	0.23	>200
[1]	0.24	0.42	>200
[2]	0.12	0.32	>200
[3]	0.5	0.31	>200

*Compound concentration (μ M) required to reduce the extracellular and intracellular (RI) HBV DNA by 50%.

[†]Compound concentration (μ M) required to reduce the viability of HepG2 cells by 50%, as determined by the MTT method.

The half-life of *N*⁴-acetyl-2'-deoxy- β -L-cytidine [2] at pH 1.2 is 45 min. Under these conditions, 70% of compound [2] is transformed into β -L-dC, which is stable at pH 1.2. Additionally, 30% of glycosidic bond breakage has been observed leading to the *N*-acylated base, which is later transformed into cytosine (Figures 5 and 6). Compared with the parent compound β -L-dC, introduction of an elec-

tron-withdrawing group on the exocyclic amine function leads to some unstability of the glycosidic bond.

The half-life of *N*⁴-[(dimethylamino)methylene]-2'-deoxy- β -L-cytidine [3] at pH 1.2 is 40 min. The mechanism of hydrolysis is shown in Figure 7. The reaction proceeds via the formation of the *N*⁴-formamide intermediate which undergoes spontaneous hydrolysis to the parent 2'-deoxy- β -L-cytidine. In this case, hydrolysis of the glycosidic bond is more difficult than with the *N*⁴-acyl derivative because of the conjugation between the formamidine double bond and the aromatic cycle of the pyrimidine base. As a consequence, no glycosidic bond breakage has been observed within up to 5 h of incubation (Figures 7 and 8).

At this stage, it seemed obvious to us to eliminate both compounds [2] and [3] as potential prodrugs of β -L-dC, due to their instability at pH 1.2 (half-life inferior to 1 h).

On the other hand, compound [1] was considered as an interesting candidate with potential improved oral bioavailability compared to β -L-dC, owing to its good stability profile at pH 1.2.

Lipophilicity

Distribution coefficients (P) were determined for the selected 3',5'-di-*O*-acetyl- β -L-dC [1] compound and for

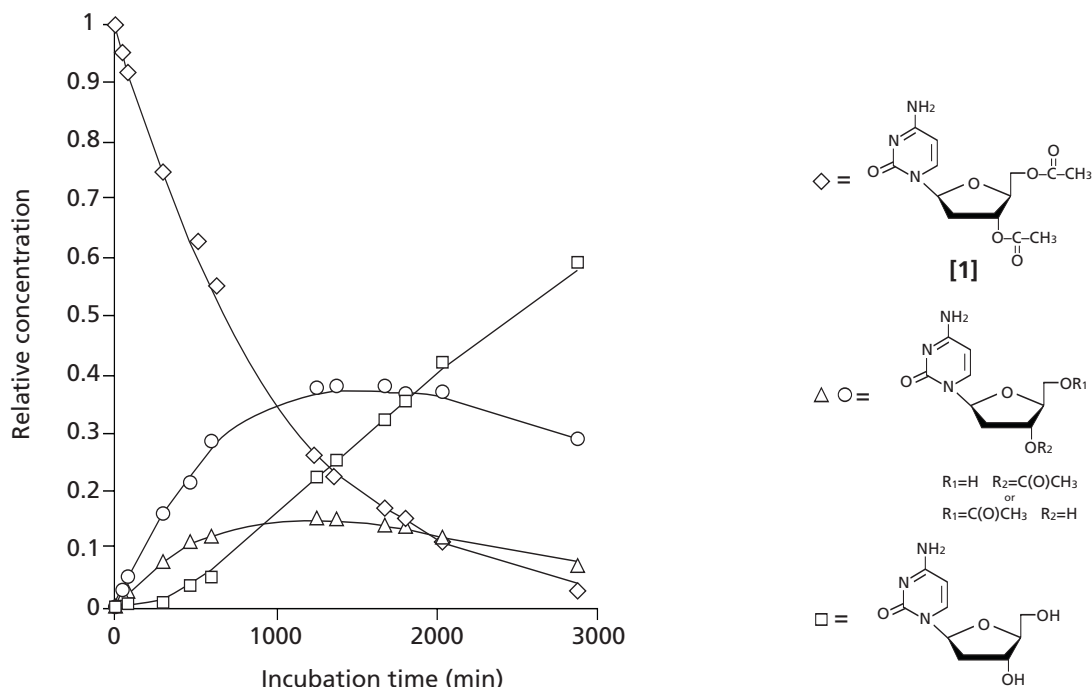
Figure 3. Kinetics curves of hydrolysis of 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [1] at pH 1.2

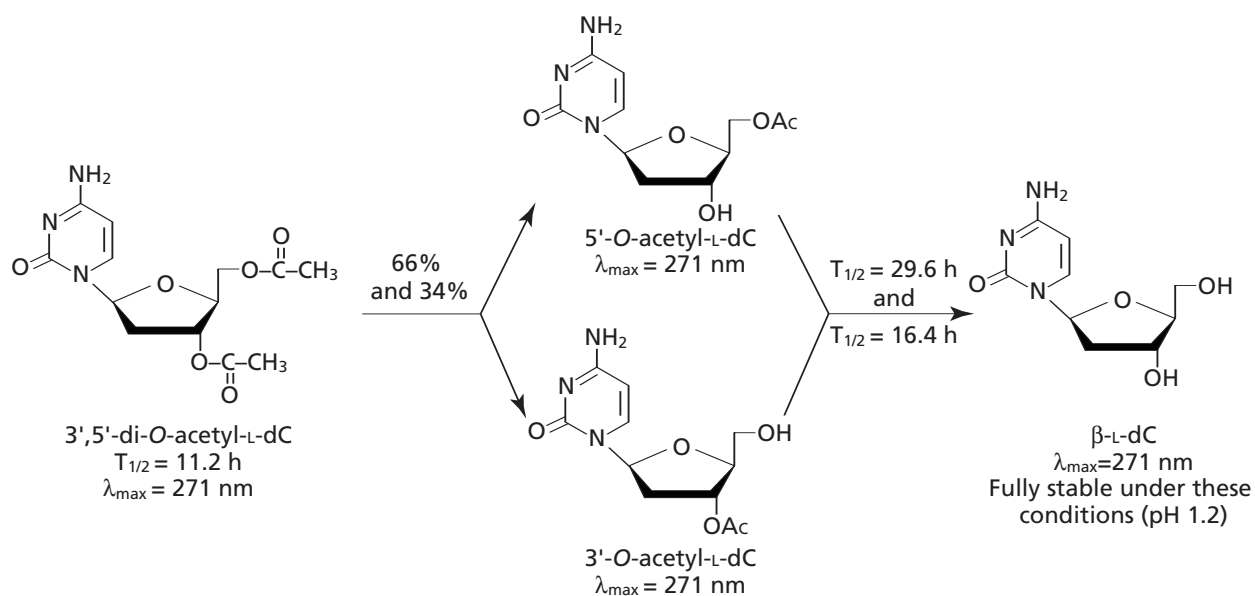
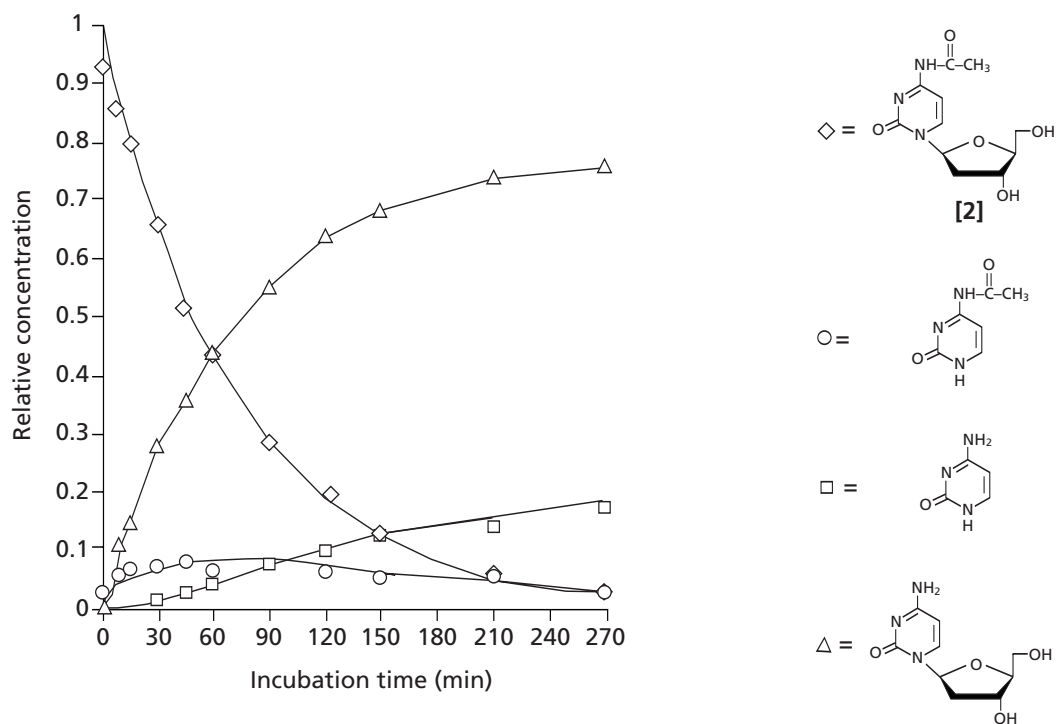
Figure 4. Proposed mechanism for the decomposition of 3',5'-di-O-acetyl-2'-deoxy- β -L-lytidine [1] at pH 1.2**Figure 5.** Kinetics curves of hydrolysis of N^4 -acetyl-2'-deoxy- β -L-lytidine [2] at pH 1.2

Figure 6. Proposed mechanism for the decomposition of *N*⁴-acetyl-2'-deoxy-β-L-cytidine [2] at pH 1.2

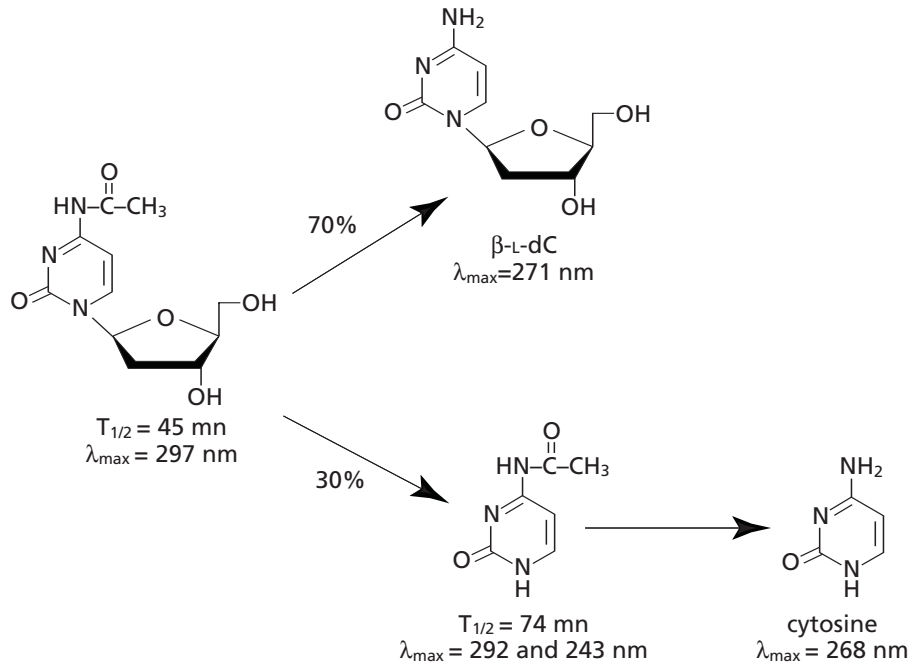


Figure 7. Kinetics curves of hydrolysis of *N*⁴-[(dimethylamino)methylene]-2'-deoxy-β-L-cytidine [3] at pH 1.2

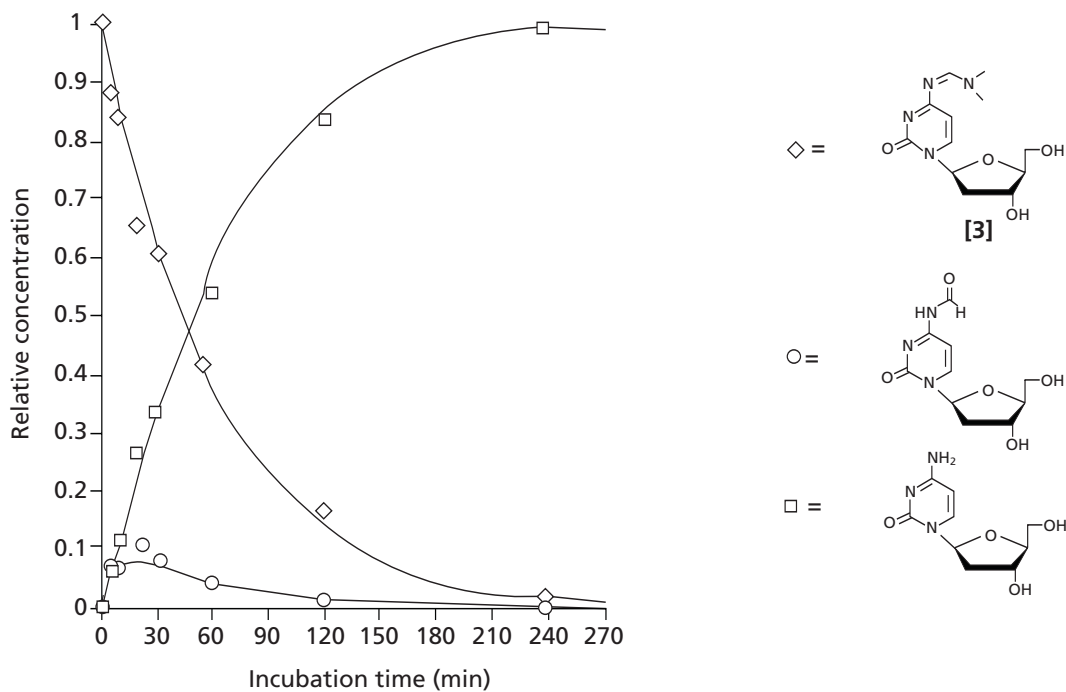
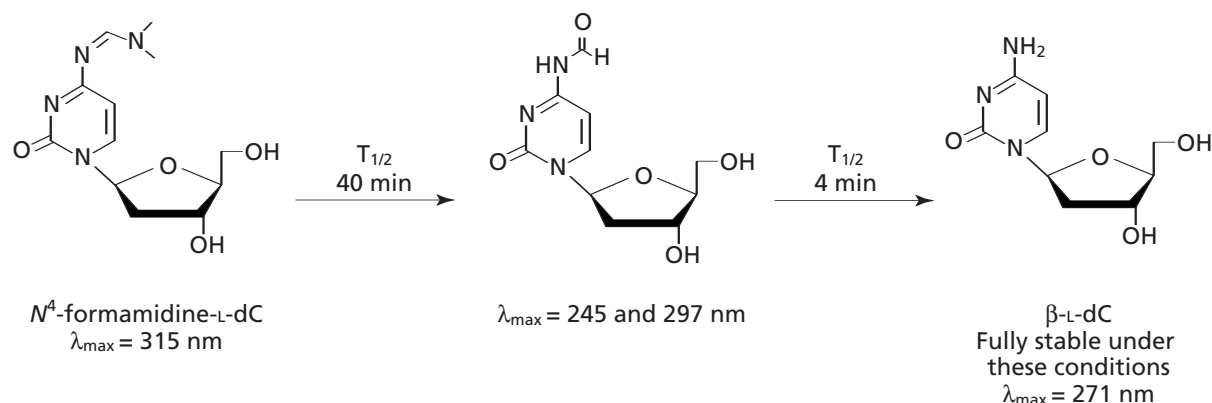


Figure 8. Proposed mechanism for the decomposition of N^4 -[(dimethylamino)methylene]-2'-deoxy- β -L-cytidine [**3**] at pH 1.2**Table 2.** Pharmacokinetic parameters of β -L-dC in cynomolgus monkeys following administration of β -L-dC (10 mg/kg) and 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [**1**] (10 mg/kg or 6.5 mg/kg as β -L-dC)

Compound dosed	Route	AUC _{0-t} ($\mu\text{M}\cdot\text{h}$)	T _{1/2} (h)	C _{max} (μM)	T _{max} (h)	CL (l/h/kg)	Vd (l/kg)	F (%)
L-dC	iv	81.1 (5.7)	1.6 (0.1)	95.7 (13)	–	0.53 (0)	1.22 (0.11)	–
	po	13.7 (4.3)	3.0 (1.3)	3.4 (1.3)	2.3 (1.5)	–	–	16.4 (5)
[1]	po	16.5 (4.8)	1.8 (0.5)	5.6 (0.3)	1.0 (0)	–	–	31.3 (7.3)

Values: mean (SD). AUC_{0-t}, Area under the plasma concentration–time curve from time 0 to t; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; T_{1/2}, terminal elimination half-life; CL, total body clearance; Vd, volume of distribution; F, oral bioavailability; iv, intravenous; po, oral administration.

the commercial β -D-dC (hydrochloride form), by using a mixture of octanol and phosphate buffer at room temperature. The P values are -0.74 and -1.41 for the di-acetylated compound [**1**] and β -D-dC, respectively. Although both compounds prefer water to octanol, acetylation of the two hydroxyl functions leads to a more lipophilic compound than the non-acetylated nucleoside.

Pharmacokinetics and oral bioavailability

Pertinent pharmacokinetic parameters of β -L-dC and 3',5'-di-*O*-acetyl- β -L-dC [**1**] in monkeys are shown in Table 2. Following intravenous (iv) administration of β -L-dC, the plasma β -L-dC concentration was the highest at the first time point taken (15 min) with a maximum plasma concentration (C_{max}) of approximately $95.7 \mu\text{M}$. The elimination was rather rapid with a terminal half-life of approximately 1.6 h. By 24 h post-dose, LdC could not be detected in plasma. The total body clearance (CL) averaged 0.53 l/h/kg and the mean apparent volume of distribution (Vd) was

1.22 l/kg, indicating that β -L-dC has a significant extravascular tissue distribution. Urinary excretion of L-dC was rapid, with 71% of the administered dose recovered within 2 h following iv administration. The total radioactivity recovery in urine was approximately 82% over 336 h. L-dC accounted for the majority (94%) of the dose recovered in the urine. The renal clearance of 0.46 l/h/kg accounted for 87% of total L-dC clearance and suggested that renal excretion was the major route of elimination. β -L-2'-deoxyuridine (L-dU) was detected in the plasma and urine, indicating that metabolic elimination of L-dC also occurred following iv administration. Low levels of L-dU were detected in plasma at the method limit of detection ($0.1 \mu\text{M}$), while L-dU accounted for 4.0% of the total dose recovered in urine. No other metabolites were detected in the plasma or urine.

Following an oral gavage, β -L-dC was rapidly absorbed and was detected at the first time point taken (15 min). The time to maximum plasma concentration (T_{max}) was approximately 2.3 h with a C_{max} of approximately $3.4 \mu\text{M}$.

Figure 9. Mean β -L-dC plasma concentration/time profile in monkeys receiving a 10 mg/kg dose of β -L-dC (A) iv and (B) per orally (po)

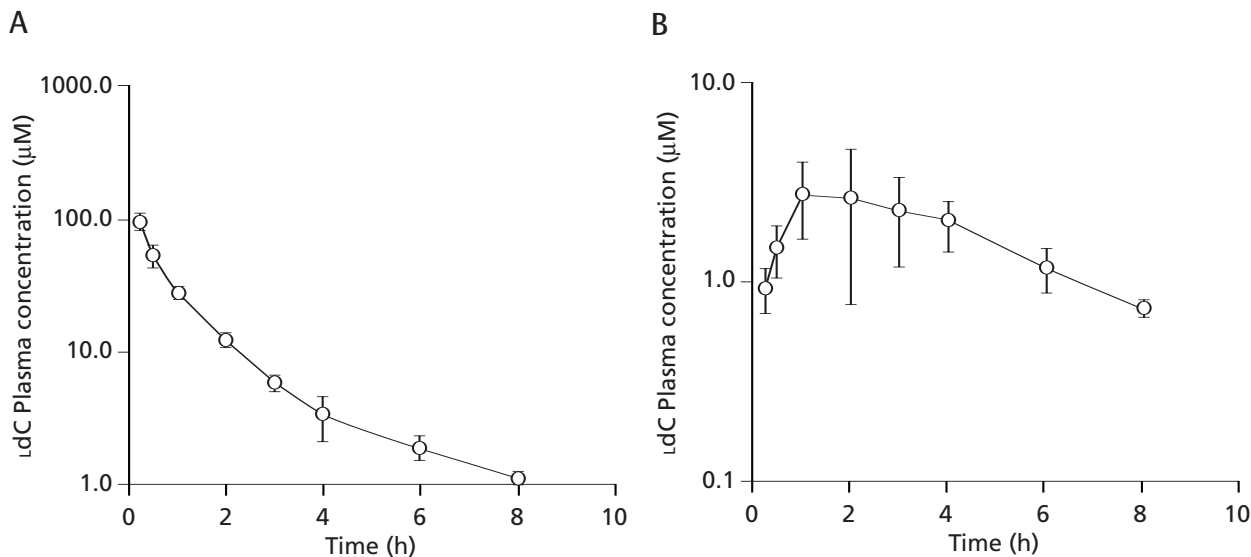
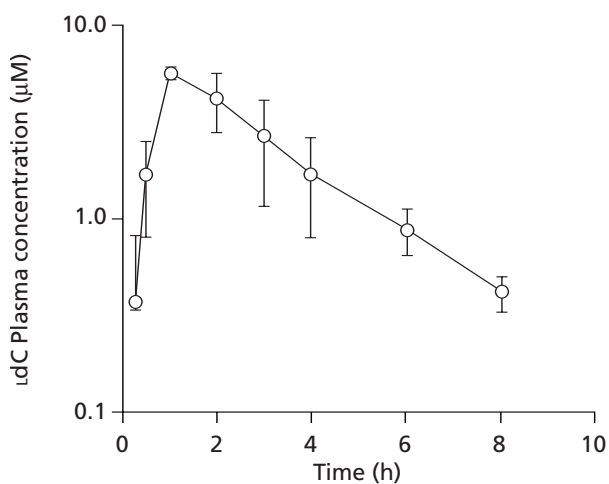


Figure 10. Mean β -L-dC plasma concentration/time profile in monkeys receiving a 10 mg/kg dose of 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [**1**] (6.3 mg/kg as β -L-dC)



The oral bioavailability averaged 16.4%. β -L-dC plasma concentration/time profiles are shown in Figure 9. L-dU was also detected in the plasma at the method detection limit, and in urine. No other metabolites were detected in

plasma or urine. Approximately 8.5% of the administered oral dose was recovered in the urine within 12 h. After 72 h, 15.5% was recovered. L-dC accounted for the majority (~69%) of drug excreted in the urine, while L-dU accounted for the remaining. Based on the total radioactivity excreted in the urine from iv and oral administration, the oral absorption of L-dC was estimated to be 18%. In monkeys receiving an oral dose of 3',5'-di-*O*-acetyl- β -L-dC [**1**], β -L-dC was the predominant species in the plasma. The biotransformation of [**1**] to β -L-dC was rapid. β -L-dC was detected at the first time point taken (15 min); its T_{\max} was approximately 1 h and C_{\max} was approximately 5.6 μ M. β -L-dC plasma concentration/time profiles are shown in Figure 10. Compound [**1**] was not detected at any time points. The apparent oral bioavailability for β -L-dC was 31.3% in monkeys receiving a 10 mg/kg dose of compound [**1**]. This is an approximately 91% increase in bioavailability over administering β -L-dC.

Discussion

The results of the present investigation demonstrate that antiviral nucleoside β -L-dC can be easily derivatized to yield compounds with more or less favourable oral absorption profiles. From a synthetic point of view, all three studied derivatives, namely 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [**1**], *N*⁴-acetyl-2'-deoxy- β -L-cytidine [**2**] and *N*⁴-[(dimethylamino)methylene]-2'-deoxy- β -L-cytidine [**3**] were easily

prepared in one step, starting from the parent nucleoside, and obtained in excellent yields. Stability studies at pH 1.2 revealed that the half-lives of the N^4 -base substituted compounds [2] and [3] were inferior to the half-life of 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [1]. So, because of its ease of synthesis and also owing to its good stability profile at acidic pHs, 3',5'-di-*O*-acetyl-2'-deoxy- β -L-cytidine [1] emerged as a promising potential candidate for an oral prodrug of β -L-dC, with favourable pharmacokinetic and physicochemical characteristics. Such a result warrants the synthesis and evaluation of other kinds of mono- and di-acylated derivatives of β -L-dC modified on the sugar moiety, and further studies on this topic will be reported later.

References

- Bhat V, Ugarkar BG, Sayeed VA, Grimm K, Kosora N, Domenico PA & Stocker E (1989) A simple and convenient method for the selective N-acylations of cytosine nucleosides. *Nucleosides & Nucleotides* **8**:179–183.
- Brainer RG, Rose WC, Dunn JA, MacDiarmid JE & Bardos TJ (1990) Synthesis of new nucleoside phosphoraziridines as potential site-directed antineoplastic agents. *Journal of Medicinal Chemistry* **33**:2596–2602.
- Bryant ML, Bridges EG, Placidi L, Faraj A, Loi A-G, Pierra C, Dukhan D, Gosselin G, Imbach J-L, Hernandez B, Juodawlkis A, Tennant B, Korba B, Cote P, Cretton-Scott E, Schinazi RF & Sommadossi J-P (2001a) Anti-HBV specific β -L-2'-deoxynucleosides. *Nucleosides, Nucleotides & Nucleic Acids* **20**:597–607.
- Bryant ML, Bridges EG, Placidi L, Faraj A, Loi A-G, Pierra C, Dukhan D, Gosselin G, Imbach J-L, Hernandez B, Juodawlkis A, Tennant B, Korba B, Cote P, Marion P, Cretton-Scott E, Schinazi R & Sommadossi J-P (2001b) Antiviral L-nucleosides specific for hepatitis B virus infection. *Antimicrobial Agents & Chemotherapy* **45**:229–235.
- Kerr SG & Kalman TI (1994) N^4 -(Dialkylamino)methylene derivatives of 2'-deoxycytidine and arabinocytidine: physicochemical studies for potential prodrug applications. *Journal of Pharmaceutical Sciences* **83**:582–586.
- Korba E & Gerin JL (1992) Use of standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. *Antiviral Research* **19**:55–70.
- Lefebvre I, Perigaud C, Pompon A, Aubertin A-M, Girardet J-L, Kirn A, Gosselin G & Imbach J-L (1995) Mononucleoside phosphotriester derivatives with *S*-acyl-2-thioethyl bioreversible phosphate-protecting groups: intracellular delivery of 3'-azido-2',3'-dideoxythymidine 5'-monophosphate. *Journal of Medicinal Chemistry* **38**:3941–3950.
- Ling R, Mutimer D, Ahmed M, Boxall E, Elias E, Dusheiko G & Harrison T (1996) Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* **24**:711–713.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *Journal of Immunological Methods* **65**:55–63.
- Pierra C, Dukhan D, Bryant M, Sommadossi J-P, Imbach J-L & Gosselin G (2000) L-Nucleosides as potent anti-HBV agents (Part II): large-scale stereospecific syntheses of β -L-2'-deoxycytidine and β -L-thymidine. *Antiviral Research* **46**:A62.
- Standring DN, Bridges EG, Placidi L, Faraj A, Loi AG, Pierra C, Dukhan D, Gosselin G, Imbach J-L, Hernandez B, Juodawlkis A, Tennant B, Korba B, Cote P, Cretton-Scott E, Schinazi RF, Myers M, Bryant ML & Sommadossi J-P (2001) Antiviral β -L-nucleosides specific for hepatitis B virus infection. *Antiviral Chemistry & Chemotherapy* **12**(Suppl. 1):119–129.
- Valette G (1998) [Biotransformation et Détermination du Caractère Lipophile de Pronucléotides à Visée Antivirale: Étude par Chromatographie Liquide Hautes Performances (C.L.H.P.) et Couplage C.L.H.P./Spectrométrie de Masse]. Montpellier: Université de Montpellier II-Sciences et Techniques du Languedoc. PhD Dissertation. In French.
- Wong DK, Cheng A, O'Rourke K, Naylor CD, Delshy AS & Heathcote J (1993) Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Annals of Internal Medicine* **119**:312–323.
- Yang H, Westland CE, Delaney WE, Heathcote EJ, Ho V, Fry J, Brosgart C, Gibbs CS, Miller MD & Xiong S (2002) Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxyl for up to 60 weeks. *Hepatology* **36**:464–473.

Received 8 June 2004; accepted 20 July 2004