INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY OF HEPATITIS B VIRUS POLYMERASE BY ß-L-D4A-TP

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Summary. – ß-L-enantiomer of 2',3'-didehydro-2',3'-dideoxyadenosine-5'-triphosphate (ß-L-D4A-TP) has previously been proven to inhibit the replication of viral DNA in the Hep G2 2.2.15 cells and in transgenic mouse harboring 1.3-fold-overlength genome of Hepatitis B virus (HBV). To study the inhibition mechanism of the nucleoside analog ß-L-D4A-TP, a polymerase reaction *in vitro* with the recombinant HBV nucleocapsids was conducted to determine the exact mode of inhibition of the HBV replication by ß-L-D4A-TP. The HBV viral DNA and viral DNA-polymerase complex formed in the polymerase reaction were assayed. The results of this study showed that ß-L-D4A-TP inhibited the replication of HBV DNA by inactivating the reverse transcriptase (RT) activity in a concentration-dependent manner. The kinetics of ß-L-D4A-TP inhibition of the RT activity was the result of an apparent competitive inhibition with dATP.

Key words: Hepatitis B virus; polymerase; reverse transcriptase; ß-L-D4A-TP

Introduction

Infection with HBV affects an estimated 2 billion people worldwide. Primary HBV infection can become persistent and continues for many years. The infection progresses into chronic hepatitis with high risk of developing liver cirrhosis and/or hepatocellular carcinoma (Srivatanakul *et al*., 2004). HBV is a hepatotropic virus with a 3.2 kb partial double-stranded circular genome. Hepadnaviruses have a complex replication cycle and HBV polymerase is involved in all phases of the replication process. After infection, a partially double-stranded DNA genome is converted into covalently closed circular DNA from which a 3.5 kb, greater-than-genome-length, (+) strand pregenomic mRNA (pgRNA) is transcribed (Mason *et al*., 1983). HBV polymerase exerts its RNA-dependent DNA polymerase activity (reverse transcriptase) to create a full-length (-) strand DNA by first reverse transcribing the pgRNA inside the newly synthesized nucleocapsid. Next, the HBV polymerase synthesizes incomplete (+) strand DNA from the (-) strand DNA template (Ganem and Varmus, 1987).

The initiation mechanism of RT is unique. While HBV polymerase uses a specific cellular tRNA as a primer, HBV polymerase utilizes a tyrosine residue located within its own N-terminus as the acceptor for the initiating deoxynucleotide residue (Bartholomeusz *et al*., 2004; Lanford *et al*., 1997). Priming is templated by a bulge sequence within a stem-loop structure called ε located near the 5'-end of the pgRNA (Nassal and Rieger, 1996; Tavis *et al*., 1994). This priming step yields a discrete 3- to 4-deoxyribonucleotide oligomer that is covalently linked to the polymerase. The polymerase-primer adduct subsequently translocates to the 3'-end of the pgRNA and binds to a complementary sequence in an element called direct repeat 1 (DR1), where the elongation of (-) strand DNA is initiated (Wang and Seeger, 1993).

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Abbreviations: 3TC-TP = lamivudine triphosphate; ß-L-D4A-TP = ß-L-enantiomer of 2',3'-didehydro-2',3'-dideoxyadenosine-5'-triphosphate; DR1 = direct repeat 1; EC50 = 50% of the inhibitory concentration; HBsAg = HBV surface antigen; HBV = Hepatitis B virus; MOI = multiplicity of infection; pgRNA = pregenomic mRNA; RT = reverse transcriptase

Finally, HBV polymerase also mediates DNA-dependent (+) strand DNA synthesis, which is primed by an RNA primer resulting from incomplete degradation of pgRNA by an RNaseH activity at the C-terminus of polymerase (Tsai *et al*., 2006). HBV polymerase is non-covalently linked to the HBV core protein that harbors RNA and DNA binding activities and plays an essential role in hepadnavirus replication and propagation (Hatton *et al*., 1992; Nassal, 1992; Yu and Summers, 1991). DR1 and the ε stem-loop structure prove important for the function of HBV polymerase (Wang and Seeger, 1992). HBV polymerase has intrinsic RNA-dependent RT, DNA-dependent DNA polymerase as well as RNaseH activity (Radziwill *et al*., 1990; Ganem and Varmus, 1987). The multifunctional properties of the HBV polymerase make this enzyme an attractive target for nucleoside antiviral therapy (Delaney *et al*., 2006; Tsai *et al*., 2006). A large number of antiviral agents have been evaluated as potential therapeutics for the chronic hepatitis. Current efforts have mostly focused on nucleoside analogs as inhibitors of the multifunctional viral polymerase (Zhou and Littler, 2006; Papatheodoridis *et al*., 2002).

The nucleoside analog ß-L-D4A-TP is structurally similar to the natural D-enantiomer of 2'-deoxyadenosine-5'-triphosphate with the exception of its configuration and inner carbon-carbon double bond content. Previous studies revealed that ß-L-D4A-TP is also a very effective and selective agent against HBV in Hep G2 2.2.15 cells (Wu *et al*., 2003; Bridges and Cheng, 1995). The compound rapidly reduced the amount of viral DNA and hepatitis B surface antigen (HBsAg) in serum of transgenic mouse without significant drug-induced liver and kidney toxicity.

The aim of the present study was to study the inhibition mechanism of the nucleoside analog ß-L-D4A-TP. A polymerase reaction *in vitro* with the recombinant HBV nucleocapsids was conducted and then the viral DNA and viral DNA-polymerase complex formed in the polymerase reaction were assayed to determine whether reverse transcriptase activity of HBV polymerase was inhibited by ß-L-D4A-TP.

Materials and Methods

Antiviral compounds. ß-L-D4A-TP and 3TC-TP (lamivudine triphosphate) were chemically synthesized at the College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, P.R. China. Chemical structures of ß-L-D4A and 3TC are shown in Fig. 1.

Cells. Insect cells Sf9 were maintained in Grace's medium supplemented with 10% fetal bovine serum at 27°C (Kost *et al*., 2005).

Construction of recombinant baculoviruses. All HBV sequences were cloned from the plasmid p3.6II containing complete genome of HBV strain adr, generously supplied by Professor Wang Yuan (Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, P.R. China) and introduced into the pFastBac Dual donor plasmid (Invitrogen) by the standard molecular cloning techniques. Nucleotide positions (nt) followed the nomenclature of Galibert *et al*. (1979) began at the unique *Eco*RI restriction site. The desired fragments HBV polymerase gene followed by 3' DR1 and 3' ε stem-loop structure (PE) (nt 2279 to 3215 and 1 to 1918) and 5' ε stem-loop structure plus HBV core protein gene (EC) (nt 1809 to 2483) were amplified by PCR. The PE construct encoding HBV polymerase was enclosed by primers creating 5' *Eco*RI and 3' *Pst*I, while EC construct encoding HBV core protein was enclosed by primers creating

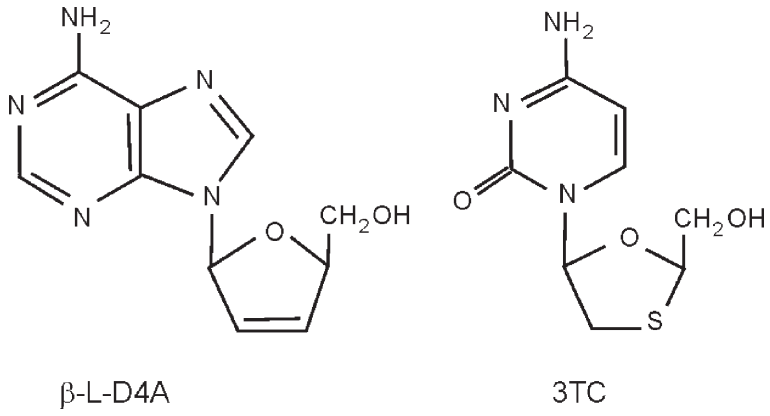

Fig. 1

Chemical structures of ß-L-D4A and 3TC

5' *Nhe*I and 3' *Kpn*I. Both constructs were sequentially subcloned into pFastBac Dual. DH10Bac competent cells were transformed with the recombinant plasmid pFastbac Dual-polymerase-core. Recombinant bacmid was isolated, purified and then Sf9 cells were transfected with recombinant bacmid using Lipofectin reagent (Gibco-BRL) according to the manufacturer's protocol. Recombinant baculoviruses were identified by the plaque assay.

Expression and purification of recombinant nucleocapsids. Sf9 cells were grown at the density of 2×10^6 cells/well in 6 well tissue culture plates and infected with the recombinant baculovirus at a MOI = 10. The cells were incubated for 72 hrs until the signs of viral infection appeared. Then the cells were harvested by centrifugation, washed 3 times with PBS, and the cell pellet was stored at -80°C. Thawed cell pellet was disrupted on ice for 30 mins in 1/10 of original volume of NP-40 lysis buffer (50 mmol/l Tris-HCl, pH 7.4, supplemented with 150 mmol/l NaCl, 5 mmol/l $MgCl_2$, 5 mmol/l DTT, 0.5% Nonidet P-40, 50 U/ml RNasin, 10% glycerol and protease inhibitors cocktail [1 mmol/l prefablock, 10 µmol/l aprotinin, 10 µg/ml pepstatin, 1 mmol/l EDTA, 100 µmol/l leupeptin]). After low-speed centrifugation (4,000 x g, 4°C, 10 mins), aliquots of 1ml lysate were immunoprecipitated with 40 µl of mouse monoclonal antibody anti-HBV core protein coupled to CNBr-activated Sepharose CL-4B (Pharmacia) at 4°C for 12 hrs. Following low-speed centrifugation (3,000 x g, 4°C, 15 mins), the Sepharose with immunocomplexed nucleocapsids, i.e. immunobeads were washed three times with PBS and prepared to polymerase reaction *in vitro*.

Polymerase reaction. 40 µl of immunobeads were resuspended in 100 µl reaction mixture containing 50 mmol/l Tris reaction buffer, pH 7.5, supplemented with 75 mmol/l NH_4Cl, 20 mmol/l $MgCl_2$, 0.5% Tween-20, 0.1 mmol/l ß-mercaptoethanol, 50 µmol/l three dNTPs (dATP, dGTP, and dCTP), 15 µmol/l dTTP, 35 µmol/l digoxigenin (DIG)-11-dUTP and various concentrations of ß-L-D4A-TP. The sample with 3TC-TP was used as a positive control, while the sample without the drug was used as a negative control. The reaction mixture was incubated at 37°C for 12 hrs.

Assay of viral DNA formed in the polymerase reaction. The products of the polymerase reaction were analyzed by agarose gel electrophoresis. The immunobeads undergoing polymerase reaction were rinsed 3 times with 50 mmol/l Tris washing buffer, pH 7.4, supplemented with 150 mmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100 and 0.02% SDS to remove the unincorporated dNTPs. The immunobeads were resuspended in five volumes 0.1 mol/l glycine elution buffer, pH 3.0 supplemented with 10% glycerol for 15 mins on ice, and then centrifuged at 3,000 rpm for 5 mins at 4°C. 200 µl of supernatant was neutralized with 13 µl of 0.8 mol/l Tris neutralization buffer, pH 8.4 supplemented with 3% Triton X-100, 80 mmol/l DTT, and 50 U/ml of RNasin. The nucleocapsids bound to immunobeads were released to the solution and collected. This procedure was repeated twice. Purified nucleocapsids were digested with proteinase K (1 mg/ml) followed by several extractions with phenol-chloroform and precipitation with ethanol. DNA partially labeled in polymerase reaction was separated by agarose gel electrophoresis followed by transfer to positively charged Hybond-N membrane (Amersham) by capillary transfer. The membranes were incubated with 150 mU/ml anti-DIG-ap-conjugate at 37°C for 2 hrs, detected by alkaline- phosphatase- catalyzed color reaction with NBT/BCIP.

Assay of viral DNA-polymerase complex formed in polymerase reaction. For analysis of polymerase-linked RT products by SDS-PAGE, the immunobeads undergoing polymerase reaction were rinsed as described above, disrupted by being boiled for 7 min in protein sample buffer (50 mmol/l Tris-Cl, pH 6.8 supplemented with 2% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mmol/l DTT). The polymerase-linked RT products were blotted to positively charged membrane. The membrane was stained with NBT/BCIP as described above.

Real-time fluorescent quantitative PCR. The real-time fluorescent quantitative PCR for quantitative determination of HBV DNA was performed for monitoring of antiviral effect of reagents blocking HBV replication. This assay is based on PCR and a non-radioactive hybridization and detection system on microwell plates (Jardi *et al*., 2001). Amount of extracted DNA in nucleocapsids subjected to polymerase reaction was determined by this assay. Inhibition rate was calculated according to the following formula: Inhibition rate in % = (amount of gene copy in negative control – amount of gene copy in sample)/amount of gene copy in negative control x 100%. Fifty percent inhibitory concentration (EC50) was calculated by Reed-Muench formula (Reed and Muench, 1938). The experiments were separately repeated for three times.

Results

Effect of ß-L-D4A-TP on the formation of viral DNA in the polymerase reaction

We expressed *in vitro* replication-competent HBV nucleocapsids in Sf9 cells using the Bac-to-Bac baculovirus expression system, which is an efficient site-specific transposition system to generate baculovirus. The nucleocapsids were purified with anti-HBV core protein antibody coupled to Sepharose from the cleared lysate of Sf9 cells infected with recombinant baculoviruses. Purified polymerase and core proteins were verified by Western blot analysis (data not shown).

To investigate the potential of ß-L-D4A-TP to inhibit replication of viral DNA in nucleocapsids, recombinant HBV nucleocapsids isolated by immunoprecipitation were subjected to the polymerase reaction that yielded abundant DIG-labeled HBV DNA. DIG-11-dUTP was used as the label and the drugs were titrated against the unlabeled dATP substrate for the A analog, ß-L-D4A-TP or against dCTP for 3TC-TP. The size of DNA extracted from nucleocapsids was diverse, with the longest chain being roughly the size for a full-length cDNA copy of PE RNA e.g. ~2.8 kbp (Fig. 2). The product profile suggested that the nucleocapsids expressed in insect cells were immature, mostly at the step of reverse transcription. In comparison with untreated control we observed that the amount of DNA from nucleocapsids treated with the drugs was decreasing in a dose-dependent manner. These results suggested that 3TC-TP as well as ß-L-D4A-TP inhibited HBV replication and reduced the amount of DNA in nucleocapsids. We confirmed that both drugs inhibited the RT activity of the polymerase in the following study.

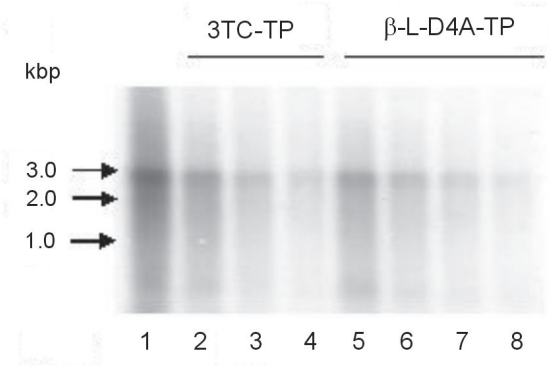

Fig. 2

Formation of HBV DNA in the polymerase reaction in the presence of ß-L-D4A-TP or 3TC-TP detected in agarose gel electrophoresis

Untreated control (lane 1), different amounts of 3TC-TP (lane 2, 3, 4 – 5, 25, 125 μmol/l, respectively), different amounts of ß-L-D4A-TP (lane 5, 6, 7, 8 – 1, 5, 25, 125 μmol/l, respectively). Size of DNA on the left.

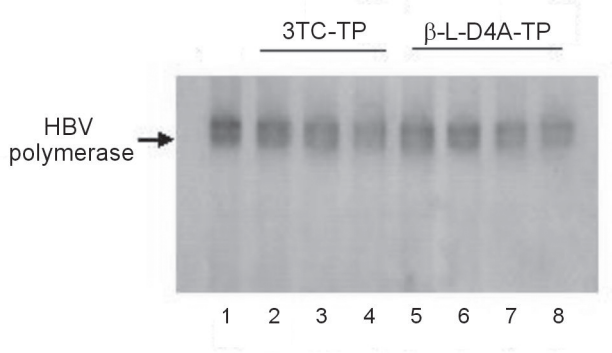

Fig. 3

Formation of HBV viral DNA-polymerase complex in the polymerase reaction in presence of ß-L-D4A-TP or 3TC-TP detected by SDS-PAGE

Untreated control (lane 1), different amounts of 3TC-TP (lane 2, 3, 4 – 5, 25, 125 μmol/l, respectively), different amounts of ß-L-D4A-TP (lane 5, 6, 7, 8 – 1, 5, 25, 125 μmol/l, respectively). HBV polymerase (arrow on the left).

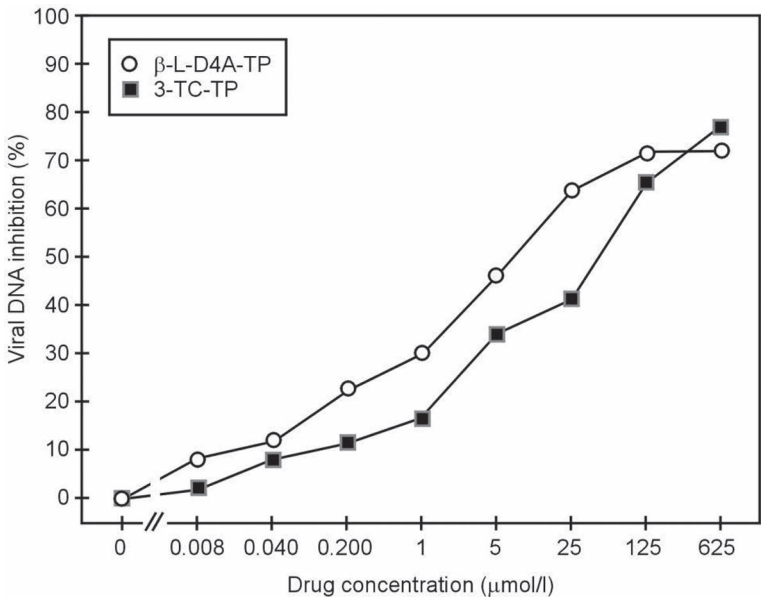

Fig. 4

Quantitative analysis of the formation of HBV viral DNA in the polymerase reaction in the presence of ß-L-D4A-TP (○) and 3-TC-TP (■) detected by real-time fluorescent PCR

Effect of ß-L-D4A-TP on the formation of viral DNA-polymerase complex in the polymerase reaction

HBV polymerase is different from the other RTs because it can use its tyrosine residue as the primer to initiate deoxynucleotide residue and synthesize (-) strand DNA covalently linked to polymerase. After polymerase reaction *in vitro*, HBV nucleocapsids were disrupted by boiling in sample buffer and subjected to SDS-PAGE. DNA-polymerase adducts moved together and a heavily labeled smear appeared at the beginning of the stained polymerase band (Fig. 3, lane 1). Presumably, the labeling at the position

of the polymerase band represented covalent linkage of a single nucleotide to polymerase in the nucleotide priming reaction, and the labeling of the higher-molecular-weight material represented the extension of this product by RT. DNA synthesized in polymerase reaction gradually diminished as the concentrations of ß-L-D4A-TP and 3TC-TP increased (Fig. 3). This result indicated that the drugs indeed inhibited the RT activity of polymerase and caused the termination of the nascent DNA chains.

Quantitative analysis of the viral DNA formation in the presence of ß-L-D4A-TP

For quantitative analysis of the formation HBV DNA, we used a real-time fluorescent PCR. As shown in Fig.4, the curves of inhibition rate in the presence of tested drugs in different concentrations showed that inhibition of the HBV DNA synthesis in the presence of ß-L-D4A-TP was more intensive when compared with the inhibition of HBV DNA synthesis in the presence of 3TC-TP at the same concentration. The EC50 for ß-L-D4A-TP and 3TC-TP was 35.30 and 44.57 μmol/l, respectively.

Discussion

Since 3TC-TP as L-configuration nucleoside analog with highly selective anti-HIV and anti-HBV activities emerged, pharmaceutical chemists have taken an active interest in L-nucleoside analogs. Many unnatural L-nucleoside derivatives were synthesized and screened (Papatheodoridis *et al*., 2002; Graciet and Schinazi, 1999; Spadari *et al*., 1998; Bolon *et al*., 1996). The antiviral activity of these compounds was investigated and the effect of enantiomeric pairs of a series of nucleoside analogs was assessed by parallel tests. It was found that most of the L-nucleoside analogs were more potent and less toxic than their corresponding D-enantiomer (Standring *et al*., 2001; Wang *et al*., 1998).

Direct comparison of the antiviral activities of ß-L-D4A-TP with those of other 2',3'-didehydro-2',3'-dideoxypurine nucleoside triphosphates demonstrated the clearly superior potency of ß-L-D4A-TP. In the Hep G2 2.2.15 cells, ß-L-D4A-TP had EC50 of 0.80 ± 0.10 μmol/l against HBV (Standring *et al*., 2001; Bolon *et al*., 1996). Structurally corresponding compound of ß-D-enantiomer was inactive. Previous studies with ß-L-D4A-TP have showed that the drug reduced both intracellular HBV replicative intermediates and viral DNA present in virions secreted into Hep G2 2.2.15 cells culture supernatant. To determine a potential of ß-L-D4A-TP *in vivo*, this compound was used against HBV in transgenic mouse model. We observed that the amount of viral DNA and HBsAg in serum of treated mice significantly decreased. Pathological and biochemical tests revealed that there was no obvious evidence for drug-induced liver and kidney toxicity.

Human deoxycytidine kinase showed a remarkable inversion of the expected enantioselectivity with ß-L-D4A-TP having better substrate efficiency than their corresponding ß-D-enantiomer. Adenosine deaminase was strictly enantioselective and favored ß-D-D4A-TP, whereas adenosine kinase and purine nucleoside phosphorylase had no apparent selective properties for the D- or L-enantiomer of ß-D4A-TP (Placidi *et al*., 2000; Pelicano *et al*., 1997).

In an attempt to explain the mechanism of the inhibition of HBV replication by the nucleoside analog ß-L-D4A-TP we conducted the polymerase reaction with recombinant HBV nucleocapsids. The presented data suggested that ß-L-D4A-TP inhibited the replication of HBV DNA by inactivation of RT activity of HBV polymerase in a concentration-dependent manner, although other modes of inhibition were not excluded. It was presumed that ß-L-D4A-TP acted at least in part as a chain terminator that was irreversibly incorporated into the nascent DNA chain. This mechanism was similar to that previously demonstrated for other nucleotide analog RT inhibitors that lack a 3'-hydroxyl group required for nucleotide addition and were DNA chain terminators such as 3TC-TP (Painter *et al*., 2004; Kodama, 2002). The kinetics of ß-L-D4A-TP inhibition of the RT activity was the result of an apparent competitive inhibition with respect to dATP. ß-L-D4A-TP was more effective than 3TC-TP, reducing synthesis of viral DNA by 50% at an inhibitor concentration of 35.30 μmol/l, followed by 3TC-TP, which inhibited synthesis by 50% when present at a concentration of 44.57 μmol/l.

Due to the serious resistance of 3TC, it is urgent to look for new anti-HBV drugs. Our studies provide an experimental basis for the clinical application of ß-L-D4A-TP in the future.

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