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Inhibition of Hepatitis B Virus DNA Polymerase by 3′-Azido-3′-Deoxythymidine Triphosphate but not by Its *Threo* Analog

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An inhibitory effect of 3′-azido-3′-deoxythymidine triphosphate on hepatitis B virus DNA polymerase was found. No effect was seen by its *threo* analog. The effect was detected at and above a concentration of 0.05 μM. The inhibition of DNA polymerase activity was the same in ten different strains of HBV. The mechanism was shown to be competitive, with an inhibition constant (Ki) of ~ 0.04 μM, and the Km value for dTTP was 0.1 μM.

Key words: hepatitis B, antiviral, reverse transcriptase

INTRODUCTION

Hepatitis B virus (HBV) belongs to a group of DNA viruses (hepadnaviruses) of which the HBV is the prototype. The hepadnaviruses have a circular DNA that is partially single stranded. Enclosed in the nucleocapsid is a viral DNA polymerase. The replication of the DNA genome is unique and has a phase with an RNA "pregenome" intermediate that is transcribed by reverse transcription to DNA [Summers and Mason, 1982]. The DNA polymerase has a double function. In the early transcription stage it functions as a reverse transcriptase, making a full length negative DNA strand on the RNA intermediate, and in the late genome maturation it copies the second positive DNA strand on the negative DNA strand.

We have shown in earlier studies that phosphonoformic acid (foscarnet), which is an effective inhibitor of reverse transcriptase [Sundqvist and Öberg, 1980], also effectively inhibits the HBV DNA polymerase [Nordenfelt et al, 1980].

Recently it has been reported that 3′-azido-3′-deoxythymidine inhibits the multiplication of HIV virus (LAV/HTLV-III) virus [Mitsuya et al, 1985] and that its

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triphosphate inhibits HIV reverse transcriptase [Vrang at al, 1987]. We have studied the effect of 3′-azido-3′-deoxythymidine triphosphate and its *threo* analog on HBV DNA polymerase activity.

MATERIALS AND METHODS

Source of HBV

Serum (10 ml) from a chronic hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg)-positive carrier (S.L.) was pelleted by ultracentrifugation, washed once in PBS, diluted in 1 ml PBS, and stored in 200 μl portions at −20°C. This material was used for the pharmacokinetic studies.

Sera from ten chronic HBsAg, HBeAg-positive carriers were used directly without pelleting. The carriers come from different parts of the world and include both ay and ad subtypes (see Table II). HBsAg and HBeAg was determined by RIA kits (Abbott Laboratories, Chicago, IL). Sub-typing was performed according to Hansson et al [1975].

DNA Polymerase Assay

The assay conditions were as previously described [Nordenfelt et al, 1980]. 25-μl test samples, 2.5 μl 10% Nonidet P-40 and 2.5 μl 3% mercaptoethanol were added and the mixture kept at room temperature for 30 min. This mixture was then added to 100 μl of a solution of 160 40 mM Tris, pH 7.5, 40 mM $MgCl_2$, 400 mM KC1, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, and 2.5 μl 32p dTTP (410 Ci/mmole, 1.0 mCi/ml) (Amersham International, England). The mixture was incubated at 37°C, and at indicated times 20-μl samples were spotted on No. 5 Munktell filter papers, size 24 mm (Stora Kopparberg, Sweden). The filters were placed for 1 hr in 5% TCA/0.1 M tetrasodium pyrophosphate, then washed three times for 10 min in the same solution, once in 5% TCA without pyrophosphate, and twice in 95% ethanol. Finally, the filters were dried and counted in a scintillation counter.

The substances studied were added to the mixture and the reaction was carried out at 37°C for 3 hr. In all assays, duplicate aliquots were taken and the results given are the mean of observed counts per minute (cpm). In the kinetic studies the reaction time was 3 hr during which time the incorporation was linear with time.

Substances Studied

1-(3′-Azido-2′,3′-dideoxy-5′-disodium triphospho-β-D-*threo*-pentofuranosyl) thymine (*threo*-AZT-TP) and 1-(3′-azido-2′,3′dideoxy-5-trisodium diphospho-β-D-*erythro*-pentofuranosyl) thymine (3-′azido-3′-deoxythymidine triphoshpate) (AZT-TP) were synthesized as described previously [Vrang et al, 1987]. *Threo*-AZT-TP was contaminated with 0.5% of AZT-TP.

RESULTS

Inhibition of HBV DNA Polymerase by AZT-TP and *Threo*-AZT-TP

The effects on the DNA polymerase activities of different concentrations of the substances, ranging from 0.01 to 1 μM, were investigated in repeated experiments, two of which are shown for each substance in Table I. As can be seen, there is a clear

TABLE I. Inhibition of HBV DNA Polymerase by AZT-TP and *Threo*-AZT-TP*

Concentration of substance (μM)	Percentage of inhibition of activity			
	AZT-TP		*Threo*-AZT-TP	
	Exp 1	Exp 2	Exp 1	Exp 2
1	80	87	16	3
0.5	68	81	12	4
0.1	34	45	7	1
0.05	21	41	7	+4
0.01	25	13	7	2

*Reaction time was 3 hr.

TABLE II. Inhibition of HBV DNA Polymerase Activity by AZT-TP at 0.2 μM Concentration in Sera From Ten Patients

Patient	Country of origin	Subtype	Percentage of inhibition
S.L. (blood-donor)	Sweden	ad	49
1 (addict)	Sweden	ay	45
2 (leukaemia)	Sweden	ad	40
3	India	ay	45
4	Gambia	ay	52
5	Lebanon	ay	63
6	India	ay	55
7	Lebanon	ay	65
8	Poland	ad	65
9 (dialysis)	Sweden	ay	54
10 (dialysis)	Sweden	ay	44

inhibition by AZT-TP at a concentration of around 0.05 μM, but no clear effect of *threo*-AZT-TP was seen even at 1 μm, the highest concentration.

Inhibition of DNA Polymerase Activity From Different HBV Isolates by AZT-TP

The polymerase activities in sera from ten different patients, chronic carriers of HBsAg and HBeAg, were compared with respect to inhibition by AZT-TP. From earlier experiments a 50% inhibition of DNA polymerase activity was found, using an HBV preparation from patient S.L., by a final concentration of 0.2 μM AZT-TP in the reaction mixture. Therefore, this concentration was used to detect more easily any strain differences in sensitivity to the substance. As can be seen in Table II there was no significant difference in sensitivity, which varied between 44 to 65% (mean 55%) for the different isolates.

Mechanism of Inhibition

The rate of inhibition was investigated in an experiment where AZT-TP to a final concentration of 1 μM was added to the reaction mixture 30 min after the start of the reaction. As shown in Figure 1, the polymerase activity was rapidly inhibited while the control reaction continued for at least 3 hr.

Figure 2 shows the Lineweaver-Burk plot for inhibition of DNA polymerase activity by AZT-TP at varying concentrations of dTTP. The results indicate that AZT-TP is a competitive inhibitor with respect to dTTP. A replot of the intercepts gave the inhibition constant Ki $\sim$ 0.04 μM, and the Km value for dTTP was 0.1 μM.

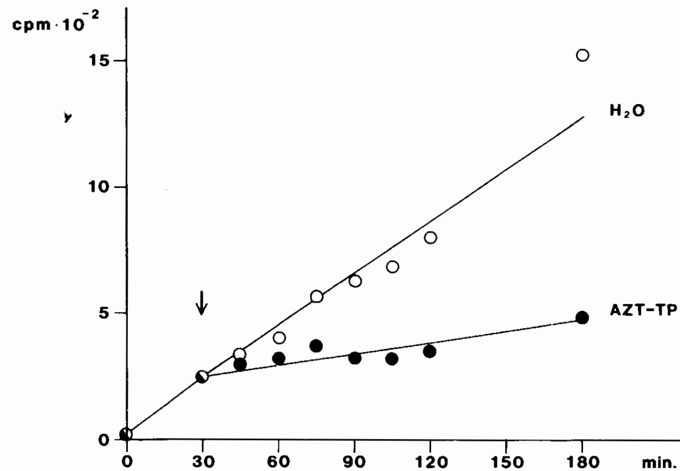

Fig. 1. Rate of inhibition of the DNA polymerase reaction by AZT-TP. To 60 μl of HBV preparation, 10 μl Nonidet P-40 and 400 μl reaction mixture were added. At the designated times after incubation, two 20-μl aliquots of the reaction mixture were removed for counting. At the time indicated by the arrow, AZT-TP (final concentration of 1 μM) or H_2O were added. Aliquots were taken immediately before and after the addition of AZT-TP or H_2O.

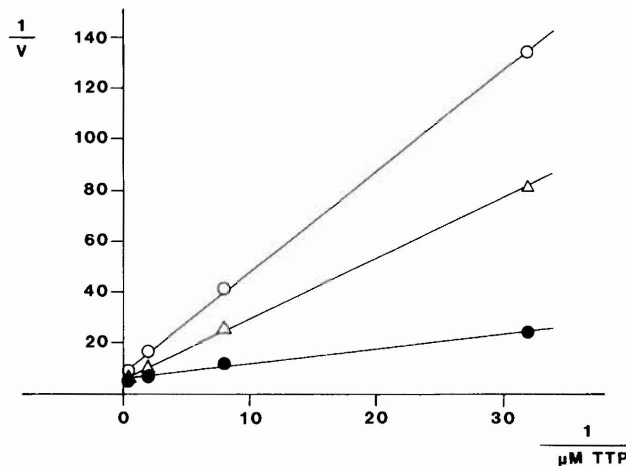

Fig. 2. Double-reciprocal plots of the hepatitis B virus DNA polymerase-catalyzed reaction with dTTP as variable substrate and AZT-TP as inhibitor. The incorporation of [^{32}P] dTTP during 3 hr was determined. AZT-TP concentrations were 0 μM, ●; 0.1 μM, △; and 0.2 μM, ○.

DISCUSSION

3′-Azido-3′-deoxythymidine has been shown to inhibit the infectivity and cytopathic effect of HIV in vitro [Mitsuya et al, 1985]. Its effect is explained by its inhibition of reverse transcriptase by its triphosphate, which acts as a competitive inhibitor [Vrang et al, 1987].

We reported earlier on the inhibition of HBV DNA polymerase activity by foscarnet [Nordenfelt et al, 1980], an effective inhibitor of reverse transcriptase from several viruses [Sundqvist and Öberg, 1979]. It was therefore anticipated that the triphosphate of 3′-azido-3′-deoxythymidine would have an inhibitory effect on the

HBV DNA polymerase, which in an early replication stage functions as a reverse transcriptase [Summers and Mason, 1982]. In this investigation, a clear inhibitive effect was found, and AZT-TP showed a competitive inhibition of HBV DNA polymerase. It gave a 50% inhibition of the activity at a concentration about 0.2 μM of AZT-TP, and a Ki value of 0.04 μM was observed. This is higher than that observed using HIV reverse transcriptase (Ki = 0.0022 μM [Vrang et al, 1986]) but lower than that for AMV reverse transcriptase (Ki = 0.48 μM). The km value for HBV DNA polymerase (0.1 μM) was lower than that for HIV reverse transcriptase (0.7 μM) and AMV reverse transcriptase (68 μM) [Vrang et al, 1987; Eriksson et al, 1987]. Fast inhibition occurred even when AZT-TP was added after the reaction had started.

However, its isomeric analog, the *threo* isomer, has a very low or no inhibitory effect. The same lack of effect of *threo*-AZT-TP has been found for the reverse transcriptase of HIV [Vrang et al, 1987], and a low effect was also found using AMV reverse transcriptase [Eriksson et al, 1987]. The explanation of this 3′-azido configuration-dependent activity is not clear, but it is likely that the azido group either directly causes steric hindrance for interaction with the enzyme or causes conformational change of the whole molecule, preventing the interaction.

AZT has already been tried in a 6 weeks treatment of patients infected with HIV [Yarchaon et al, 1986]. No serious toxic side effects were noted at the lower dosages, but at 10 mg/kg orally every 4 hr the white-cell counts were depressed.

At present no therapy has been proven to cure acute, chronic, or fulminant HBV infection. Therapy trials of this medically important infection include the compound ara-A, which as a triphosphate is inhibitory to HBV DNA polymerase [Thomas and Scully, 1985; Sherlock and Thomas, 1985; Alexander and Williams, 1986]. Recent attempts [Hedin et al, 1986] to treat fulminant hepatitis B with foscarnet indicate a beneficial effect, but further studies are necessary.

Obviously AZT is a new antiviral candidate that should be tried in this connection, even though it seems to be less inhibitory to HBV DNA polymerase than to HIV reverse transcriptase. Furthermore, a search for substances with possible beneficial effects on HBV infection should be directed toward substances with an inhibitory effect on reverse transcriptase.

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