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Inhibition of the replication of hepatitis B virus in vitro by a novel 2,6-diaminopurine analog, β-LPA

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Abstract

Antiviral therapy of chronic hepatitis B remains a major clinical problem worldwide. Like lamivudine, nucleoside analogs have become the focus of investigation of anti-hepatitis B virus (anti-HBV) drugs. Here, β-LPA is a novel 2,6-diaminopurine analog found to possess potent anti-HBV activity. In HepG2.2.15 cell line, β-LPA had a 50% effective concentration (EC_{50}) of 0.01 μM against HBV, as determined by analysis of secreted and intracellular episomal HBV DNA. Levels of HBV surface antigen (HBsAg) and e antigen (HBeAg) in drug-treated cultures revealed that β-LPA had no significant inhibitory effects on HBsAg and HBeAg. β-LPA didn't show any cytotoxicity up to 0.4 μM with a 50% cytotoxic concentration (CC_{50}) of 50 μM. Furthermore, treatment with β-LPA resulted in no apparent inhibitory effects on mitochondrial DNA content. Considering the potent inhibition of HBV DNA synthesis and no obvious toxicity of β-LPA, this compound should be further explored for development as an anti-HBV drug.

Keywords: Hepatitis B virus; Nucleoside analog; Antiviral therapy; Chronic hepatitis B

The hepatitis B virus (HBV) is a major global health problem. According to the World Health Organization, over 350 millions people (≈5% of the world population) are chronically infected with HBV. Although safe and effective vaccination for HBV is available for developing countries [1–3], there is still no effective treatment for the millions of chronically infected individuals [4]. Consequently, long-term infection with HBV could lead to cirrhosis, and hepatocellular carcinoma [5,6]. In light of these facts, it is evident that the discovery and development of novel antiviral agents for the treatment of HBV is an extremely important undertaking.

HBV is a hepatotropic virus with a 3.2-kb partial double stranded circular genome with four overlapping reading frames encoding the viral polymerase, capsid, surface proteins, and a transcriptional transactivator protein termed "X". Following infection, the viral genome is converted in the nucleus of infected hepatocytes to a supercoiled covalent closed circular DNA (cccDNA) from which a 3.5-kb pregenomic mRNA is transcribed. HBV replicates inside the newly synthesized nucleocapsid by first reverse transcribing the pregenomic mRNA via the viral RNA-dependent DNA polymerase to create a full-length negative-sense DNA strand. Next, the HBV polymerase exerts its DNA-dependent DNA polymerase activity to synthesize the usually incomplete positive-sense DNA strand from the negative-sense strand template [7]. The multifunctional properties of the HBV polymerase make this enzyme an attractive target for nucleoside antiviral therapy.

Treatment of chronic hepatitis B remains a clinical challenge. Antiviral treatment of chronic hepatitis B relies currently on immune modulators such as interferon alpha and its pegylated form, and viral polymerase inhibitors that belong to the nucleoside and nucleotide analog family. Unfortunately, interferon alpha therapy is associated with several side effects, and the response rate for those receiving treatment has been unsatisfactory [8,9]. Because of the slow kinetics of viral clearance and the spontaneous viral gen-

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ome variability, viral mutants resistant to nucleoside analogs may be selected [10,11]. However, the development of new antiviral agents is rapidly improving the offing of therapy of chronic hepatitis B.

In this report we describe the in vitro properties of β-LPA (Fig. 1), a novel 2,6-diaminopurine analog possessing excellent potency against HBV with little cytotoxicity or mitochondrial toxicity.

Materials and methods

Compounds. β-LPA was synthesized by ourselves with the help of Pharmaceutic College of Wuhan University and identified by infrared, mass spectra, nuclear-magnetic resonance. Lamivudine and 2′3′-dideoxycytidine (ddC) were provided by Professor Cheng YC (School of Medicine, Yale University, New Haven, CT, USA). The chemicals were dissolved in phosphate-buffered saline (PH 7.4) and sterilized by filtration before addition to cell cultures.

Determination of anti-HBV activity in HepG2.2.15 cells. HepG2.2.15 cells (human hepatoma cell line HepG2 stably transfected with HBV genome, kindly provided by Prof. Y.C. Cheng) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 ml/l fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418 at 37 °C in a humidified incubator with 5% CO_2. The cells were incubated at a density of 5×10^4/ml per well in 12-well culture plates. The compounds studied were added to the medium 2 days after the inoculation. The cells were grown with various concentrations of drugs for 9 days with changes of drug-containing medium everyday. On day 11, the culture medium was harvested. An aliquot of the culture medium (5 μl) was used for estimation of HBsAg and HBeAg. The remaining medium was processed to obtain virions by a polyethylene glycol precipitation method [12]. 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 non-treated 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.

Quantitative assay of HBsAg and HBeAg. HBsAg and HBeAg in the culture medium were determined according to the protocols supplied by the manufacturer (Sino-American Biotechnology Company, China). 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 1 N sufuric acid. The A490 of the final reaction was read. Appropriate positive and negative controls were assayed along with the samples.

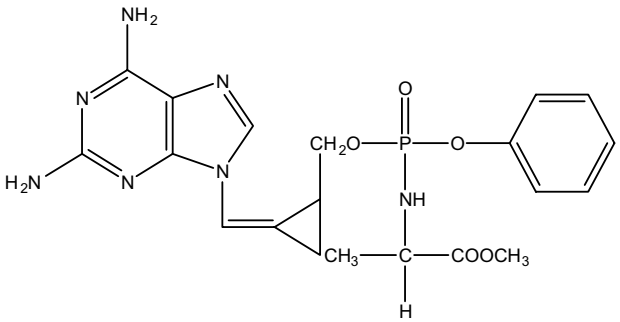

Fig. 1. Chemical structure of β-LPA. β-LPA is a 2,6-diaminopurine analog with a methyl phenyl phosphoro-L-alaninate moiety.

Analysis of cellular toxicity. Cells were inoculated at a density of 5×10^3/ml per well in 96-well tissue culture plates. After 24 h in culture, the cells were treated with various concentrations of β-LPA or lamivudine in DMEM for 3 days. Then 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were performed using the cell titer kit™ (Promega) following the standard procedure with the following exceptions. At the time points described as illustrations, 10 μl MTT reagent was added per well, allowed to incubate for 30 min, after which 100 μl stop/lysis buffer was added. Plates were sealed with Parafilm™ 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 cyto-Fluor microplate reader (PE Biosystems, Foster City, CA). The data were normalized (A570–A750 nm). Then the mean absorbance (6 wells/concentration) was plotted against drug concentration and the CC_{50} value was calculated.

Effect of β-LPA on mitochondrial DNA. Since some of the nucleoside analogs used in the treatment of HBV, such as FIAU [13], could affect liver function, especially mitochondrial function, a detailed analysis of the effect of β-LPA on mitochondrial DNA synthesis was carried out. HepG2.2.15 cells were cultured as above and after 24 h in culture, treatment with the compounds was initiated. β-LPA was added at concentrations of 0.08 and 0.4 μM in DMEM. Cultures treated with 0.4 μM ddC were maintained in parallel as positive controls for damage of mitochondrial DNA. Blank control was also set. The addition of the compounds was repeated every 3 days until day 9. At this point, cellular DNA was isolated according to the standard protocols and digested with restriction enzyme BamHI. 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 ^{32}pdCTP.

Results

Inhibition of HBV DNA replication by β-LPA

Inhibition of HBV DNA replication by β-LPA was evident as demonstrated by the amount of DNA obtained from the secreted viral particles (Fig. 2A) as well as from the intracellular episomal particles (Fig. 2B). Concentrations of β-LPA ranging from 0.0032 to 0.4 μM produced a dose-dependent inhibition. Quantitation of the data for extracellular HBV DNA from a set of experiments run in triplicate is shown in Fig. 2C, lamivudine, was used as a positive assay control. Analysis of the intracellular episomal HBV DNA reflected similar trends in inhibition.

Levels of HBsAg and HBeAg in drug-treated cultures

Measurements of the levels of viral surface antigen and e antigen from the media of cultures treated with β-LPA revealed that β-LPA had no significant inhibitory effect on HBsAg and HBeAg at low concentrations, but had marked effect of reducing HBsAg and HBeAg at 0.4 μM, significantly different from the blank group ($P < 0.05$ or $P < 0.01$, respectively) (Shown in Table 1). Inhibition rate = (P/N value of blank control-P/N value of treated)/(P/N value of blank control-2.1) × 100%.

Cellular toxicity of β-LPA

β-LPA did not show evident toxicity to HepG2.2.15 cells even at a concentration of 0.4 μM, but at high concen-