

Inhibition of hepatitis B virus by a novel L-nucleoside, β -L-D4A and related analogues

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Abstract

AIM: To explore the inhibition of β -L-D4A on hepatitis B virus (HBV) in 2.2.15 cells derived from HepG2 cells transfected with HBV genome.

METHODS: 2.2.15 cells were plated at a density of 5×10^4 per well in 12-well tissue culture plates, and treated with various concentrations of β -L-D4A for 6 days. In the end, 5 μ l of medium was used for the estimation of HBsAg and HBeAg, the other medium was processed to obtain virions by a polyethylene glycol precipitation method. At the same time, intracellular DNA was also extracted and digested with HindIII. Both DNAs were subjected to Southern blot, hybridized with a 32 P-labeled HBV probe and autoradiographed. Intensity of the autoradiographic bands was quantitated by densitometric scans of computer and ED₅₀ was calculated. Then Hybond-N membrane was washed and rehybridized with a 32 P-labeled mtDNA-specific probe, and effect of β -L-D4A on mitochondrial DNA was studied. 2.2.15 cells were also seeded in 24-well tissue culture plates, and cytotoxicity with different concentrations was examined by MTT method. ID₅₀ was calculated. Structure-activity relationships between D2A and D4A were also studied as above.

RESULTS: Autoradiographic bands were similar between supernatant and intracellular HBV DNA. Episomal HBV DNA was inhibited in a dose-dependent manner. ED₅₀ was 0.2 μ M. HBsAg or HBeAg was not apparently decreased, and inhibition of mitochondrial DNA was not obvious. The experiment of cytotoxicity gained ID₅₀ at 200 μ M.

CONCLUSION: β -L-D4A possesses potent inhibitory effects on the replication of HBV *in vitro* with little cytotoxicity and mitochondrial toxicity, TI value is 1000. It is expected to be developed as a new clinically anti-HBV drug.

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INTRODUCTION

Like interferon, nucleoside analogues have become the focus of investigations of anti-HBV drugs^[1]. However, most anti-HBV nucleoside analogues tested to date have at best only transient and limited effects in a small percentage of the general population of HBV-infected individuals and exist moderately to seriously side effects^[2,3]. Vidarabine, ribavirin, acyclovir, ganciclovir, famciclovir, fialuridine, etc. have not been used widely. In recent years, considerable interest has been focused on the use of 2', 3'-dideoxynucleosides (DDNs) for the treatment of chronic HBV infection^[4]. DDNs are phosphorylated to triphosphate in cells, which in turn specifically inhibits the viral polymerase, terminates the elongation of HBV DNA, showing potent anti-HBV activities with relatively low cellular toxicities^[5].

Lamivudine as one of DDNs has been used widely in clinic, and has a rapidly potent anti-HBV effect, but there is a rebound of HBV DNA after treatment, and drug resistance and viral mutants may appear after a long-term treatment with lamivudine^[6]. Thus it is important to search for more effective agents against HBV, even with an improved therapeutic index. In this report, 2', 3'-dideoxy-2', 3'-dideoxyadenine (β -L-D4A), a novel L-Nucleoside was demonstrated to effectively block the production of HBV in 2.2.15 cells *in vitro*. Mitochondrial effects and cytotoxicity were also investigated to evaluate the potential use of these compounds in treatment of HBV infection.

MATERIALS AND METHODS

Compounds and agents

β -L-D4A was synthesized by ourselves with the help of Pharmaceutic College of Wuhan University and identified by infrared, mass spectra, nuclear-magnetic resonance. Lamivudine, ddC', D-D4A, L-D2A, D-D2A were provided by Professor Cheng YC (School of Medicine, Yale University, New Haven, CT). All compounds were dissolved in phosphate-buffered saline (pH 7.4).

Determination of anti-HBV activity in 2.2.15 cells

2.2.15 cells (clonal cells derived from hepG2 cells that were transfected with a plasmid containing HBV DNA) that could secrete hepatitis B virions, kindly provided by Prof. Cheng, were incubated in DMEM medium with 10 % (vol/vol) fetal bovine serum, 100 IU/mL penicillin and 100 μ g/mL streptomycin at 37 °C in a moist atmosphere containing 5 % CO₂/95 % air. The cells were inoculated at a density of 5×10^4 /ml per well in 12-well tissue culture plates. The compounds studied were added to the medium 3 days after the inoculation. The cells were grown in the presence of drugs for 9 days with changes of medium every 3 days. On the 12th day, the culture medium was harvested. An aliquot of the culture medium (5 μ l) was used for estimation of HBV surface antigen (HBsAg) and HBV e antigen (HBeAg). The remaining medium was processed to obtain virions by a polyethylene glycol precipitation method^[7]. The viral DNA recovered from the secreted particles was subjected to Southern blot analysis. Cellular DNA was isolated according to the standard protocols. Inhibition of viral DNA replication was determined by comparison of the viral DNA from drug-treated

and nontreated cultures. The level of inhibition was determined by hybridization of the blots to an HBV-specific probe followed by autoradiography. Quantitation of the autoradiographs was performed by density scanning with a computer software.

Effect of β -L-D4A on mitochondrial DNA

Since some of the nucleosides used in the treatment of HBV, such as FIAU^[8], could affect liver function, especially mitochondrial function, a detailed analysis of the effect of β -L-D4A on mitochondrial DNA synthesis was carried out. 2.2.15 cells were cultured as above and after 24 h in culture, treatment with the compounds was initiated. β -L-D4A was added at concentrations of 0.4 μ M and 10 μ M in DMEM. Cultures treated with 0.4 μ M 2'-3'-dideoxycytidine (ddC) were maintained in parallel as positive controls for damage of mitochondrial DNA. Blank control was also set. Cellular DNA was isolated according to the standard protocols and digested with restriction enzyme BamH I. Hybridizations and detection of the mitochondrial DNA were done according to the laboratory manual of molecular cloning. The probe was cytochrome oxidase III DNA labeled by ³²p dCTP.

Cytotoxicity

Cells were inoculated at a density of 5×10^3 /ml per well in 24-well tissue culture plates. After 24 h in culture, the cells were treated with various concentrations of β -L-D4A in DMEM for 3 days. Then 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were performed using the cell titer kitTM (Promega) following the standard procedure with the following exceptions. At the time points described as illustrations, 15 μ l MTT reagent was added per well, allowed to incubate for 30 minutes, after which 130 μ l stop/lysis buffer was added. Plates were sealed with ParafilmTM and left overnight at room temperature to allow solubilization of the formazan salt product. Absorbance was measured at 750 nm and 570 nm using a Thermomax (Molecular Devices, San Jose, CA), or a cytoFluor microplate reader (PE Biosystems, Foster City, CA). The data were normalized (A₅₇₀-A₇₅₀ nm) and the mean absorbance (5 wells/concentration) was plotted against drug concentration. The ID₅₀ values were calculated as described above.

Determination of effects of β -L-D4A on HBsAg and HBeAg

HBsAg and HBeAg in the culture medium were determined according to the protocols supplied by the manufacturer. Essentially, the culture medium was appropriately diluted with phosphate-buffered saline and absorbed on the surface of plates coated by antibody to HBsAg or HBeAg. After an incubation period, the plates were washed and incubated in orthophenylene diamine. After a 30-min incubation, the reaction was terminated by adding 1N sulfuric acid. The A₄₉₀ of the final reaction was read. Appropriate positivity and negativity were assayed along with the samples.

RESULTS

Structure-activity relationships

The 2.2.15 cell line was used to evaluate the antiviral activities of ddA analogues: D-D4A, L-D2A, D-D2A (Structures are shown in Figure 1). The antiviral effects were measured by an analysis of extracellular HBV DNA. The experiment revealed that the inhibition of HBV DNA was more strong by L-isomer than by D-isomer and much more notable by L-D4A than by L-D2A (Figure 2).

Dosage-activity relationships

Inhibition of HBV DNA replication by β -L-D4A was evident as demonstrated by the amount of DNA obtained from the

secreted viral particles as well as from the intracellular episomal particles. Concentrations of β -L-D4A ranging from 0.08 μ M to 10 μ M produced a dose-dependent inhibition (Figure 3). The intensity of autoradiographic bands for extracellular HBV DNA was quantitated by densitometric scans of computer and ED₅₀ was calculated at 0.2 μ M (Figure 4). Analysis of the intracellular episomal HBV DNA reflected similar trends in inhibition (Figure 5).

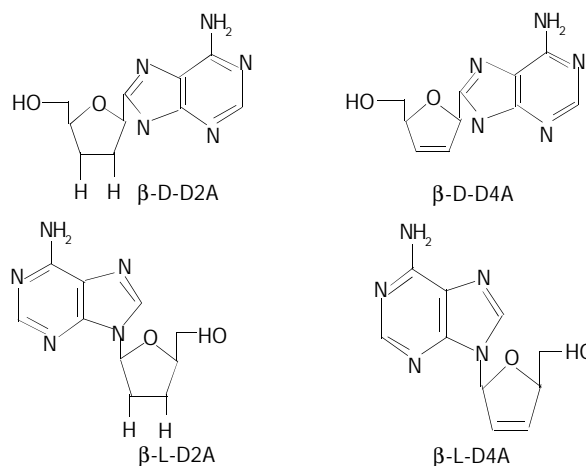


Figure 1 Structures of β -L-D4A and analogues.

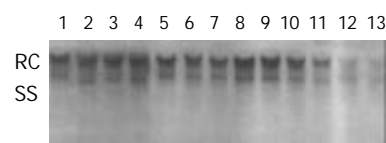


Figure 2 Inhibition of isomers of D4A and D2A on HBV DNA replication in supernatant. Lane 1 as negative control; lanes 2, 3, 4 as D-D2A at 2 μ M, 4 μ M and 8 μ M, respectively; lanes 5, 6, 7 as L-D2A at 2 μ M, 4 μ M and 8 μ M, respectively; lanes 8, 9, 10 as D-D4A at 2 μ M, 4 μ M and 8 μ M, respectively; lanes 11, 12, 13 as L-D4A at 2 μ M, 4 μ M and 8 μ M, respectively. RC: relaxed circular HBV DNA; SS: single-stranded HBV DNA.

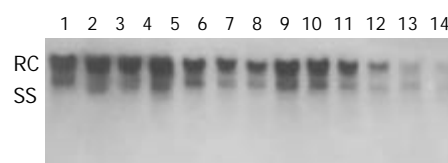


Figure 3 Inhibition on replication of extracellular HBV DNA by β -L-D4A. lanes 1, 2 as negative control; lanes 3, 4 as lamivudine at 1 μ M; lanes 5, 6 as β -L-D4A at 10 μ M; lanes 7, 8 as β -L-D4A at 2 μ M; lanes 9, 10 as β -L-D4A at 0.4 μ M; lanes 11, 12 as β -L-D4A at 0.08 μ M; lanes 13, 14 as blank control (HepaG₂). RC: relaxed circular HBV DNA; SS: single-stranded HBV DNA.

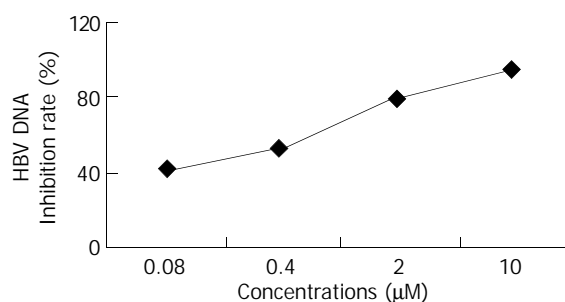


Figure 4 Inhibition on replication of extracellular HBV DNA by β -L-D4A.

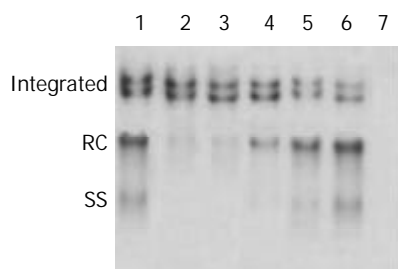


Figure 5 Inhibition on replication of intracellular HBV DNA by β -L-D4A. lane 1 as negative control; lane 2 as lamivudine at 1 μ M; lane 3 as β -L-D4A at 10 μ M; lane 4 as β -L-D4A at 2 μ M; lane 5 as β -L-D4A at 0.4 μ M; lane 6 as β -L-D4A at 0.08; lane 7 as blank control (HepaG2). RC: relaxed circular HBV DNA; SS: single-stranded HBV DNA.

Effect of β -L-D4A on mitochondrial DNA

Hybridization of intracellular DNA to a cytochrome oxidase III was done to evaluate the effect of β -L-D4A on mitochondrial DNA. Mitochondrial DNA levels treated with 0.4 μ M and 10 μ M β -L-D4A were nearly similar to the blank control, but were obviously lower for ddC at 0.4 μ M (Figure 6).



Figure 6 Toxicity of mitochondrial DNA. lane 1 as blank control; lane 2 as β -L-D4A at 0.4 μ M; lane 3 as β -L-D4A at 10 μ M; lane 4 as ddC at 0.4 μ M.

Cytotoxicity of β -L-D4A

β -L-D4A did not show evident toxicity to 2.2.15 cells even at a concentration of 10 μ M, but at high concentrations it had cytotoxicity, with 50 % inhibitory concentration (ID_{50}) of 200 μ M. The data are shown in Table 1 and Figure 7.

Table 1 Effect-dosage relationship of inhibition of cell growth by β -L-D4A

Dosage (μ M)	<i>n</i>	A value ($\bar{x} \pm s$)	Inhibition rate (%)
50	3	0.84 \pm 0.22	22.3
100	3	0.75 \pm 0.16	36.0
150	3	0.73 \pm 0.12	37.2
200	3	0.67 \pm 0.21	50.4
250	3	0.53 \pm 0.15	73.5
300	3	0.43 \pm 0.25	89.2
0	3	2.01 \pm 0.23	0

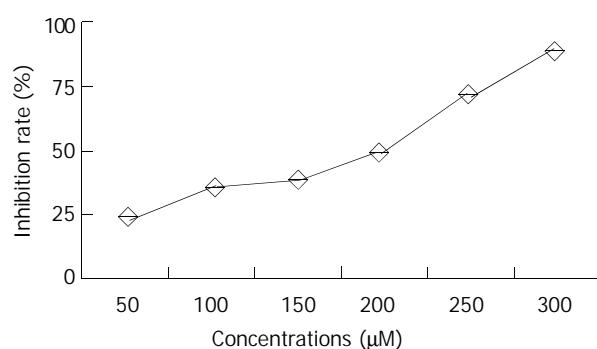


Figure 7 Effect of β -L-D4A on cell growth curve.

Effects of β -L-D4A on HBsAg and HBeAg

Measurements of the levels of viral surface antigen and e antigen from the media of cultures treated with β -L-D4A revealed that β -L-D4A had no significant inhibitory effect on HBsAg and HBeAg at low concentrations, but had marked effect of reducing HBsAg and HBeAg at 2 μ M and 10 μ M, significantly different from the blank group ($P < 0.05$ or $P < 0.01$, respectively) (Shown in Table 2).

Table 2 Effect-dosage relationship of inhibition of HBsAg and HBeAg by β -L-D4A

Dosage(μ M)	<i>n</i>	HBsAg		HBeAg	
		P/N value ($\bar{x} \pm s$)	Inhibition rate(%)	P/N value ($\bar{x} \pm s$)	Inhibition rate (%)
0.08	3	2.96 \pm 0.18	13.7	4.98 \pm 0.23	5.5
0.4	3	2.84 \pm 0.22	22.3	4.97 \pm 0.15	5.7
2	3	2.75 \pm 0.16 ^a	36.0	4.88 \pm 0.25	9.4
10	3	2.73 \pm 0.12 ^a	37.2	4.02 \pm 0.33 ^b	37.5
Blank	3	3.09 \pm 0.21	0	5.27 \pm 0.27	0

^a $P < 0.05$; ^b $P < 0.01$ vs blank control.

DISCUSSION

One rational approach to the development of drugs for the treatment of HBV infection in patients is to identify those compounds that specifically inhibit HBV DNA replication^[9,10]. Since deoxynucleoside analogues were found to be effective on a variety of viruses, several compounds have been tested *in vitro* and *in vivo* against hepadnaviruses^[9-12]. The selectivity of these compounds against HBV in general is assessed by their relative potency against HBV versus cellular toxicity^[13-16]. Cytotoxicity studies are usually conducted by growth retardation assays that measure cell growth in the presence of compounds tested for three or four generations^[17]. This growth retardation assay could not, however, detect delayed cytotoxicity of deoxynucleoside analogues such as ddC that have an effect on cellular mtDNA synthesis. Since mitochondria play an important role in organ function, it was hypothesized that the delayed toxicity as shown by peripheral neuropathy (e.g., observed in patients treated with ddC analogs) could be due to decreases in mtDNA^[18]. In this study, in addition to assessing their anti-HBV activities, compounds were examined for their effects on 4-day cell growth and on mtDNA.

Deoxynucleosides such as D2A and D4A can exist as (+)- or (-)- enantiomers. From the results we got, we could see that (-)-isomers were superior to (+)-isomers, and β -L-D4A was the most potent inhibitor of HBV replication in 2.2.15 cells, which could almost completely block HBV DNA replication at a concentration of 4 μ M. However, (+)-isomers were almost inactive against HBV replication when tested up to 8 μ M, mechanism of which might be in that the compounds could be deaminated intracellularly to the inactive analogues as reported before^[19].

β -L-D4A was the most potent inhibitor of HBV replication in 2.2.15 cells from our results. No effects on mtDNA were observed. Cell growth retardation with the administration of β -L-D4A at low concentrations was not evident, but at high concentrations, β -L-D4A began to show cytotoxicity in a dose-dependent manner with 50 % inhibitory concentration (ID_{50}) of 200 μ M. Since β -L-D4A does not inhibit mtDNA synthesis at concentrations that inhibit virus production, the delayed toxicity, such as peripheral neuropathy, associated with the treatment of ddC^[20] may not occur. In addition, β -L-D4A is not toxic to proliferating cells at concentrations that completely block the synthesis of HBV virion, suggesting that acute bone

marrow toxicity may not be a concern. The study on dosage-activity relationships showed that β -L-D4A could inhibit HBV DNA replication in a markedly dose-dependent manner with 50 % inhibitory concentration (ED_{50}) of 0.2 μ M. As the therapeutic index (TI value) was equal to ID_{50}/ED_{50} , we got the TI value of β -L-D4A at 1 000. TI of lamivudine was 750 as reported in another article^[21]. Therefore β -L-D4A like lamivudine possesses potent anti-HBV replication effect and has a higher TI value. In addition to that, β -L-D4A shows inhibition of expression of HBV antigens at high concentrations, indicating that β -L-D4A may be able to decrease the levels of HBsAg and HBeAg with a long term use.

About the mechanism of action of β -L-D4A, we think it is likely the inhibition of viral DNA polymerase, chain-termination resulted from incorporation into elongated DNA strand, or both. The mechanism needs to be further explored.

On the basis of the study of nucleoside analogs against HIV, human immunodeficiency virus resistant to ddC is not cross resistant to the thymidine analog zidovudine^[22]. This permits the possibility of HBV treatment with β -L-D4A in the event of resistance to cytosine analogs as lamivudine. Furthermore, possibilities of a combined therapy of β -L-D4A and lamivudine for HBV can be explored.

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