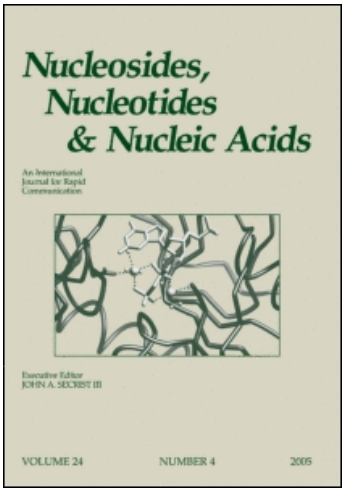

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ANTI-HBV SPECIFIC β-L-2'-DEOXYNUCLEOSIDES

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ANTI-HBV SPECIFIC β-L-2′-DEOXYNUCLEOSIDES

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ABSTRACT

A unique series of simple unnatural L-nucleosides that specifically inhibit hepatitis B virus (HBV) replication has been discovered. These molecules have in common a hydroxyl group in the 3′-position (3′-OH) of the β-L-2′-deoxyribose

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sugar that confers antiviral activity specifically against hepadnaviruses. Replacement of the 3′-OH broadens activity to other viruses. Substitution in the base decreases antiviral potency and selectivity. Human DNA polymerases and mitochondrial function are not effected. Plasma viremia is reduced up to 8 logs in a woodchuck model of chronic HBV infection. These investigational drugs, used alone or in combination, are expected to offer new therapeutic options for patients with chronic HBV infection.

INTRODUCTION

Since the Food and Drug Administration (FDA) approved lamivudine for the treatment of HIV infection in the United States in 1996 and for HBV in 1998, intensive studies on additional "unnatural" L-nucleosides as antiviral agents against HIV, HBV, herpesviruses, including EBV, and as anticancer agents have been conducted [1]. Now, through an extensive structure–activity analysis, we have found that the 3′-OH group of the β-L-2′-deoxyribose of the β-L-2′-deoxynucleoside series confers unique specificity for anti-HBV activity. In this chemical series, β-L-2′-deoxycytidine (L-dC), β-L-thymidine (L-dT), and β-L-2′-deoxyadenosine (L-dA) had the most potent, selective and specific antiviral activity against HBV replication.

The β-L-2′-deoxynucleoside Series Is Specific for Hepatitis B Virus

The structure–activity relationship (SAR) established among the β-L-2′-deoxycytidine, -thymidine and -deoxyadenosine series are presented in Table 1. Substitution of a halogen atom at the 5-position (R1) in the pyrimidine ring of L-dC, without modification of the deoxyribose sugar (e.g., β-L-2′-deoxy-5-fluorocytidine, L-5-FdC; β-L-2′-deoxy-5-chlorocytidine, L-5-CldC), decreased the potency against HBV but did not affect the antiviral specificity for HBV. In contrast, analogs of L-dC which lacked the 3′-OH group (R3) on the deoxyribose sugar (e.g., β-L-2′,3′-dideoxycytidine, L-ddC; β-L-2′,3′-dideoxy-3′-thiacytidine, 3TC; β-L-2′,3′-didehydro-2′,3′-dideoxycytidine, L-d4C) lost antiviral specificity for HBV and showed activity against HIV. Similarly, replacement of the 3′-OH group with a 3′-fluoro moiety (e.g., β-L-2′,3′-dideoxy-3′-fluorocytidine, L-3′-FddC) eliminated the antiviral specificity although antiviral potency against HBV and HIV was retained.

In addition, substitutions at the 5-position (R1) of the pyrimidine base of β-L-2′,3′-dideoxycytidine lacking the 3′-OH group (e.g., β-L-2′,3′-dideoxy-5-fluorocytidine, L-5-FddC; β-L-2′,3′-dideoxy-5-chlorocytidine, L-5-CldC; β-L-2′,3′-dideoxy-3′-thia-5-fluorocytidine, FTC; β-L-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine, L-d4FC; β-L-2′,3′-dideoxy-3′-fluoro-5-fluorocytidine, L-3′-F-5-FddC; β-L-2′,3′-dideoxy-3′-azido-5-fluorocytidine, L-3′-azido-5-FddC) further affected the antiviral potency of these analogs against HBV, as well as HIV. These studies suggest that the 3′-OH of the β-L-2′-deoxyribose of L-dC plays a crucial

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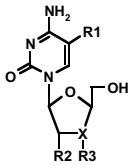
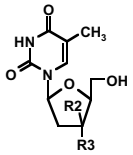
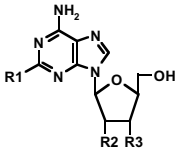
Table 1. Structure–Activity Relationships of L-dC, L-dT and L-dA Analogs

	R1	R2	R3	X	EC_{50} (μM)[a] Anti-HBV 2.2.15 Cells	Anti-HIV PBM Cells	
L-dC	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	
L-dT		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	
L-dA	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	

[a]Antiviral 50% effective concentrations (EC_{50}) were determined as described in the Methods section. The greater than symbol (>) is used to indicate the highest concentration at which the compounds were tested. Values are presented as means of at least three independent experiments. Anti-HIV data for L-ddC, 3TC, FTC, L-5-FddC, L-d4FC from references [2–4]. L-d4T, L-ddA and L-d4A data from references [5, 6].
[b]nd, not determined.

role in inhibiting virus replication possibly by specific interaction with the HBV DNA polymerase.

The structure–activity relationships for the L-dT and L-dA series (Table 1) were similar to that observed for the L-dC series. The specific anti-HBV activity of L-dT and L-dA was lost upon removal or substitution of the 3′-OH group (R3).

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β-L-2′-deoxy-xylo-thymidine (L-xylo-dT), which is identical to L-dT except for the 3′-OH group in the opposite orientation (R2), also lost anti-HBV activity, further emphasizing the importance of the 3′-OH group in the interaction with the HBV DNA polymerase. An L-dT analog with a fluorine substitution at the 2′ upposition (L-FMAU, β-L-2′-deoxy-2′-fluoro-5-methyl-arabinofuranosyl uracil) has been reported to have activity against both HBV and EBV [7]. Thus, it is possible that modification of the 2′-position in addition to the 3′-position of L-dT may also change antiviral specificity for HBV.

Substitution at the 2-position (R1) on the purine base of L-dA (e.g., β-L-2′-deoxy-2-chloroadenosine, L-2-CldA) had a negative effect on anti-HBV activity. The analogs of L-dA lacking the 3′-OH group with or without further modification of the deoxyribose sugar lost specificity and were not as potent against HBV. The marginal antiviral activity of β-L-2′,3′-dideoxyadenosine (L-ddA), despite its potent inhibitory activity against both HIV reverse transcriptase (HIV-RT) and woodchuck hepatitis (WHV) DNA polymerase (Placidi et al., Antimicrob. Agents Chemother., submitted, 2000), can be explained by the low intracellular concentrations of the phosphorylated form due to rapid and extensive catabolism [8]. This conclusion is also supported by recent studies that demonstrated potent antiviral activity of an L-ddA 5′-monophosphate prodrug (β-L-2′,3′-dideoxy-adenosine-5′-monophosphate-tbutyl-S-acyl-2-thioethyl; L-ddAMP-bis-(tbutyl-SATE)). The prodrug form decreases the intracellular catabolism of the parent molecule [*Antiviral Therapy* **3** (suppl. 3) abstr. A22, 1998] and releases the 5′-monophosphate derivative inside the cell. When used in this pronucleotide form, L-ddA was active against both HIV and HBV, further supporting the importance of the 3′-OH group for antiviral specificity. As in the L-dC and L-dT series, unmodified β-L-2′-deoxyadenosine most potently and specifically inhibited HBV replication.

To further assess their antiviral specificity, L-dC, L-dT and L-dA were screened against 15 different RNA and DNA viruses (Table 2).

The β-L-2′-deoxynucleosides inhibited hepadnavirus replication as previously defined by the SAR but 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 woodchuck hepatitis B virus (WHV) is described later using an *in vivo* model of chronic hepatitis B virus infection. Thus, the unmodified β-L-2′-deoxynucleosides, L-dC, L-dT and L-dA, are uniquely specific for the hepadnaviruses HBV, DHBV, and WHV.

Selectivity of β-L-2′-deoxynucleosides

Since long-term treatment is expected for chronic HBV infection, drug selectivity is a critical issue. Toxic side-effects have been a major issue limiting the clinical use of some nucleoside analogs [9–12]. The 5′-triphosphates of L-dC, L-dT and L-dA did not inhibit human DNA polymerases α, β and γ at concentrations up to 100 μM. Krayevsky and coworkers also reported that the 5′-triphosphates

Table 2. Antiviral Activity and Cytotoxicity Levels of L-dC, L-dT and L-dA

		EC_{50} (μM)[b]			CC_{50} (μM)		
Virus[a]	Cell line	L-dC	L-dT	L-dA	L-dC	L-dT	L-dA
HBV	2.2.15	0.10	0.80	0.10	>2000	>2000	>1000
DHBV	PDH	0.0007	0.054	0.0009	nd[c]	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
Parainfluenz 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 L-dC, L-dT and L-dA was confirmed using a panel of viruses by the NIH NIAID Antiviral Research and Antimicrobial Chemistry Program.

[b]Antiviral 50% effective concentrations (EC_{50}) and 50% cytotoxic concentrations (CC_{50}) for HBV and HIV-1 were determined as described in the Methods section. 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.

[c]nd, not determined.

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of L-dC and L-dT were not substrates for human DNA polymerases [13]. L-dC, L-dT and L-dA had no cytotoxic effect on the human hepatoma cell line 2.2.15 (CC_{50} values $>1{,}000$ μM), in primary human peripheral blood mononuclear cells (PBMC), human foreskin fibroblasts (HFF), or other cell types of mammalian and avian origin (Table 2). In addition, studies by Verri et al. demonstrated that L-dC was not cytotoxic toward lymphoblastoid T cells [14]. Human bone marrow stem cells in primary culture have been shown to be a good predictor of potential nucleoside analog-induced hematotoxicity in patients [15, 16]. Granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) precursors exposed to L-dC, L-dT and L-dA in clonogenic assays at concentrations up to 50 μM were not affected. These results suggest that L-dC, L-dT and L-dA are highly selective and their phosphorylated forms will be non-toxic *in vivo*.

L-dC, L-dT and L-dA were efficiently metabolized (activated) to their respective 5′-triphosphate derivatives in HepG2 cells and human hepatocytes in primary culture [*Antiviral Therapy* **4** (suppl. 4) abstr. A122, 1999]. Earlier studies reported limited intracellular activation of L-dT [17, 18]. Together with the potent *in vitro* antiviral activity, this data suggests that like other nucleoside analogs, the intracellular phosphorylated form was responsible for inhibition of the viral polymerase. Furthermore, the 5′-triphosphates of L-dC, L-dT and L-dA each inhibited WHV DNA polymerase with a 50% inhibitory concentration (IC_{50}) of 0.24−1.82 μM. In addition, exposure of HepG2 cells to L-dC led to a second 5′-triphosphate derivative, i.e., β-L-2′-deoxyuridine 5′-triphosphate (L-dUTP) which also inhibited WHV DNA polymerase with an IC_{50} of 5.26 μM [*Antiviral Therapy* **4** (suppl. 4) abstr. A119 and A122, 1999]. Similar to β-L-cytidine analogs [14, 19–21], L-dC was not a substrate for cytosolic cytidine deaminase which suggested that the 5′-monophosphate metabolite of L-dC may be susceptible to deamination through deoxycytidylate deaminase. The inhibition of HBV replication by these β-L-2′-deoxynucleosides and inhibition of hepadnaviral polymerase by their corresponding 5′-triphosphates suggested that, like most nucleoside analogs, L-dC, L-dT and L-dA may act by inhibiting the reverse transcription of HBV pregenomic RNA. Demonstration that L-dNTP analogs inhibit HBV reverse transcriptase/DNA polymerase activity does not preclude other mechanisms of action. Inhibition of other important activities of the polymerase (which include RNaseH activity, priming of reverse transcription and co-ordination of intracellular virion assembly), or the possibility of internal incorporation of L-dNMP into viral DNA as a mechanism of inhibition are currently under investigation.

β-L-2′-deoxynucleosides Have No Effect on Mitochondrial Function or Morphology

Nucleoside analogs used in AIDS therapy, such as zidovudine (AZT, β-D-3′-azido-3′-deoxythymidine), stavudine (d4T, β-L,2′,3′-didehydro-2′,3′-dideoxythymidine) didanosine (ddI, β-D-,2′,3′-dideoxyinosine) and zalcitabine (ddC,

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β-D-2′,3′-dideoxycytidine), have shown clinically limiting delayed toxicities such as peripheral neuropathy, myopathy, and pancreatitis [9–12]. This nucleoside analog-related cellular toxicity has been attributed to decreased mitochondrial DNA (mtDNA) content and altered mitochondrial function leading to increased lactic acid production [22–28]. Concomitant morphological changes in mitochondria (e.g., loss of cristae, matrix dissolution and swelling, and lipid droplet formation) can be observed with ultrastructrual analysis using transmission electron microscopy [24, 28, 29]. In HepG2 cells incubated with 10 μM FIAU (fialuridine, 1,2′-deoxy-2′-fluoro-1-β-D-arabinofuranosly-5-iodo-uracil), a substantial increase in lactic acid production was observed (Table 3). Electron micrographs of these cells showed the presence of enlarged mitochondria with morphological changes consistent with mitochondrial dysfunction. Lamivudine (10 μM) did not effect mitochondrial structure or function. Using similar conditions, exposure of HepG2 cells to 10 μM L-dC, L-dT or L-dA for 14 days had no effect on lactic acid production, mitochondrial DNA content or morphology (Table 3).

Table 3. Effect of L-dC, L-dT and L-dA on Mitochondria in HepG2 Cells

Compound	Conc. (μM)	Cell Density	L-Lactate	mtDNA	Lipid Droplet Formation	Mitochondrial Morphology
		% of Control				
Control		100	100	100	neg[a]	normal
L-dC	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	neg	normal
L-dT	0.1	103 ± 7	102 ± 2	103 ± 4	nd[b]	nd
	1.0	106 ± 8	99 ± 2	101 ± 7	nd	nd
	10	97 ± 7	105 ± 2	97 ± 4	neg	normal
L-dA	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	neg	normal
Lamivudine[c]	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	neg	normal
FIAU[c]	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 L-dT, L-dC or L-dA for 14 days. Mitochondrial morphology, lipid droplet formation and intracellular mtDNA levels were assessed as described in the Methods section. Values are presented as means and standard deviations of three independent experiments.

[a]neg, negative.

[b]nd, not determined.

[c]Data from reference [12, 27].

In Vivo Antiviral Activity and Safety

The woodchuck model of chronic hepatitis B virus infection has proven to be a predictor of the antiviral activity and safety of antiviral drug candidates for the treatment of human chronic HBV infection [30, 31]. L-dC, L-dT and L-dA were given orally to woodchucks once daily at 10 mg/kg/day. The serum levels of WHV DNA during 4 weeks of drug treatment and 8 weeks of post-treatment follow-up were determined 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). The WHV DNA replication was significantly inhibited within the first few days of treatment and was maintained throughout the treatment period. Notably, serum WHV DNA levels (HBV viremia) decreased up to 8 logs to below the limit of detection by PCT in the L-dT treated animals and decreased by 6 logs in the L-dC treated animals (Fig. 1). The oral bioavailability of LdT is 3 times that of L-dC in the woodchuck. WHV DNA levels rebounded to near pre-treatment levels by 8 weeks following drug withdrawal. Viral rebound was detected within the first week post-treatment. In addition a decline in WHV surface antigen as measured using the method of Cote et al [32] paralleled the marked

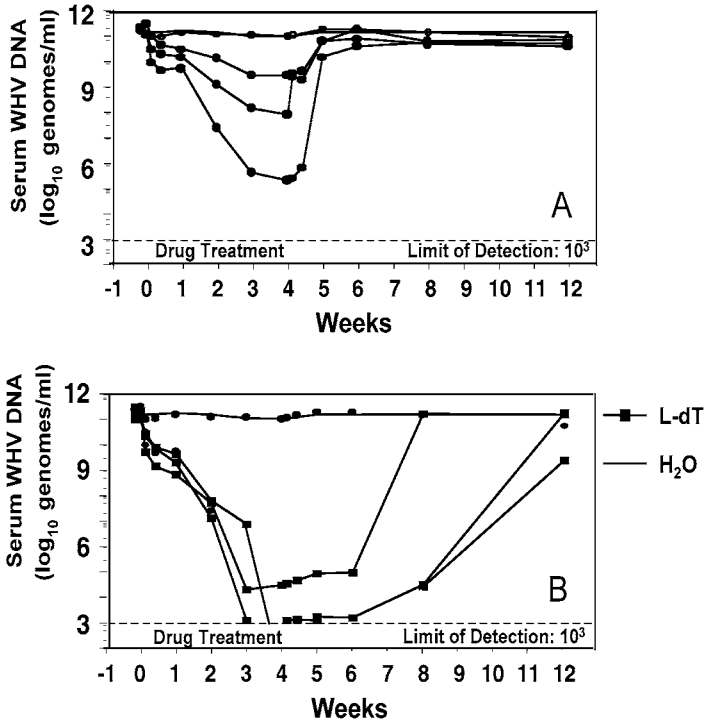

Figure 1. Woodchuck hepatitis virus serum levels in animals treated 4 weeks with L-dC (panel A), L-dT (panel B) and 8 weeks post-treatment. Data are presented for individual animals administered 10 mg/kg/d orally (n = 3) and untreated control animals (n = 4).

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reduction in viral load. The onset of the response was delayed by at least one week but continued to fall for several weeks after drug removal.

The cytidine analog lamivudine (10 mg/kg/d), used for comparison to the L-dC treatment group, reduced the HBV genome equivalents/ml in serum by 0.5 log. This weak effect is consistent with previous studies using similar doses of lamivudine [33]. Higher doses (40–200 mg/kg) are required to produce significant antiviral activity in this model [34]. The low activity of lamivudine in the woodchuck model has been explained in part by the low conversion of lamivudine and other cytidine analogs to their active 5′-triphosphate forms in woodchuck liver compared to that in human liver. In addition, the oral bioavailability of lamivudine in woodchucks was reported to be 18%–54%, whereas the oral bioavailability observed in humans was 82% [35, 36].

The woodchuck model was also valuable for the preclinical toxicological evaluation of nucleoside analogs. This model identified the delayed severe hepatocellular toxicity induced by FIAU in humans not seen in preclinical evaluation in rats, dogs or monkeys [30, 37]. The FIAU-induced toxicity observed in woodchucks including significant weight loss, wasting and hepatocellular damage seen on liver biopsy, was identified beginning 6–8 weeks from onset of treatment and was similar to that observed in the treated HBV-infected patients [30, 38]. Using this model we found in additional studies that the unmodified β-L-2′-deoxynucleosides L-dC, L-dT and L-dA were well tolerated and caused no drug-related toxicity through 12 weeks of treatment and 4 weeks of follow-up.

In summary, this series of β-L-2′-deoxynucleosides has in common the presence of a hydroxyl group in the 3′-position that determines specific antiviral activity against hepadnavirus. In the woodchuck model of chronic HBV infection, oral administration reduced serum viral load by as much as 10^8 genome equivalents/mL without toxicity. These β-L-2′-deoxynucleosides are highly attractive clinical development candidates for the treatment of chronic HBV infection.

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