Antiviral Chemistry & Chemotherapy **12** (Suppl. 1):119–129

Antiviral β-L-nucleosides specific for hepatitis B virus infection

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Three simple, related nucleosides, β-L-2′-deoxycytidine (LdC), β-Lthymidine (LdT), and β-L-2′-deoxyadenosine (LdA), have been discovered to be potent, specific and selective inhibitors of the replication hepatitis B virus (HBV), as well as the closely related duck and woodchuck hepatitis viruses (WHV). Structure–activity relationship analysis indicates that the 3′-OH group of the β-L-2′-deoxyribose of the β-L-2′-deoxynucleoside confers specific anti-hepadnavirus activity. The simple nucleosides had no effect on the replication of 15 other RNA and DNA viruses, and did not inhibit human DNA polymerases (α, β and γ) or compromise mitochondrial function. The nucleosides are efficiently converted intracellularly into active triphosphate metabolites that have a long half-life. Once-daily oral administration of these compounds in the woodchuck efficacy model of chronic HBV infection reduced viral load by as much as 10^8 genome equivalents/ml serum and there was no drug-related toxicity. In addition, a decline in WHV surface antigen (WHsAg) paralleled the decrease in viral load. This class of nucleosides displays an excellent overall safety profile. The first compound, LdT, has already entered clinical trials and LdC, currently being developed as a prodrug, is expected to enter the clinic in the near future. These compounds have the potential for use in combination therapy with the goal of achieving superior viral suppression and diminishing the onset of resistance.

Keywords: LdA, LdC, LdT, woodchuck hepatitis virus, metabolism, pharmacokinetics, safety, IND, clinical trials

Introduction

With an estimated 350 million individuals worldwide chronically infected with hepatitis B virus (HBV), the elimination of persistent HBV replication and the prevention of the associated progression to chronic active hepatitis, cirrhosis and hepatocellular carcinoma (HCC) remains an important therapeutic goal. Currently, the only approved options for the treatment of chronic HBV infection are interferon-α (IFN-α) and lamivudine (3TC, β-L-2′,3′-dideoxy-3′-thiacytidine). IFN-α therapy is characterized by a low response rate and significant drug-associated side-effects. Lamivudine, a β-L-nucleoside analogue, was approved initially for the treatment of HIV, and has more recently been approved for HBV. However, a complete HBV antiviral response to 3TC, as assessed by HBe seroconversion, is seen in only a minority of patients after 1 year of therapy. Also, 3TC-resistant HBV virus is now recognized in 16–32% of HBV-infected patients after 1 year of treatment and in as much as 58% after 2–3 years (Lai *et al*., 1998; Dienstag *et al*., 1999). In addition, 3TC therapy poses a special dilemma in the substantial population of HIV–HBV co-infected patients. In the co-infection setting, the efficacy of the current HIV combination therapies and the resulting marked decrease in HIV-related mortality has led to HBV-related liver disease

emerging as the major threat to the patient's long-term survival. The control of HIV replication in co-infected patients with combination therapies that include 3TC thus risks the development of 3TC-resistant HBV for which there is presently no good treatment option.

Several nucleoside analogues are currently under evaluation for the treatment of HBV infection. Two recent candidates, famciclovir (an oral prodrug of penciclovir) and lobucavir, have been discontinued due to limited clinical activity against HBV and adverse side-effects, respectively. Candidates still in clinical trials include adefovir dipivoxil (an oral prodrug of PMEA), which may be beneficial against 3TC-resistant mutants, as well as emtricitabine [(-)-FTC], L-FMAU and entecavir (BMS-200475).

In this report, we summarize the findings on a series of three structurally simple β-L-nucleosides, LdA, LdC and LdT, that were recently identified as potent, selective and highly specific inhibitors of HBV replication. These nucleosides have been described in detail in a number of recent reports (Benzaria *et al*., 2001; Bridges *et al*., 2001a,b; Bryant *et al*., 2001a,b; Cretton-Scott *et al*., 2001a,b; Juodawlkis *et al*., 2001; Pierra *et al*., 2001). Each compound has been shown to exhibit an excellent safety profile in pre-clinical testing. The first member of this series to reach clinical trials is LdT. A dose-escalation study in HBV-infected patients is currently in progress under a US IND (Investigational New Drug application).

Results

The β-L-nucleosides, LdA, LdC and LdT, are specific and selective inhibitors of hepadnaviruses

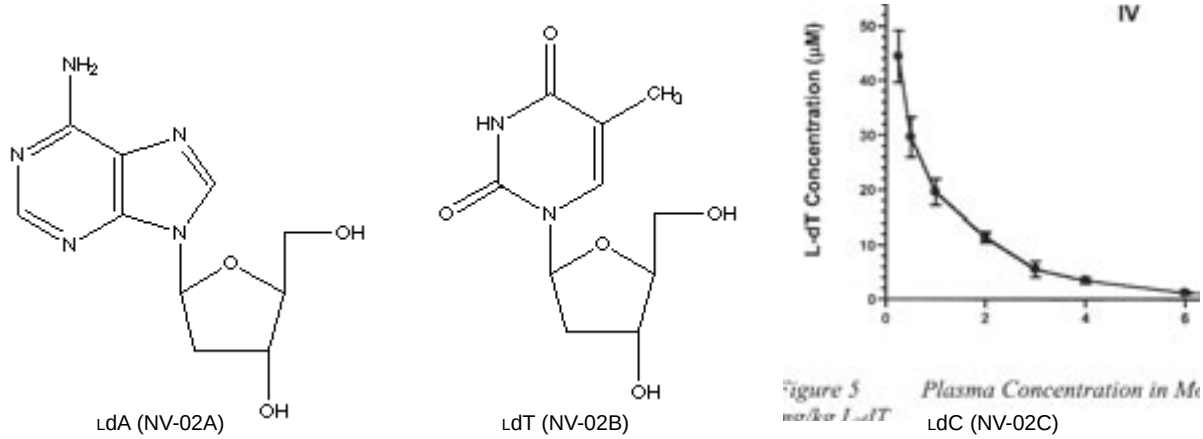
The structures of the β-L-nucleosides, LdA, LdC, and LdT, which are collectively known as the Novirio NV-02 series, are shown in Figure 1. These molecules are simple in structure and closely resemble the natural deoxynucleosides dA, T and dC. Unlike most nucleoside analogues, they exhibit no chemical modifications and differ from their natural nucleoside counterparts only with respect to the spatial relationship of their base and sugar moieties, having an L-configuration versus the D-configuration of the natural deoxynucleosides.

An extensive structure–activity analysis of Novirio's nucleoside collection identified the NV-02 series molecules as the most potent, selective and specific inhibitors of HBV replication in the HepG 2.2.15 tissue culture assay. The structure–activity relationships (SAR) established among the β-L-2′-deoxycytidine, -thymidine and -deoxyadenosine series are presented in Table 1, which compares the antiviral activity of these molecules against HBV and HIV.

The data reveal LdC, LdT and LdA to be potent inhibitors of HBV replication (EC_{50}s in the 100–250 nM range) with excellent specificity, as shown by their lack of activity against HIV. Closer examination of the SAR shows that the key to obtaining specific inhibitors of HBV is the hydroxyl (-OH) group in the 3′-position of the β-L-2′-deoxyribose sugar. This is most clearly seen in the LdC series, where only compounds retaining the 3′ OH moiety (for example, β-L-2′-deoxy-5-fluorocytidine, L-5-FdC and β-L-2′-deoxy-5-chlorocytidine, L-5-ClC) are specific inhibitors of HBV. Conversely, replacement of the 3′-OH (R3) on the deoxyribose sugar resulted in several instances in molecules with good activity against both HBV and HIV, reflecting a loss of antiviral specificity (for example, β-L-2′,3′-dideoxycytidine, L-ddC; β-L-2′,3′-dideoxy-3′-thiacytidine, 3TC; β-L-2′,3′-didehydro-2′3′-dideoxy-cytidine, L-d4C).

In the LdA series, the activity of β-L-2′,3′-didehydro-2′,3′-dideoxy-5-fluoroadenosine (L-d4A) against both HIV and HBV, again shows that specificity is lost along with the R3 OH group. Similarly, in the LdT series,

Figure 1. Structures of β-L-nucleoside compounds

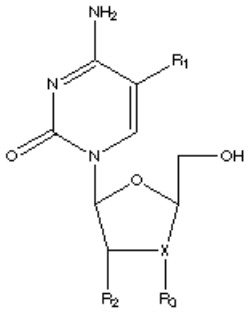
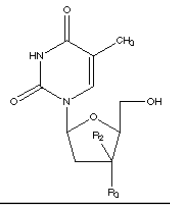
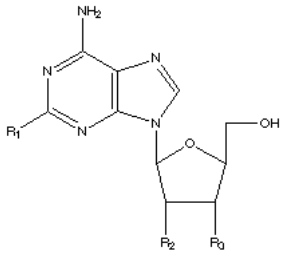


LdA (NV-02A) LdT (NV-02B) LdC (NV-02C)

Figure 5 Plasma Concentration in M

Table 1. Structure–activity relationship of β-L-2′-deoxynucleosides

					EC_{50} (μM)	
					anti-HBV	anti-HIV
	R1	R2	R3	X	2.2.15 cells	PBM cells
LdC	H	H	OH	CH	0.24±0.08	>200
L-5-FdC	F	H	OH	CH	5	>100
L-5-CldC	Cl	H	OH	CH	10	>100
L-ddC	H	H	H	CH	0.1	0.26
3TC	H	H	–	S	0.05±0.01	0.002
L-3′-azido-5-FddC	F	H	N_3	CH	0.11±0.09	0.05
L-3′-FddC	H	H	F	CH	0.5	82
FTC	F	H	–	S	0.04	0.008
L-5-ClddC	Cl	H	H	CH	10	>100
L-d4C	H	–	–	CH	<0.1	1.0
L-d4FC	F	–	–	CH	<0.1	0.034
L-3′-F-5-FddC	F	–	F	CH	4	>100
L-5-FddC	F	–	–	CH	0.10±0.05	0.021
LdT		H	OH		0.19±0.09	>200
L-ddT		H	H		>10	>100
L-3′-FddT		H	F		>10	>100
L-3′-azido-ddT		H	N_3		>10	>100
L-3′-amino-ddT		H	NH_2		>10	>10
L-d4T		–	–		>10	>100
L-xylo-dT		OH	H		>10	>10
LdA	H	H	OH		0.10–1.9	>10
L-2-CldA	Cl	H	OH		>10	>10
L-ddA	H	H	H		5	>10
L-d4A	H	–	–		0.80±0.10	0.38
L-3′-azido-ddA	H	H	N_3		5	>10
L-3′-amino-ddA	H	H	NH_2		>10	>10
L-3′-fluoro-ddA	H	H	F		>10	>100
L-ddAMP-bis(tbutylSATE)	H	H	H		0.08±0.03	0.002
L-3′-azido-d4A	H	–	N_3		>10	>100

EC_{50}, antiviral 50% effective concentration. The symbol (>) is used to indicate the highest concentration at which the compounds were tested. Values represent the means of at least three independent experiments. Anti-HIV data for L-ddC, 3TC, FTC, L-5-FddC, L-d4FC from references (Schinazi et al., 1992; Gosselin et al., 1994; Shi et al., 1999). L-d4T, L-ddA and L-d4A data from references (Bolon et al., 1996; Gosselin et al., 1997).

specificity is only seen in the presence of the 3′-OH group. In this case, only LdT itself is active, while closely related molecules are not, suggesting that the 3′-OH group plays a role in determining the affinity of the molecule for the HBV polymerase.

To further assess their antiviral activity and specificity, LdC, LdT and LdA were screened against 15 different RNA and DNA viruses (Table 2). The striking finding was that all three β-L-2′-deoxynucleosides inhibited HBV replication as well as the replication of the closely related duck HBV (DHBV), yet had no activity against HIV-1, HSV-1, HSV-2, VZV, EBV, HCMV, adenovirus type-1, influenza A and B, measles virus, parainfluenza type-3, rhinovirus type-5 and RSV type-A at concentrations as high as 200 μM. Potent antiviral activity against the WHV, using an *in vivo* model of chronic HBV infection, is described later in this report. Thus, the unmodified β-L-2′-deoxynucleosides, LdC, LdT and LdA, exhibit an unusual degree of specificity for inhibiting members of the small family of hepadnaviruses, HBV, DHBV and WHV.

The majority of nucleoside analogues with antiviral activity inhibit the viral replication step via direct interaction of their 5′-triphosphate metabolites with the respective viral polymerase. Consistent with this idea, the 5′-triphosphates of the β-L-2′-deoxynucleosides, LdC, LdA and LdA inhibit the WHV DNA polymerase in *in vitro* assays with 50% inhibitory concentration (IC_{50}) values of 0.24–1.82 μM (data not shown). By analogy with other nucleoside analogues, LdC, LdT and LdA likely inhibit the reverse transcription of pre-genomic RNA and/or the synthesis of HBV second-strand DNA. They may, moreover, cause obligate chain termination of DNA synthesis by internal incorporation of L-dNMP into viral DNA. Additionally, it is possible that these compounds may inhibit other important activities of the polymerase (which include RNaseH activity, the hepadnavirus-specific

Table 2. Antiviral activity of LdC, LdT and LdA

		EC_{50} (μM)			CC_{50} (μM)		
Virus[a]	Cell line	LdC	LdT	LdA	LdC	LdT	LdA
HBV	2.2.15	0.24	0.19	0.10	>2000	>2000	>1000
DHBV	PDH	0.87	0.18	0.15	ND	ND	ND
HIV-1	PBMC	>200	>200	>200	>200	>200	>200
HSV-1	HFF	>20	>200	>100	>60	>200	>100
HSV-2	HFF	>100	>100	>100	>100	>100	>100
VZV	HFF	>100	45.2	>100	>100	18.6	>100
EBV	Daudi	>50	>50	5.7	>50	>50	23.1
HCMV	HFF	>100	>100	>100	>100	>100	>100
Adenovirus type-1	A549	>100	ND	>100	>100	ND	>100
Influenza A	MDCK	>100	>100	>100	>100	>100	>100
Influenza B	MDCK	>100	>100	>100	>100	>100	>100
Measles	CV-1	>100	>100	>100	>100	>100	>100
Parainfluenza type-3	MA-104	>100	>100	>100	>100	>100	>100
Rhinovirus type-5	KB	>100	ND	>100	>100	ND	>100
RSV type-A	MA-104	>100	>100	>100	>100	>100	>100

[a]The specific antiviral activity of LdC, LdT and LdA was confirmed using a panel of viruses tested by the NIH NIAID Antiviral Research and Antimicrobial Chemistry Program.
EC_{50}, antiviral 50% effective concentration; CC_{50}, 50% cytotoxic concentration. PDH, primary duck hepatocytes; PBMC, peripheral blood mononuclear cells; HFF, human foreskin fibroblast; Daudi, Burkitt's B-cell lymphoma; A549, human lung carcinoma; MDCK, canine kidney epithelial cells; CV-1, African green monkey kidney fibroblast cells; MA-104, Rhesus monkey kidney epithelial cells; KB, human nasopharyngeal carcinoma.
ND, not determined.

priming of reverse transcription and the co-ordination of intracellular virion assembly). Finally, we note the possibility that LdC, LdT and LdA may differ in their precise mechanism of action. Further analysis of the complete mechanism of action of these compounds is underway.

In addition to antiviral specificity, the selectivity of antiviral drugs becomes a critical factor in determining whether they will ultimately be suitable for use in human patients. This is particularly true when long-term therapy is required, as is the case for chronic HBV infection. Toxic side-effects, primarily related to non-selective interaction with cellular polymerases, have been a major limitation for the clinical use of several nucleoside analogues (Faulds & Brogden, 1992; Whittington & Brogden, 1992; Wilde & Langtry, 1993; Hurst & Noble, 1999).

When tested in *in vitro* polymerase assays using purified human DNA polymerases α, β and γ, the 5′-triphosphates of LdC, LdT and LdA did not inhibit enzymatic activity at concentrations up to 100 μM (data not shown). Krayevsky and co-workers also reported that the 5′-triphosphates of LdC and LdT were not substrates for human DNA polymerases (Semizarov *et al*., 1997). Thus, these compounds are highly selective for viral versus host polymerases.

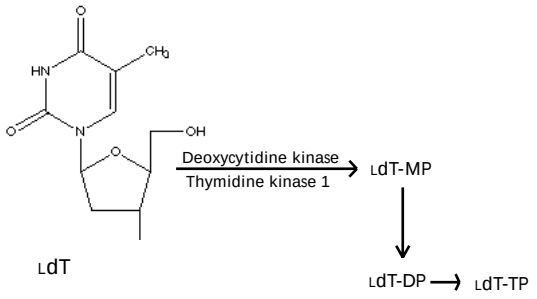
Further evidence for the selectivity of these compounds comes from the lack of cytotoxicity seen for the NV-02 nucleosides (Table 2), implying a lack of effect on host cell functions. When tested against 10 different cell lines, LdC, LdT and LdA mostly showed little or no evidence of cytotoxicity at concentrations greater than 100 μM. In particular, LdC, LdT and LdA had no cytotoxic effect on primary human peripheral blood mononuclear cells (PBMC), human foreskin fibroblasts (HFF), or other cell types of mammalian origin (Table 2). In addition, studies by Verri *et al*. (1997) demonstrated that L-dC was not cytotoxic toward lymphoblastoid T cells. Finally, these compounds were found not to be cytotoxic in the human hepatoma cell line 2.2.15 (CC_{50} values >2500 μM); the CC_{50} values for LdT and LdC are ~3 mM in this cell line. The excellent safety profile of these compounds will be discussed in more detail later in this report.

Intracellular activation, metabolism and pharmacology

Metabolic pathways have been worked out for LdT and LdC based on extensive intracellular accumulation and decay data, and on competition experiments using the corresponding endogenous D-nucleosides. These pathways are summarized in Figure 2 for LdT and Figure 3 for LdC. LdT is converted into the triphosphate (TP) form by redundant cellular kinases, whereas dCTP formation utilizes only deoxycytidine kinases.

LdC, LdT and LdA are metabolized efficiently (activated) to their respective 5′-triphosphate derivatives in HepG2 cells and human hepatocytes in primary culture (Placidi *et al*., 1999). The phosphorylation pattern in primary hepatocytes of human and animal origin is qualitatively and quantitatively similar for the activated

Figure 2. Proposed metabolic pathway for LdT



5′-TP form of each compound. This is in contrast to earlier studies reporting limited intracellular activation of LdT (Spadari *et al*., 1992; Focher *et al*., 1995).

The metabolic profiles obtained after a 24-h exposure of HepG2 cells to 10 μM [^{3}H]-LdT and [^{3}H]-LdC are shown in Figure 4. LdT was efficiently converted into the active triphosphate form, which reached a peak concentration of just below 30 μM at 24 h. The mono- and diphosphate forms were present intracellularly at much lower levels. For LdC, the triphosphate form again accumulated efficiently, reaching a maximal intracellular concentration of 70 μM, but the metabolic pathway is more complex. Along with L-dCTP, a second 5′-triphosphate derivative, corresponding to β-L-2′-deoxyuridine 5′-triphosphate (LdUTP), was formed. Similar to other β-L-cytidine analogues (Chang *et al*., 1992; Furman *et al*., 1992; Martin *et al*., 1997; Verri *et al*., 1997), LdC was not a substrate for cytosolic cytidine deaminase; thus, deoxycytidylate deaminase acting on LdC-5′-monophosphate is presumed to explain the formation of this metabolite. Another metabolite corresponding to a choline form of dCDP was also detected in HepG 2 cells. However, the important point is that these metabolites are minor; their formation does not significantly diminish the concentration of the active LdCTP species.

Metabolic decay experiments revealed the apparent intracellular half-lives of the LdT-5′-TP and LdC-5′-TP to be long, in other words ≥14 h. Thus, even after 24 h, the intracellular TP concentrations were well in excess of the estimated IC_{50} values (about 0.24–1.82 μM for the WHV DNA polymerase) and remained above the IC_{90} values (about 5μM).

In summary, the efficient conversion of LdT and LdC into high concentrations of the respective triphosphate forms, coupled with the long half-lives of the triphosphates, creates a favourable scenario for HBV antiviral therapy.

Pharmacokinetic profiles

The pharmacokinetic profile of LdT in the cynomologous monkey is presented in Figure 5. Following intravenous administration, plasma concentrations of LdT declined in a bi-exponential manner and to undetectable levels after 8 h. The terminal phase half-life was ~1.5 h in monkeys and somewhat longer (~3.5 h) in woodchucks. The total clearance was higher in monkeys (~0.60 l/h/kg) than in woodchucks (~0.30 l/h/kg). The volume of distribution at steady state (Vss) indicated good tissue distribution in both species. Oral absorption of L-dT was slow in monkeys and in woodchucks, with peak concentrations occurring 1–4 h after dosing. The absolute oral bioavailability (%F) for L-dT reached 68.6% in monkeys and 38.3% in woodchucks.

The %F of LdC was lower and more variable than LdT in woodchucks (9.6%) and monkeys (16.4%). To improve oral absorption, a series of ester prodrugs was synthesized. The %F of the 3′,5′ valine ester prodrug of LdC, which was selected for development, increased at least fourfold in monkeys compared to LdC.

Figure 3. Proposed metabolic pathway for LdC

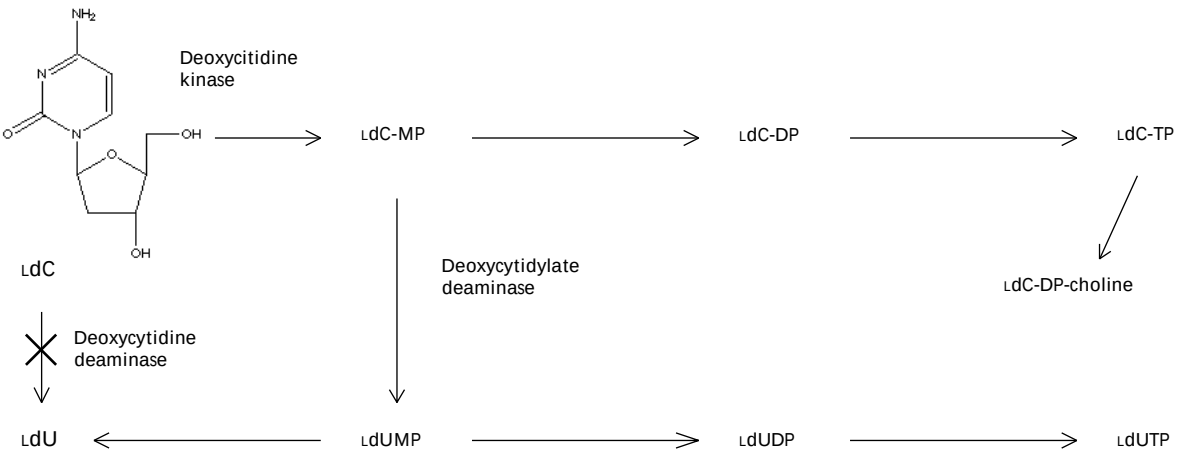


Table 3. Human bone marrow toxicity of LdA, LdT and LdC in granulocyte macrophage progenitor and erythrocyte precursor cells

Compound	CFU-GM IC_{50} (μM)	BFU-E IC_{50} (μM)
LdA	>40	>10
LdT	>40	>10
LdC	>40	>10
Zidovudine	1.9±1.2	0.6±0.5

CFU-GM, granulocyte macrophage progenitor cells, colony forming units; BFU-E, erythrocyte precursor cells, burst forming units.

Antiviral activity in the woodchuck chronic hepatitis model

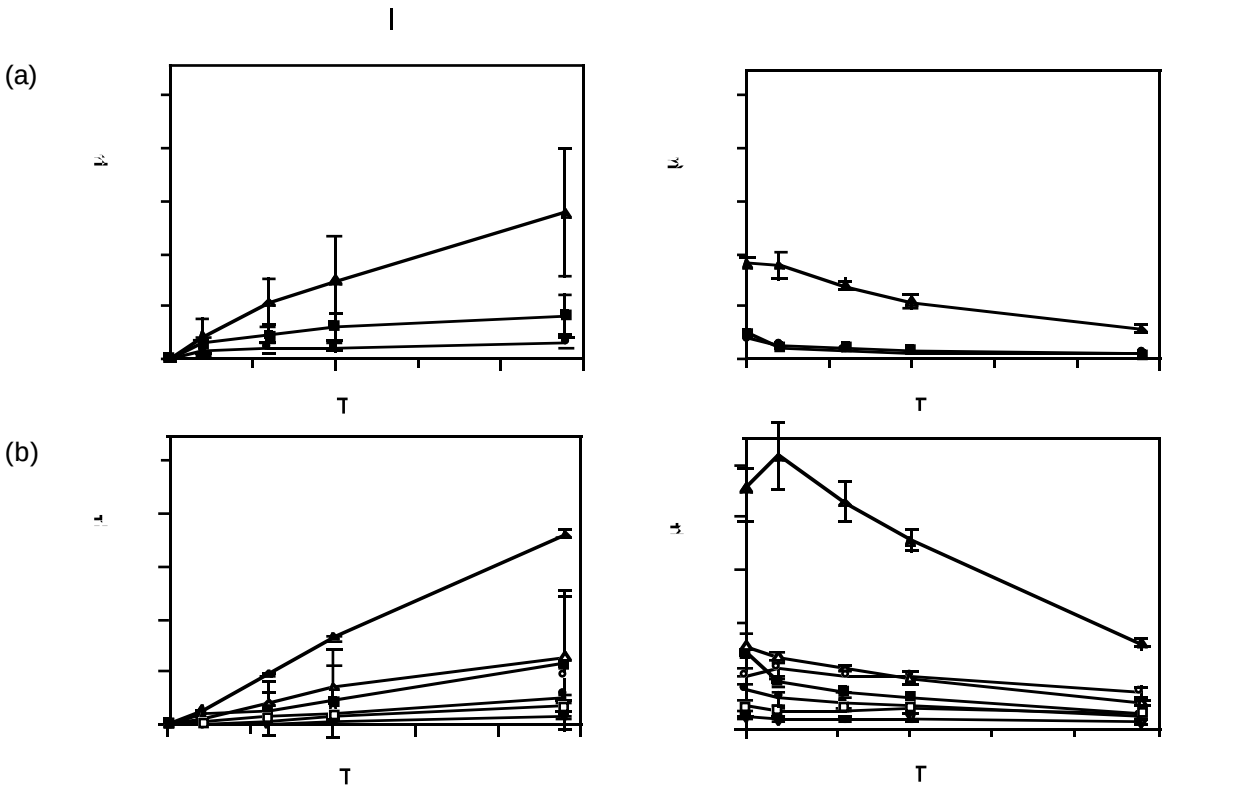
Woodchucks chronically infected with WHV are widely accepted as a model of HBV infection and have proven useful in the evaluation of anti-HBV agents. This model has been shown to be a positive predictor of antiviral activity as well as safety for the treatment of human chronic HBV infection (Korba *et al*., 1990, 2000; Tennant *et al*., 1998).

The study design for assessing efficacy in this model involved 4 weeks of daily treatment (three animals per group) with 10 mg/kg/day LdT or LdC (delivered by oral gavage) and 8 weeks of follow-up. The study included two control arms: a placebo arm and a 10 mg/kg/day 3TC treatment arm. Serum levels of WHV DNA were determined throughout the study by DNA dot-blot hybridization (detection limit, approximately 10^7 genome equivalents/ml serum) and by quantitative PCR (detection limit, 300 genome equivalents/ml serum).

WHV DNA replication was significantly inhibited within the first few days of treatment with either LdT or LdC, whereas placebo levels remained unaffected (data not shown). Most notably, serum WHV DNA levels (WHV viraemia) decreased up to 8 log to below the limit of detection by PCR in the LdT treated group (see below) and decreased by 4–6 log in the LdC-treated animals (data not shown). WHV DNA levels rebounded to near pre-treatment levels by 8 weeks following drug withdrawal.

In contrast, the cytidine analogue 3TC (10 mg/kg/day)

Figure 4. Intracellular accumulation and decay of metabolites after 24 h exposure of HepG2 cells to 10 μM LdT or LdC



(a) Closed square, LdTMP; closed triangle, LdTTP; closed circle, LdTDP.
(b) Closed square, LdCMP; closed triangle, LdCTP; closed circle, LdCDP; closed inverted triangle, LdUDP; open square, LdUMP; open triangle, LdCDP choline; open circle, LdUTP.

Table 4. Effect of LdC, LdT and LdA on mitochondria in HepG2 cells

Compound	Conc. (μM)	Cell density (% of control)	L-Lactate (% of control)	mtDNA (% of control)	Lipid droplet formation	Mitochondrial morphology
Control		100	100	100	Negative	Normal
LdC	0.1	102±12	100±4	105±11	ND	ND
	1.0	100±6	101±6	99±10	ND	ND
	10	101±10	101±2	107±8	Negative	Normal
LdT	0.1	103±7	102±2	103±4	ND	ND
	1.0	106±8	99±2	101±7	ND	ND
	10	97±7	105±2	97±4	Negative	Normal
LdA	0.1	103±14	99±3	97±14	ND	ND
	1.0	102±14	102±3	92±8	ND	ND
	10	100±14	103±5	88±18	Negative	Normal
Lamivudine[a]	0.1	101±2	99±5	107±8	ND	ND
	1.0	99±1	101±3	96±9	ND	ND
	10	99±1	98±3	98±10	Negative	Normal
FIAU[a]	0.1	83±6	119±5	101±2	ND	ND
	1.0	73±9	134±9	118±5	ND	ND
	10	37±10	203±13	86±4	Positive	Abnormal

HepG2 cells were treated with the indicated concentrations of LdT, LdC or LdA for 14 days. Values are presented as means and standard deviations of three independent experiments.
ND, not determined.
[a]Data from reference (Lewis *et al*., 1992; Wilde & Langtry, 1993).

reduced the HBV genome equivalents/ml in serum by only 0.5–1.0 log. This limited effect is consistent with previous studies using similar doses of 3TC (Genovesi *et al*., 1998). Higher doses (40–200 mg/kg) of this drug are required to produce significant antiviral activity in this model (Mason *et al*., 1998). The low activity of 3TC in the woodchuck model has been ascribed in part to poor absorption and in part to the low conversion of 3TC and other cytidine analogues to their active 5′-triphosphate forms seen in rodent/woodchuck liver compared to that in human liver. The oral bioavailability of 3TC in woodchucks has been reported to be 18–54 versus 82% in humans (van Leeuwen *et al*., 1992; Rajagopalan *et al*., 1996). With these caveats in mind, the performance of LdC in the woodchuck is surprisingly good, suggesting that the ester prodrug of LdC, which had an oral bioavailability of four times that of LdC in the monkey, should have good potency against HBV in human patients.

Figure 5. Plasma concentration in monkeys after (a) intravenous or (b) oral administration of 10 mg/kg LdT. The data are the mean (±SD) from three animals per group.

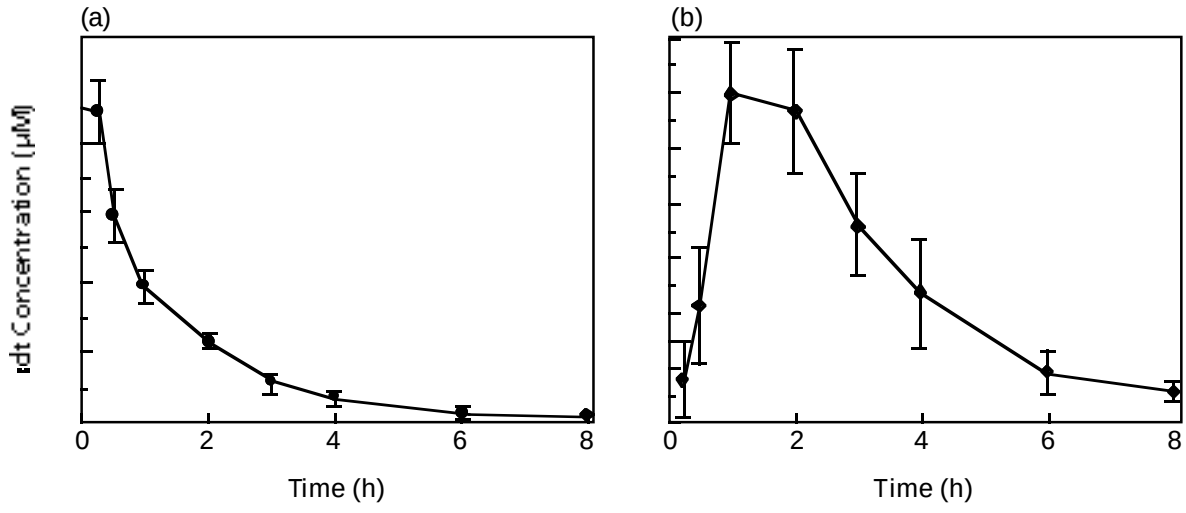
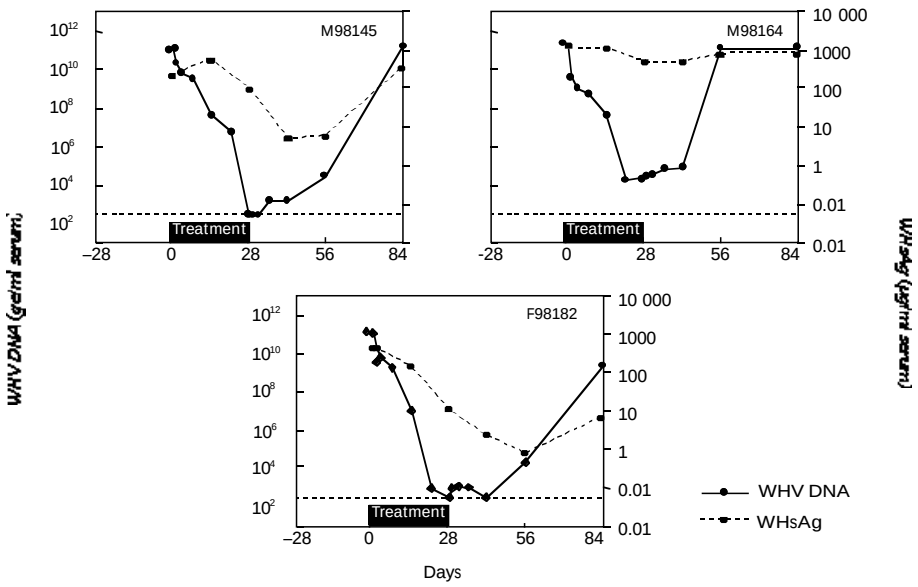


Figure 6. Reduction of serum virus load and WHsAg in the woodchuck chronic HBV model



For the LdT-treated animals, which showed the most marked reduction in viral load, we also observed a decline in WHV surface antigen as measured using the method of Cote *et al*. (1993). The data are summarized in Figure 6. The strength of the surface antigen response broadly paralleled the viral load response, but the onset of the surface antigen response was delayed by at least 1 week compared to the reduction in viral load. Surface antigen levels continued to fall for several weeks after drug removal before rebounding. This result is intriguing, since a correlation has been demonstrated in this model between HBsAg reduction and the clearance of cccDNA from infected hepatocytes (Cote *et al*., 1993).

In a separate 12-week study in the woodchuck, the combination of 1 mg/kg/day LdT and 1 mg/kg/day LdC reduced viral load to levels significantly lower than either agent alone. This combination of LdT and LdC (each at one-tenth the concentration of monotherapy) reduced viral load to the limit of detection (300 genome equivalents/ml serum). Following drug removal, the time to viral rebound was markedly prolonged when LdT and LdC were administered in combination. A dramatic decrease in hepatitis B surface antigen, as a marker of viral replication, was also seen (data not shown). In both the 28-day study and the 12-week study, no toxicity was seen at the highest dose tested.

Safety profile of LdT, LdC and LdA

As discussed earlier in this report, long-term therapy is expected for chronic HBV infection. Thus, the nucleoside safety profile is a critical issue, particularly since clinically limiting side-effects have been well documented for some nucleoside analogues (Faulds & Brogden, 1992; Whittington & Brogden, 1992; Wilde & Langtry, 1993; Hurst & Noble, 1999). The lack of inhibitory activity of the LdT, LdC and LdA triphosphates has been discussed earlier, along with the lack of cytotoxicity in a number of different mammalian cell lines. Described briefly below are additional safety studies that have been performed with these compounds and in particular with the lead compound, LdT.

Human bone marrow stem cells in primary culture have been shown to be a good predictor of potential nucleoside analogue-induced haematotoxicity in patients (Sommadossi *et al*., 1989; Faraj *et al*., 1994). Granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) precursors exposed to LdC, LdT and LdA in clonogenic assays which routinely detect the cellular toxicity of zidovudine (AZT, β-D-3′-azido-3′-deoxythymidine) were not affected (Table 3). These results suggest that LdC, LdT and LdA are highly selective and their phosphorylated forms will be non-toxic *in vivo*.

Nucleoside analogues used in AIDS therapy, such as AZT, stavudine (d4T, β-L-2′,3′-didehydro-2′,3′-dideoxythymidine) didanosine (ddI, β-D-2′,3′-dideoxyinosine) and zalcitabine (ddC, β-D-2′,3′-dideoxycytidine), have shown clinically limiting delayed toxicities, such as peripheral neuropathy, myopathy and pancreatitis (Faulds & Brogden, 1992; Whittington & Brogden, 1992; Wilde & Langtry, 1993; Hurst & Noble, 1999). These adverse

effects are attributable to decreased mitochondrial DNA (mtDNA) content and/or altered mitochondrial function leading to increased lactic acid production and hepatic steatosis (Chen & Cheng, 1989; Dalakas *et al*., 1990; Lewis *et al*., 1992; Cui *et al*., 1995, 1996, 1997; Pan-Zhou *et al*., 2000). Concomitant morphological changes in mitochondria (for example, loss of cristae, matrix dissolution and swelling, and lipid droplet formation) can be observed with ultrastructrual analysis using transmission electron microscopy (Cui *et al*., 1996; Lewis *et al*., 1996; Pan-Zhou *et al*., 1998). For example, fialuridine (FIAU, 1,2′-deoxy-2′-fluoro-1-β-D-arabinofuranosly-5-iodo-uracil) toxicity was shown to be associated with an irreversible intracellular event that decreased mitochondrial respiratory function, resulting in decreased mitochondrial ATP production and fatty acid metabolism. This form of mitochondrial toxicity can be initially identified in cell culture by increased lactic acid production and intracellular lipid droplet formation. In HepG2 cells incubated with 10μM FIAU, a substantial increase in lactic acid production was observed (Table 4). Electron micrographs of these cells showed the presence of enlarged mitochondria with morphological changes consistent with mitochondrial dysfunction (data not shown). Lamivudine (10 μM) did not affect mitochondrial structure or function. Using similar conditions, exposure of HepG2 cells to 10μM LdC, LdT or LdA for 14 days had no effect on lactic acid production, mitochondrial DNA content or morphology (Table 4).

In acute (50–2000 mg/kg single oral dose) and subacute (500–2000 mg/kg/day orally for 28 days) toxicology studies in rats and monkeys there were no overt signs of toxicity, nor were there any LdT related effects on body weight, food consumption, or clinical pathology parameters (haematology and serum chemistry). In addition, there were no macroscopic lesions observed at necropsy, nor were there any microscopic findings on histomorphological analysis attributable to LdT. Based on the results of these studies, the no observed adverse effect level (NOAEL) for LdT following a single oral dose, or repeated dosing for 28days by oral gavage in the Sprague–Dawley rat and cynomologus monkey was 2000 mg/kg.

In normal healthy woodchucks or woodchucks chronically infected with HBV, no toxicity was observed during acute (10 mg/kg single dose intravenous and per oral) and subacute (28 days at 10 mg/kg/day orally and 12weeks at 1 mg/kg/day orally) studies. There was no weight loss in the LdT treatment groups compared to control animals. Clinical pathology parameters (haematology and serum chemistry) were in the normal range and end of treatment liver biopsies in the 12-week study showed no evidence of fatty change (microvesicular steatosis).

Genotoxicity assays have been completed on LdT and LdC, Neither compound was mutagenic in the *S. typhimurium* or *E. coli* plate incorporation mutagenicity assay at concentrations up to a maximum of 5000 μg/plate. There was no evidence of chromosomal aberrations in the Chinese hamster ovary (CHO) assay after exposure to LdT or LdC at concentrations up to a maximum of 5000 μg/ml (20.6 mM). In the mouse micronucleus assay, LdT and LdC were not clastogenic to male or female animals (maximum dose tested 2000 mg/kg).

Discussion

Three structurally simple β-L-2′-deoxynucleosides, LdA, LdC, and LdT, have been identified as highly specific and selective inhibitors of hepadnaviral replication. Unlike essentially all nucleoside analogues currently in the clinic, LdA, LdC, and LdT are not chemically modified. They differ from their natural nucleoside counterparts only with respect to the spatial relationship of their base and sugar moieties, having an L-configuration versus the D-configuration of the natural deoxynucleosides. For all three nucleosides, the presence of a hydroxyl group in the 3′ position appears to be the key to the high degree of specificity they display towards HBV versus other viruses.

In addition to their *in vitro* activity against HBV, LdT and LdC are potent inhibitors of WHV replication in the woodchuck efficacy model of chronic HBV infection. At a once-daily oral dose of 10 mg/kg/day), LdC is considerably more efficacious than 3TC at reducing serum viral titres, while LdT in this model reduced WHV viral titres by as much as 8 log. This level of antiviral suppression rivals that seen with any other compound. The excellent absolute %F of LdT and of the val-LdC prodrug, coupled with the long (~15 h) half-lives of the respective triphosphates in cells, suggests that these compounds will be suitable for once daily dosing in people.

A salient characteristic of LdT, the first member of this series, is its excellent safety profile. LdT exhibits very low cellular or mitochondrial toxicity and does not inhibit cellular polymerases. Based on its structure, LdT is expected to act as an obligate chain terminator of DNA synthesis, as is seen with other L-nucleosides such as 3TC. Thus, LdT will not incorporate into cellular DNA. Consistent with this idea, LdT is negative in all genotoxicity assays. Most impressively, no adverse events were seen in rats or monkeys treated with LdT for 28 days at doses as high as 2 g/kg/day.

The stellar safety profile of LdT, coupled with its antiviral potency, has led to its recent entry into a Phase I/II clinical trial in HBV infected patients under a US IND. An ester prodrug of LdC is expected to begin clinical trials early in 2001. It is also anticipated that these new

nucleosides will be used in combination (in other words LdT and LdC or LdT and 3TC) to further reduce chronic HBV replication and prevent the selection of resistant virus.

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