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Inhibition of RNA- and DNA-dependent duck hepatitis B virus DNA polymerase activity by nucleoside and pyrophosphate analogs

Bengt Löfgren[1], Erik Nordenfelt[1] and Bo Öberg[2]

[1]*Virology Section, Department of Medical Microbiology, University of Lund, Lund, Sweden and* [2]*Medivir AB, Stockholm, Sweden*

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Summary

The DNA polymerase of hepadnaviruses has two different functions during virus replication. It acts both as an RNA-dependent DNA polymerase (reverse transcriptase) and as a DNA-dependent DNA polymerase. Duck hepatitis B virus (DHBV) preparations were used to investigate the inhibitory effects of selected compounds on these two enzyme activities. The reverse transcriptase activity was represented by an actinomycin D-resistant, phosphonoformate-sensitive DNA polymerase activity isolated from DHBV-infected duck livers. DHBV from serum was used as the source of the DNA-dependent DNA polymerase activity. Pyrophosphate and nucleoside triphosphate analogs were assayed for their inhibitory effects on the two enzyme preparations. A marked inhibition was obtained with 3′-fluoro-2′,3′-dideoxythymidine 5′-triphosphate, acyclovir triphosphate, 2′,3′-dideoxythymidine 5′-triphosphate, 2′,3′-dideoxyguanosine 5′-triphosphate and 2′,3′-dideoxycytidine 5′-triphosphate. The two thymidine analog triphosphates showed a markedly lower inhibitory effect on the reverse transcriptase activity than on the DNA-dependent DNA polymerase activity. This was in analogy with earlier findings with 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate. Among the tested pyrophosphate analogs only phosphonoformate was inhibitory.

Duck hepatitis B virus; DNA-dependent DNA polymerase; Reverse transcriptase; Pyrophosphate analog; 2′,3′-Dideoxynucleoside 5′-triphosphate analog

Correspondence to: B. Löfgren, Department of Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden.

Introduction

The duck hepatitis B virus (DHBV), as well as the human hepatitis B virus, belongs to the Hepadnaviridae. This virus group has an interesting replication strategy. The viral DNA acts as a template for the synthesis, by cellular enzymes, of an RNA pre-genome. The viral DNA polymerase first acts as a reverse transcriptase copying the viral RNA pre-genome to a DNA strand of negative polarity, and then as a DNA-dependent DNA polymerase producing an incomplete positive DNA strand (Summers and Mason, 1982; Gust et al., 1986). There is no convincing evidence that the two different DNA polymerase reactions are catalyzed by more than one enzyme.

We have used DHBV as a model in the search for DNA polymerase inhibitors with potential effect on hepatitis B virus replication. The inhibitory effects of pyrophosphate and nucleoside triphosphate analogs were tested with the isolated enzymes in vitro. The effect on the RNA-dependent reaction (i.e. the reverse transcriptase activity) was compared to the effect on the DNA-dependent reaction. We have reported earlier that the inhibition of the two activities by 3′-azido-2′,3′-dideoxythymidine triphosphate (AZT-TP) differs substantially (Löfgren et al., 1988). The purpose of the present investigation was to test the validity of this phenomenon with other DNA polymerase inhibitors, and to detect compounds with high inhibitory effect on hepadnavirus reverse transcriptase activity.

Materials and Methods

Virus preparations

DHBV-infected ducks. Embryos from white ducks in a Swedish breeding colony were infected by injection of duck hepatitis B virus into the yolk sac. The virus was a kind gift from Dr J. Omata, Chiba University, Japan. A couple of chronically DHBV-infected ducks was used as a source of congenitally infected ducklings.

DHBV preparation. Sera from congenitally DHBV-infected ducks were stored at −20°C. Serum samples were diluted in phosphate-buffered saline (PBS) and centrifuged at 35 000 rpm for 3.5 h at +4°C in a SW-41 rotor. The pellets were washed with 3 ml PBS, and the centrifugation repeated. The final virus pellets were redissolved in PBS to 1/40 of the original volume and stored at −20°C. For determination of inhibitory curves a common pool of five preparations were used.

DHBV core preparation. An actinomycin D-resistant, phosphonoformate-sensitive DNA polymerase activity was isolated from DHBV-infected duck livers as described by Summers and Mason (1982) with some modifications, as described by Tao et al. (1988). Congenitally DHBV-infected ducks were sacrificed at an age of about one week. The liver was removed, immediately frozen in liquid nitrogen and

then stored at −70°C. Portions of about 2 g liver tissue were cut into pieces in extraction buffer and thoroughly disrupted in a glass homogenizer. The extraction buffer contained 20 mM Tris-HCl, pH 7.4, 7 mM $MgSO_4$, 50 mM NaCl, 0.1% 2-mercaptoethanol, 100 μg/ml bovine serum albumin and 0.25 M sucrose. Cell debris was removed by centrifugation at 10000 rpm for 20 min at +4°C in a Kontron TST 55.5 swing-out rotor. The supernatant was layered on top of two 29 ml linear gradients of 15–30% sucrose in extraction buffer. The gradients were centrifuged at 27000 rpm for 3 h and 50 min at +4°C in a Sorvall T-865 fixed angle rotor. Three milliliter fractions were collected from the bottom via a cannula inserted from the top, and assayed for DNA polymerase activity as described below. In most experiments the peak of DNA polymerase activity was found in the fourth fraction. The three fractions with the highest activity were pooled, and the activity was pelleted by centrifugation of 4.5 ml portions at 27000 rpm for 12 h at +4°C in a TST 55.5 rotor. The pellets were redissolved in a total of 0.9 ml extraction buffer and stored in small portions at −20°C.

DNA polymerase assay

The method used was that of Nordenfelt et al. (1980) with minor modifications, as described earlier (Tao et al., 1988). The same procedure was used for the serum-derived DHBV and for the core preparation. No synthetic template was added. Virus preparations were diluted in PBS so that the final cpm values should be approximately equal for the DHBV and the core preparation. They were pretreated with 1/8 volume of 6% 2-mercaptoethanol and 10% Nonidet P40 in distilled water, respectively, for 10–30 min at room temperature. Then, 25 μl-portions were incubated at 37°C for 3 h together with 25 μl reaction mixture and 25 μl water or a solution of the substance studied. For IC_{50} determinations, however, an incubation time of 60 to 90 min was used as the reaction showed a good linearity during this time interval. Optimal pH and concentrations of Mg^{2+} and K^+ were determined in separate experiments. Final concentrations during the reaction were: Tris-HCl, pH 8.0, 100 mM; $MgCl_2$ 20 mM; KCl 200 mM; dATP, dCTP, dGTP and dTTP (Sigma) 100 μM. The nucleoside triphosphate corresponding to the compound tested was replaced by its radiolabeled analog (α-^{32}P-NTP) at a final concentration of 0.1 μM (ethanol/water solution, >400 Ci/mmol, Amersham, U.K.). In inhibition experiments using the DHBV core preparation, actinomycin D (Sigma) was included at a final concentration of 100 μg/ml. After incubation, 60 μl-portions were spotted on 24-mm filter papers (Munktell, Sweden), and DNA was precipitated in cold 5% trichloroacetic acid with 0.1 M sodium pyrophosphate. Filters were washed three times in the same solution, once without sodium pyrophosphate, and twice in 99.5% ethanol. After drying, activity was measured in a liquid scintillation counter. When no inhibitor was included, the conditions described here resulted in cpm values between 2000 and 25000 for both preparations.

Characterization of virus preparations

Sensitivity to foscarnet (phosphonoformate) was used to assay the hepadnavirus specificity of the DNA polymerase activity in all virus preparations (Nordenfelt et al., 1980). The serum-derived DHBV preparations and the core preparations showed equally high sensitivity to foscarnet in the absence of actinomycin D. Inhibition by actinomycin D, however, differed according to the expected nature of the dominating DNA polymerase templates. A typical pattern of inhibition by foscarnet and actinomycin D is shown in Table 1.

To confirm the RNA nature of the template in the core preparation the inhibitory effect of ribonuclease A pretreatment was investigated. Ribonuclease A (Sigma) was heated to 100°C for 5 min, added to the 2-mercaptoethanol/Nonidet P40 pretreatment solution and incubated with the two different virus preparations for 15 min at 37°C. The DNA polymerase assay was then performed as described above (without actinomycin D). Final concentrations of ribonuclease A in the pretreatment solution were 0.1 and 1.0 mg/ml, respectively. The results are presented in Table 1.

The K_m value for TTP was approximately 0.1 μM for both preparations, as calculated from Lineweaver-Burk plots.

Compounds studied

Pyrophosphate analogs Foscarnet (phosphonoformate) was from Astra Alab AB, Sweden. The other pyrophosphate analogs (see Table 2) were synthesized by Drs K. Eklind, B. Gotthammar, N.-G. Johansson, S. Kovacs and B. Lindborg, according to methods to be published.

Nucleoside analogs 3′-fluoro-2′,3′-dideoxythymidine 5′-triphosphate (FLT-TP) was prepared by Dr L. Vrang (Bazin et al., 1989). Acyclovir triphosphate (ACV-TP) was prepared by Dr A. Larsson, and (R)-9-(3,4-dihydroxybutyl)-guanine triphosphate (DHBG-TP) was prepared by Dr A. Lindberg according to published methods. Selenazole 5′-triphosphate, thiazofurin 5′-triphosphate and ribavirin 5′-

TABLE 1

Percent inhibition of DNA polymerase activities in serum-derived DHBV preparations and DHBV core preparations; foscarnet and actinomycin D, respectively, were included in the reaction mixture, while RNAase A was included in the pretreatment solution; mean values from two determinations are presented

	DHBV preparation (from serum)	DHBV core preparation (from liver)
Foscarnet, 10 μM	70	70
Actinomycin D, 100 μg/ml	85	40
RNAase A, 0.1 mg/ml	2	18
RNAase A, 1.0 mg/ml	2	35

triphosphate were gifts from Dr R.K. Robbins, α,β-Methylene-guanosine 5′-triphosphate was from P-L Biochemicals, and β-τ-methylene-2′-deoxyguanosine 5′-triphosphate was from Miles. The following nucleoside analogs were from Sigma: 2′,3′-dideoxyadenosine 5′-triphosphate (ddATP), cordycepin 5′-triphosphate, adenosine-N^1-oxide 5′-triphosphate, periodate-oxidized adenosine 5′-triphosphate, periodate-oxidized and borohydride-reduced adenosine 5′-triphosphate, 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP), 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP), guanosine-5′-tetraphosphate, periodate-oxidized guanosine-5′-triphosphate, 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) and periodate-oxidized uridine-5′-triphosphate.

Results

Screening of compounds

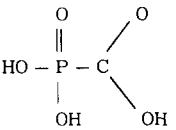
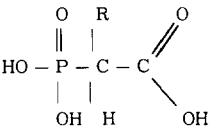
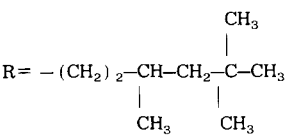
Different compounds were screened for their inhibitory effect on the DNA polymerase activity in the two enzyme preparations. Pyrophosphate analogs were screened at 100 μM using any of the four ^{32}P-dNTPs to measure the enzyme activity. Nucleoside analog triphosphates were tested at a final concentration of 10 μM, using the corresponding ^{32}P-dNTP. The triphosphates of selenazole, thiazofurin and ribavirin can be viewed both as dATP and dGTP analogs and were tested separately with, as substrate, ^{32}P-dATP and ^{32}P-dGTP, respectively. Only substances with an inhibitory effect of >50% on any of the two preparations at these concentrations were subjected to further investigation. As can be seen from Table 2, none of the pyrophosphate analogs tested showed significant inhibition, except for foscarnet which was included as a reference substance. Of the 19 nucleoside analogs tested, five showed an inhibition of more than 50% at 10 μM, namely ddCTP, ddGTP, ddTTP, ACV-TP and FLT-TP (Table 3).

Comparison of inhibition on core and serum preparations

For the effective compounds inhibition of the DNA polymerase activity in the serum-derived DHBV preparation (representing the DNA-dependent DNA polymerase reaction) was compared to the inhibition of the DHBV core activity (representing the reverse transcriptase reaction). For this purpose, different concentrations of the compounds were tested on the two enzyme preparations in parallel. For each compound at least two experiments were performed. Inhibition curves obtained from these experiments were used to determine the concentration of compound to give 50% inhibition (IC_{50}). Examples of inhibition curves are shown in Figs. 1 and 2, and IC_{50} values (mean of two determinations) are presented in Tables 2 and 3. As can be seen from these Figures and Tables, the inhibition of the activity of the two enzyme preparations differed substantially for ddTTP and FLT-TP. These two compounds showed a markedly lower inhibition of the reverse transcriptase activity (core preparation) than of the DNA-dependent DNA poly-

TABLE 2

Effect of pyrophosphate analogs on DHBV DNA polymerase activities

Compound	IC_{50} (μM)		Ratio
	Serum preparation	Core preparation	IC_{50} serum/IC_{50} core
 (Foscarnet)	4.0	4.0	1.0
			
R= -OH	>100	>100	
R= $-(CH_2)_4-CH_3$	>100	>100	
R= $-(CH_2)_6-CH_3$	>100	>100	
R= $-(CH_2)_8-CH_3$	>100	>100	
R= $-(CH_2)_{10}-CH_3$	>100	>100	
	>100	>100	
R= $-(CH_2)_3$ 	>100	>100	
R= $-(CH_2)_3$ 	>100	>100	
R= $-(CH_2)_2$ 	>100	>100	
R= $-(CH_2)_{11}$-Br	>100	>100	
r= $-(CH_2)_{10}$-COOH	>100	>100	

merase reaction (serum preparation). None of the compounds tested was markedly more effective against the reverse transcriptase activity than against the DNA-dependent DNA polymerase activity.

TABLE 3

Nucleoside triphosphate analogs with effect on DHBV DNA polymerase activities; IC_{50} values for the DNA-dependent (serum preparation) and the RNA-dependent (core preparation) reactions are shown; none of the dATP analogs tested showed an IC_{50} of 10 μM or less

Compound	IC_{50} (μM)		Ratio
	Serum preparation	Core preparation	IC_{50} serum/IC_{50} core
dCTP analogs			
ddCTP	6	7	0.9
dGTP analogs			
ddGTP	1.4	1.0	1.4
ACV-TP	0.5	0.5	1.0
dTTP analogs			
ddTTP	0.4	4.1	0.1
FLT-TP	0.04	0.4	0.1
AZT-TP*	0.1	4	0.025

*Results reported earlier (Löfgren et al., 1988), included for comparison.

FLT-TP was the most effective compound tested and, with respect to the effects on the reverse transcriptase activity, was followed (in order to decreasing inhibition) by ACV-TP, ddGTP, foscarnet, ddTTP, and ddCTP (ddATP was not inhibitory at 10 μM). The ratios between the IC_{50} values for the serum- and core-derived preparations are shown in Tables 2 and 3. Results obtained earlier for 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate (AZT-TP) are included in Table 3 for comparison.

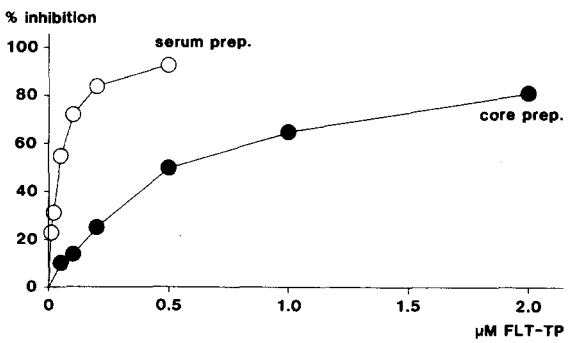


Fig. 1. Percent inhibition of DNA-dependent (serum preparation), ○—○, and RNA-dependent (core preparation), ●—●, DHBV DNA polymerase activities by different concentrations of 3′-fluoro-2′,3′-dideoxythymidine 5′-triphosphate. Reactions with the two preparations were run simultaneously for 75 min with a substrate concentration (^{32}P-dTTP) of 0.1 μM.

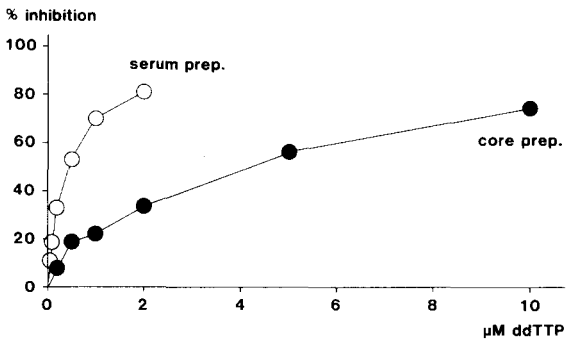


Fig. 2. Percent inhibition of DNA-dependent (serum preparation), ○—○, and RNA-dependent (core preparation), ●—●, DHBV DNA polymerase activities by different concentrations of 2′,3′-dideoxythymidine 5′-triphosphate. reactions with the two preparations were run simultaneously for 75 min with a substrate concentration (^{32}P-dTTP) of 0.1 μM.

Discussion

During the replication of hepadnaviruses the RNA pregenome is transcribed to a negative DNA strand by a viral reverse transcriptase. A positive DNA strand is then formed by a viral DNA-dependent DNA polymerase (Summers and Mason, 1982; Gust et al., 1986). Since the reverse transcription can not be effected by cellular enzymes, and since the production of new DNA strands is required for the synthesis of new complete virus particles, the reverse transcriptase is a crucial enzyme in the virus replication.

In the assays reported here the endogenous RNA and DNA molecules of the DHBV preparations served as templates for the DNA polymerase reactions. Thus, analogs to all four nucleotides could be tested in the same system. The two enzyme activities of the DHBV preparations showed different sensitivity to the thymidine triphosphate analogs. This points to the dual function of the hepadnavirus DNA polymerase. However, the possibility that the RNA- and DNA-dependent reactions are catalyzed by two different enzymes can not be ruled out. Indeed, Bavand and Laub (1988) recently described two proteins, associated with hepatitis B virus particles, which showed reverse transcriptase activity. They proposed that the larger protein may be a precursor of the minor one. Offensperger et al. (1988) investigated the inhibitory effect of different compounds on replicative complexes isolated from DHBV-infected duck liver. They found an IC_{50} of 4 mM for chloroquine and of 0.2 μM for suramin. Using synthetic templates they found no difference in inhibition of the reverse transcriptase activity and DNA-dependent DNA polymerase activity.

The results of the present study show that, at least for nucleoside analogs, the inhibitory effect on the reverse transcriptase activity may be different from the inhibitory effect on the DNA-dependent DNA polymerase reaction. Thus, the effects on both activities should be measured when screening for potentially hepad-

navirus DNA polymerase inhibitors. Of the substances tested FLT-TP showed the highest inhibitory potency. This was valid for both enzyme preparations. The findings should prompt in vivo experiments with 3′-fluoro-2′,3′-dideoxythymidine in DHBV-infected ducks. Further investigations on compounds with high inhibitory effect on the DHBV enzyme should also deal with toxicity, rate of phosphorylation in cell systems, and comparison with the inhibitory effects of these compounds on human HBV DNA polymerase.

Acknowledgements

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