ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1995, p. 1430–1435 Vol. 39, No. 7
0066-4804/95/$04.00+0

Mechanism of Inhibition of Duck Hepatitis B Virus Polymerase by (−)-β-L-2′,3′-Dideoxy-3′-Thiacytidine

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Received 28 December 1994/Returned for modification 23 February 1995/Accepted 2 May 1995

We have used the endogenous reverse transcriptase reaction of viral core particles from duck liver to elucidate the mechanism of inhibition of duck hepatitis B virus (DHBV) replication by the nucleoside analog (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC). As is the case in human immunodeficiency virus replication, 3TC–5′-triphosphate (3TC-TP) acts as a chain terminator for the DNA polymerase activities. The results of several different experiments support this conclusion, which explains the potent activity of 3TC against the hepadnaviruses. In isolated DHBV core particles, 3TC-TP inhibited the reverse transcriptase in a manner that resembled competitive inhibition with respect to dCTP. However, the kinetics of inhibition was not linear on a double-reciprocal plot for the highest concentrations of 3TC-TP and the lowest concentration of dCTP. This anomaly would be expected if binding to the nucleotide site was followed by DNA chain termination. Calculations that used only the linear part of the curve yielded a K_i of 0.78 ± 0.10 μM 3TC-TP. The inhibition of core particles incubated in vitro with 3TC-TP was not reversed by removal of the free inhibitor. 3TC-TP inactivated the reverse transcriptase activity in a concentration-dependent manner. The K_m of the chain termination reaction was calculated at 0.71 ± 0.05 μM. Similar competitive kinetics and irreversible inhibition were also obtained on the endogenous DNA polymerase from viral particles from serum, suggesting that 3TC-TP also acts as a chain terminator of the DNA-directed DNA polymerase of DHBV replication. Core particles purified from DHBV-infected hepatocytes treated with 3TC also showed irreversible inhibition of the endogenous reverse transcriptase activity, indicating that chain termination is the mechanism of inhibition in the presence of the normal deoxynucleotide pools within cells. Endogenous sequencing of duck liver viral core particles with 3TC-TP as a chain terminator shows a pattern of bands similar to that obtained with ddCTP, and [^{32}P]3TC-TP labeled the growing DNA chain at the C position.

The nucleoside (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC [lamivudine]) is a deoxycitidine analog in which the 3′ carbon is replaced by a sulfur atom with loss of the 3′ OH group necessary for the elongation of the DNA chain. 3TC was shown to be an effective inhibitor of human immunodeficiency virus (HIV) replication (7, 13, 28, 33), and it was recently approved for compassionate use as an anti-HIV agent.

Previous work showed that 3TC is also a very effective agent against human hepatitis B virus (5, 10, 32) and duck hepatitis B virus (DHBV [32]). In vivo and in infected hepatocytes, 3TC rapidly reduces the amount of viral DNA within days of the start of treatment, and it does not show significant toxicity (reference 5 and our unpublished results). 3TC is currently undergoing phase II clinical trials as an antiviral agent for human hepatitis B virus.

For both HIV and hepadnaviruses, the mechanism of action of 3TC requires phosphorylation to 3TC–5′-triphosphate (3TC-TP), which in turn specifically inhibits the viral polymerases (3, 6, 15). The (−) enantiomer is more potent and less toxic than the (+) enantiomer (6, 8, 25, 27). The specificity is conferred by the much lower affinity of 3TC-TP for the cellular α- and β-polymerases (15, 16). Using recombinant HIV reverse transcriptase (RT) and synthetic RNA and DNA templates, it has been shown that 3TC-TP inhibits HIV by acting as a chain terminator which is irreversibly incorporated into the nascent DNA chain (16, 26, 35).

The mechanism of inhibition of hepadnaviruses involves inhibition of the viral polymerase (5, 7, 8), but the precise mode of action is still unknown. Hepadnaviruses replicate by a multistep mechanism that begins with reverse transcription of pregenomic RNA. The DNA synthesis is initiated by a primer protein which directly binds the first nucleotide (dGTP) of the DNA minus strand (2, 24, 34). The DNA plus strand is then synthesized with the minus strand as a template to yield the mature, partially double-stranded DNA virus. This process occurs inside the capsid formed when the core protein of the virus encapsidates the pregenomic RNA. The viral polymerase is produced with little abundance by the infected cells, and numerous attempts to express the cloned, fully active enzyme have failed. However, hepadnavirus RT and DNA polymerase activities can be demonstrated, with endogenous polymerase assays, in isolated replicating core particles from DHBV-infected liver or in mature virions from sera. These assays rely on the incorporation of radioactive nucleotides into the incomplete viral DNA (17). By using replicating cores extracted from congenitally infected ducks, we determined that 3TC-TP inhibits virus replication by acting as a chain terminator of both the RT and DNA polymerase activities of the enzyme.

MATERIALS AND METHODS

Chemicals. 3TC was obtained from Janet Cameron, Glaxo Group Research, Greenford, Middlesex, United Kingdom, and 3TC-TP was prepared according to the method of Ludwig and Eckstein (20). The purity was greater than 90% as measured by nuclear magnetic resonance and high-performance liquid chromatography (HPLC) analysis. Chemicals were purchased from Sigma unless otherwise indicated. Deoxy- and dideoxynucleotide triphosphates and proteinase K were purchased from Boehringer Mannheim. α-^{32}P-labeled deoxynucleotides (3,000 Ci/mmol) and $^{32}P_i$ (6,000 Ci/mmol) were purchased from Dupont, NEN, Mississauga, Canada.

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Synthesis of [α-^{32}P]3TC-TP. The chemicals required for the synthesis of [α-^{32}P]3TC-TP were prepared as follows. Hexamethylphosphoramide was distilled and stored over 4A molecular sieves. Triethylamine was refluxed with *p*-toluenesulfonyl chloride, filtered, and distilled prior to distillation from potassium hydroxide. Trichloroacetonitrile (Aldrich) and acetonitrile (Aldrich HPLC grade) were used without further purification. For chromatographic charcoal, finely powdered Norit A was boiled with 6 M HCl and washed thoroughly with water. It was partly inactivated by treatment with a 10% solution of octanol in ethanol for 15 min and then washed with water, dried at 100°C, and stored in a sealed bottle. Tributylammonium PP_i was prepared according to the method of Moffatt (23) and used as a 0.5 M solution in dimethylformamide.

[α-^{32}P]3TC-TP was synthesized as follows. A mixture of 3TC (5 mg [20 μmol]), pyridinium trifluoracetate (2 mg [10 μmol]), and 0.1 ml (1.0 mCi) of $^{32}P_i$ in water was dried by evaporation of acetonitrile on a rotoevaporator attached to a vacuum pump. The residue was dissolved in 0.2 ml of hexamethylphosphoramide and further dried by two successive evaporations with 0.5 ml of acetonitrile. The residue was treated with 15 μl (0.1 mmol) of triethylamine and 10 μl (0.1 mmol) of trichloroacetonitrile, and the stoppered flask was incubated at 32°C for 45 min. The reaction mixture was coevaporated with 0.5 ml of acetonitrile and then treated with 5 mg of 1,1′-carbonyldiimidazole.

The flask was returned to the rotoevaporator and held under vacuum for 3 h before 0.1 ml of a 2% solution of methanol in acetonitrile was added. After 10 min at room temperature, the reaction solution was evaporated to dryness, and tributylammonium PP_i in dimethylformamide (0.2 ml) was added to the flask. The reaction mixture was stirred overnight, diluted with 0.05 M triethylammonium bicarbonate solution, and applied to a column (0.75 by 2 cm) of DEAE-Sephadex. The column was washed with 20 ml of 0.1 M triethylammonium bicarbonate, and then the desired [α-^{32}P]3TC-TP was eluted with 10 ml of 0.25 M triethylammonium bicarbonate. The eluant was evaporated and then successively evaporated with 50% ethanol–water until no evidence of triethylammonium bicarbonate remained. The contents of the flask were then taken up in a small amount of water and applied to a small plug of charcoal (100 mg) in a Pasteur pipette. The reaction flask was washed with water, and the washings were applied to the charcoal. The small column was washed with 10 ml of water before the nucleotide was eluted with 0.3 ml of 50% ethanol–5% ammonia. The specific activity of [α-^{32}P]3TC-TP was 6,000 Ci/mmol, and the radiochemical purity was greater than 90%, as measured by thin-layer chromatography (TLC). The impurities were phosphate, PP_i, and 3TC-DP.

Preparation of core particles. Core particles were purified from the livers of Pekin ducks congenitally infected with DHBV by sucrose gradient sedimentation as previously described (30), except that a discontinuous sucrose gradient (15, 22.5, and 30%) was used. In order to remove the nucleotide-degrading enzymes, core particles were further purified by gel exclusion column chromatography with the matrix Bio-Gel A-150 (Bio-Rad). The Bio-Gel A-150 was packed in a 3-ml plastic column with cotton to hold the matrix. This was critical, because the frits used in some commercial columns did not allow passage of the core particles. The column was equilibrated with polymerase buffer (see composition below) and used at 4°C. Core particles from the sucrose gradient were loaded on the Bio-Gel A-150 column and eluted with polymerase buffer. Fractions of approximately 100 μl were collected. Typically, endogenous polymerase activity eluted in fractions 15 to 22, approximately where particles with a size of 8×10^6 Da would be expected to elute from this column.

TLC was performed to detect degradation of deoxynucleotide triphosphates. α-^{32}P-labeled nucleotides were spotted on polyethyleneimine cellulose TLC plates (Polygram Cel 300PEI/Uv254; Macherey-Nagel) and developed with 0.1 M LiCl–1 M acetic acid for 15 min, 1 M LiCl–1 M acetic acid for 45 min, and 1.5 M LiCl–1 M acetic acid for 90 min. Cold standards were included with each assay and were detected by UV absorption. The intensities of the radioactive spots were estimated with a PhosphorImager (Fuji) or an optical densitometer (Joyce-Loebl).

Core particles from serum of congenitally infected ducks were purified as follows. Duck serum was clarified by being spun at $8,000 \times g$ for 10 min, layered on a 20% sucrose cushion (in 150 mM NaCl–50 mM Tris [pH 8.0]–10 mM EDTA), and spun at 30,000 rpm for 16 h in a Beckman SW40 ultracentrifuge rotor. The pellet was resuspended in EB buffer (10 mM Tris [pH 7.5], 25 mM NaCl, 0.25 M sucrose, 7 mM $MgCl_2$, 0.1% 1-mercaptoethanol, 100 mg of bovine serum albumin per ml) and used for the endogenous polymerase activity assay as described below.

Endogenous polymerase reaction. The endogenous polymerase reaction was measured by using a procedure similar to the one reported earlier (17, 30). The polymerase buffer contained 20 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris (pH 7.4), and 0.1% Nonidet P-40 (0.2% for virions from serum). [α-^{32}P]dCTP was added as a tracer with 50 μM (each) cold dGTP, dATP, and dTTP. For the RT assay, actinomycin D was added at a concentration of 100 μg/ml to specifically inhibit the DNA-dependent polymerase activity. The total incorporation of [α-^{32}P]dCTP in core particles was reduced by 20 to 30% in the presence of 100 μg of actinomycin D per ml. The endogenous polymerase reaction produced mainly single-stranded DNA species, as detected by agarose gel electrophoresis followed by Southern transfer and autoradiography. This was in agreement with previously published results (30).

Kinetics and calculations. Inhibition kinetics was determined by measuring the initial rate of endogenous polymerase activity in the presence of various concentrations of [α-^{32}P]dCTP and the remaining four nucleotides (50 μM [each]). A range of dCTP concentrations from 0.015 to 0.5 μM was prepared by mixing cold dCTP and [α-^{32}P]dCTP at a constant specific activity of 16,650 cpm/fmol. The kinetics parameters k_m and V_{max} were calculated by fitting the experimental points through the Michaelis-Menten kinetics equation or by linear regression on a double-reciprocal plot by using an application for Macintosh computers, DeltaGraphPro 3.0 (DeltaPoint, Inc., Monterey, Calif.). The competitive k_i was calculated from the Michaelis-Menten parameters determined in the presence and absence of inhibitor.

Irreversible inactivation of isolated core particles. Core particles were incubated under the conditions used for the endogenous RT assay in the presence of various concentrations of 3TC-TP and 50 μM (each) dGTP, dATP, and dTTP. After incubation, the reaction mixture was washed twice with polymerase buffer by filtration through Ultrafree −MC centrifugal filters (Millipore). This filtration diluted the nucleotide pool 200-fold, and about 75% of the RT activity was recovered. The eluted core particles were used for the endogenous RT assay to determine the percentage of inhibition (*I*) by 3TC-TP with respect to the control. The initial rate of inactivation was calculated assuming an exponential-to-plateau time course of inactivation, which is the expected kinetics for enzyme inactivation in the presence of a constant concentration of inhibitor. The equation is

$$I = 100(1 - e^{-kt})$$

where k can be calculated as

$$k = \ln (1 - I/100)/t$$

and where the initial rate is the first derivative of equation 1 for

$$t = 0$$

$$dI/dt = 100k$$

Irreversible inactivation of core particles in cell culture. Primary hepatocyte cultures were prepared from 1-week-old congenitally infected ducks as previously described (31). Triplicate cultures in 100-mm-diameter tissue culture plates were incubated in the presence and absence of 3TC and 2 μg of cycloheximide per ml. Core particles from the hepatocytes were prepared by polyethylene glycol precipitation as previously described (18, 29), except that the DNase digestion step was omitted. The pellet containing the core particles was resuspended in 50 μl of EB buffer and used immediately in the endogenous RT assay.

Endogenous sequencing. Endogenous sequencing was performed as previously described (24), with 0.13 μM α-^{32}P-labeled dGTP, dATP, and dTTP (3,000 Ci/mmol) and 0.13 μM cold dCTP. [α-^{32}P]dCTP was not used, because there was evidence that some commercial preparations produce smears on acrylamide gels. Samples were treated with 10 μg of RNase A (Sigma) per ml for 5 min at 37°C before being loaded on the sequencing gel (34).

Incorporation of ^{32}P-labeled 3TC-TP into the growing DNA chain. Core particles from duck liver were incubated under the conditions used for the endogenous polymerase reaction, except that [α-^{32}P]3TC-TP was substituted for dCTP. After 1 h of incubation, the reaction was stopped with 200 μl of lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 1% lauryl sarcosine, 200 μg of proteinase K per ml [Boehringer Mannheim]). The samples were incubated overnight at 37°C and extracted twice with an equal volume of 1:1 phenol-chloroform. The DNA was precipitated with 100 mM NaCl–10 μg of tRNA–2 volumes of ethanol at −20°C. After being dried, the DNA was resuspended in 5 μl of sequencing gel loading buffer (50% formamide, 5 mM EDTA, 0.025% bromophenol blue, 0.025% xylenecyanol blue) and run on a 15% polyacrylamide–7 M urea sequencing gel for 6 h at 1,450 V in 1× Tris-borate-EDTA buffer. The gel was dried and autoradiographed.

RESULTS

Removal of nucleotide-degrading enzymes. Core preparations obtained by sucrose gradient sedimentation (as described in Materials and Methods) contained various enzyme activities that degraded a substantial portion of nucleotide triphosphates during the endogenous polymerase assay, as determined by TLC (not shown). Enzymes against dATP were particularly active and completely degraded 0.015 μM [α-^{32}P]dATP in less than 5 min (not shown). This resulted in instability of the polymerase activity and weak inhibition of the polymerase by the nucleotide triphosphate analogs. To obviate these problems, core preparations were subjected to further purification through a Bio-Gel A-150 column (see Materials and Methods), and the endogenous polymerase reaction was carried out in the presence of 100 μM 5′ adenylylimidodiphosphate, a nonhydrolyzable analog of ATP known to inhibit ATPase activities (36). Degradation of nucleotide triphosphates was measured by in-

TABLE 1. Degradation of nucleotide triphosphates in purified core preparations

Nucleotide	% degradation of[a]:					
	Triphosphate		Diphosphate		Monophosphate	
	0 min	30 min	0 min	30 min	0 min	30 min
C	97.0	96.0	2.7	3.2	0.0	0.0
T	97.6	94.7	2.4	5.2	0.0	0.0
G	95.7	83.4	3.9	15.8	0.3	0.8
A	98.6	86.1	1.1	13.3	0.0	0.5
3TC[b]	87.1	88.9	12.9	11.9	0.0	0.0

[a] Values represent percentages of nucleotide species, measured by PhosphorImager analysis of the radioactive spots on polyethyleneimine TLC.

[b] The intensities of radioactive spots of 3TC were estimated by optical densitometry.

cubating α-^{32}P-labeled nucleotide triphosphates under the same conditions used for the endogenous RT assay in all of the subsequent experiments presented in this paper (i.e., dCTP was added at 0.015 μM [the lowest concentration used in the kinetics experiments] and dATP, dGTP, and dTTP were added at 50 μM each). After incubation of the nucleotide triphosphates in the presence of purified core preparation, no degradation of dCTP, dTTP, and 3TC-TP was detected, but about 15% of the dGTP and dATP was converted to dGDP and dADP, respectively. Table 1 summarizes these data and shows the percentages of the three phosphorylated species of nucleotides present before (0 min) and after (30 min) incubation in the presence of DHBV core preparation. However, for the subsequent experiments presented in this paper, dGTP and dATP were added at a concentration of 50 μM, which is in great excess of the K_m for the nucleotides (0.1 to 0.2 μM) (14, 21).

The time course of the incorporation of [α-^{32}P]dCTP in purified core particles was linear for at least 20 min at both 0.015 μM ($R^2 = 0.997$, $n = 3$) and 0.5 μM ($R^2 = 0.999$, $n = 3$) dCTP (not shown). These are the lowest and the highest concentrations of dCTP used in the kinetics studies presented in this work.

Kinetics of inhibition of RT by 3TC-TP. Using the purified core preparation, we have studied the kinetics of 3TC-TP inhibition of RT activity of DHBV polymerase. For these experiments, purified DHBV core was incubated with different concentrations of [α-^{32}P]dCTP (from 0.015 to 0.5 μM) in the absence or in the presence of 3TC-TP. The K_m for dCTP was calculated from these experiments at 0.10 ± 0.05 μM ($n = 9$).

The kinetics of inhibition of dCTP incorporation by 3TC-TP resembled competitive inhibition, with an increase in K_m and little change in V_{max}. However, at high concentrations of 3TC-TP and at low concentrations of dCTP, the kinetics was not linear on a double-reciprocal plot. For this reason, the competitive K_i could not be measured with confidence. By considering only the points corresponding to concentrations of dCTP higher than 0.06 μM, linear competitive kinetics was obtained, and a K_i of 0.78 ± 0.10 μM ($n = 4$) was calculated.

3TC-TP at the concentrations of 1 and 10 μM did not significantly inhibit RT activity when kinetics experiments were done in the presence of an excess (50 μM) of dCTP and different concentrations of [α-^{32}P]dTTP (data not shown). Moreover, 5 μM 3TC-TP did not inhibit the incorporation of 0.1 μM dATP, dGTP, or dTTP. This indicates that the inhibition by 3TC-TP is mediated by the binding at the nucleotide triphosphate site of the polymerase molecule at the C position.

Similar results were obtained for the viral DNA-directed and DNA polymerase activity (i.e., on the DNA second-strand synthesis) with viral particles extracted from the serum of congenitally infected ducks. The K_m for dCTP was 0.08 ± 0.02 μM ($n = 3$), and the apparent K_m in the presence of 2 μM 3TC-TP was 0.84 μM. The kinetics of inhibition was compatible with a competitive mechanism, and the K_i was calculated at 0.20 μM.

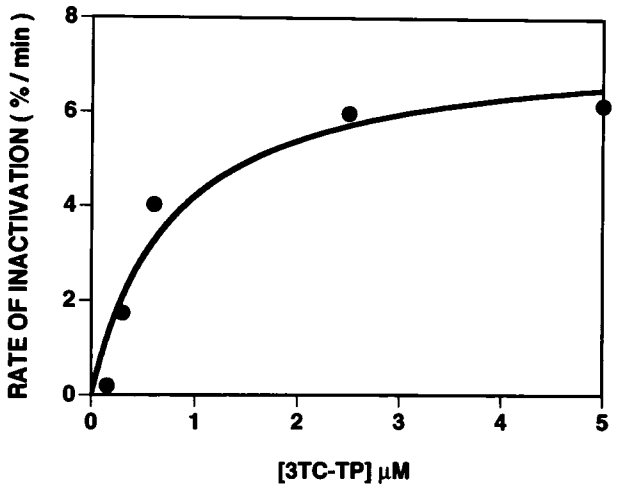

FIG. 1. Irreversible inhibition of endogenous RT activity by 3TC-TP. A core preparation from duck liver was incubated for 20 min in the presence of the indicated concentration of 3TC-TP and 50 μM (each) dGTP, dATP, and dTTP. At the end of the incubation, free nucleotides were separated from the core particles and endogenous RT activity was measured. The percentage of inhibition was calculated with respect to that of a control sample that was incubated in the absence of 3TC-TP and then processed with the other samples. The initial rate of inactivation was calculated as the derivative at time zero of an exponential-to-plateau time course of inactivation (see Materials and Methods). For this experiment, K_m was 0.70 μM and V_{max} was 7.4% inactivation per min.

Irreversible inhibition of core particles by 3TC-TP. Nonlinear kinetics of inhibition is expected if 3TC-TP is incorporated into the nascent DNA chain, and by stopping elongation, it thus inactivates the RT contained in the core particles. In kinetics terms, this is equivalent to a decrease in the enzyme concentration. To show that this is the case for inhibition by 3TC, DHBV core particles from liver were incubated for 20 min in the presence of various concentrations of 3TC-TP (from 0.2 to 5 μM) and 50 μM (each) dATP, dTTP, and dGTP. The residual RT activity was measured after removal of free 3TC-TP, as described in Materials and Methods. Figure 1 shows one of the experiments in which the initial rate of inactivation increases with the concentration of 3TC-TP. Assuming a Michaelis-Menten equation, the K_m of this inactivation reaction was calculated at 0.71 ± 0.05 μM ($n = 3$). Inactivation of the DNA-directed and DNA polymerase activity could also be obtained in mature virions from duck serum, incubated in the presence of 10 μM 3TC-TP under the same conditions described for the inactivation of liver cores.

Figure 2 shows that the irreversible inactivation of core particles can be obtained in hepatocytes in culture treated with the nucleoside 3TC. For this experiment, various concentrations of 3TC were added to the medium of hepatocyte cultures prepared from congenitally infected ducks. After 3 h of incubation, cells were harvested, and the core particles were extracted. The method of extraction involves precipitation of core particles from the cytoplasmic extract, which removes the intracellular and extracellular nucleotides and nucleosides. An aliquot of the extracted core particle preparation was used for dot blot hybridization to measure the amount of DHBV DNA in each cell extract. There was no significant difference be-

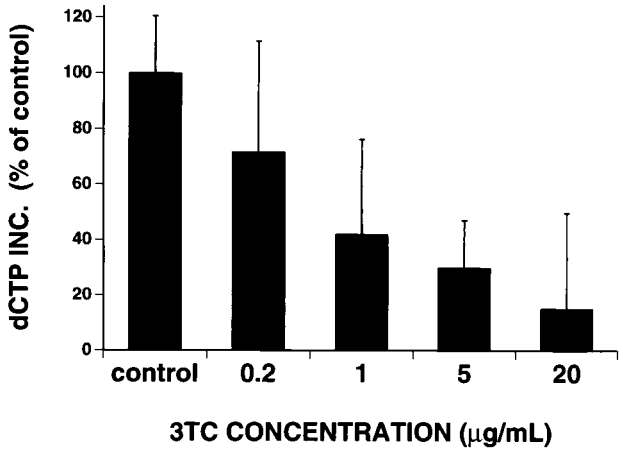

FIG. 2. Irreversible inactivation by 3TC of core particles in isolated duck hepatocytes. Hepatocytes from congenitally infected ducks were incubated for 3 h in the presence of 3TC added to the culture medium. Cycloheximide was present during the incubation at a concentration of 2 μg/ml. RT activity was measured in the core particles isolated from the hepatocytes as described in Materials and Methods. Each bar represents the average of three replicates. INC., incorporated.

tween control and treated cells (not shown), indicating that the inhibition of activity seen with 3TC is not due to a reduction in the number of intracellular core particles. The experiment illustrated in Fig. 2 was carried out in the presence of 2 μM cycloheximide. Cycloheximide was added to prevent synthesis of viral proteins that could produce de novo active core particles during incubation with 3TC. Cycloheximide had no effect on the RT activity of core particles in untreated (no 3TC) cells (not shown).

Endogenous sequencing. In order to confirm that 3TC-TP was actually terminating the DNA chain at the C position, we performed an endogenous sequencing reaction with core particles from duck liver. Endogenous sequencing is an endogenous RT assay in the presence of each of the four dideoxynucleotides as chain terminators. Figure 3 shows the results of chain termination with ddGTP, ddATP, ddTTP, and ddCTP as well as 3TC-TP. The band pattern in the 3TC-TP lane is almost identical to the pattern obtained with ddCTP. The sequence obtained from this experiment is shown to the left of Fig. 3 and corresponds to the DNA strand synthesized during the RT step, from nucleotide 2442 to nucleotide 2398 of the published sequence of DHBV 16 (GenBank DNA sequence database). This sequence is found 95 nucleotides from the replication initiation site as defined by Lien et al. (19).

In order to demonstrate physical incorporation of 3TC-TP in the growing DNA chain, the endogenous RT assay was performed in the presence of α-^{32}P-labeled 3TC-TP and an excess of the other three nonradioactive nucleotide triphosphates. The DNA was then purified and run on a sequencing gel to detect bands that arise because of the chain termination effect of 3TC-TP. Figure 4 shows that radioactive bands appeared in the samples incubated with [α-^{32}P]3TC-TP for increasing times (lanes 1 to 3). This incorporation was reduced by the presence of 1 μM cold dCTP (not shown). Lane C shows endogenous labeling of core particle DNA with [α-^{32}P]dCTP in the presence of a 200-fold excess of ddCTP. The bands formed as a result of the chain termination due to ddCTP seem to match those formed by 3TC-TP, which suggests the incorporation of 3TC-TP at the C positions of the nascent DNA chain.

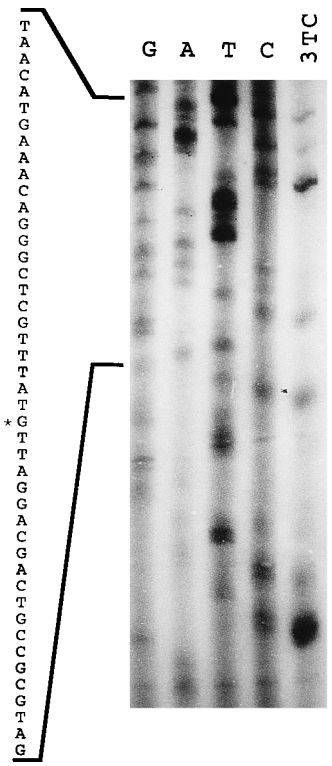

FIG. 3. Endogenous sequencing of DHBV core particles. Core particles were incubated in the presence of 0.13 μM (each) ^{32}P-labeled dGTP, dATP, and dTTP and cold dCTP and in the presence of one of the dideoxynucleotide triphosphates as indicated at the top of each lane. Actinomycin D was added at a concentration of 100 μg/ml to inhibit second-strand DNA synthesis. The 8% acrylamide–8 M urea gel was run for 3 h at 2,000 V and autoradiographed for 15 h at −70°C. The nucleotide sequence is shown to the left of the gel. The asterisk indicates the position that differs from the published sequence of DHBV 16.

DISCUSSION

The data presented in this paper show that 3TC-TP inhibits the replication of DHBV DNA by acting as a chain terminator of the viral polymerase. This mechanism is similar to that previously demonstrated for 3TC-TP inhibition of HIV replication (16, 26, 35).

The kinetics of 3TC-TP inhibition of the RT activity is the result of an apparent competitive inhibition with respect to dCTP. Other chain terminators display competitive inhibition kinetics (see, for example, reference 11). However, because chain termination by 3TC-TP causes cessation of DNA synthesis in the core particle, a deviation from the Michaelis-Menten model is expected, especially at a low concentration of dCTP or a high concentration of 3TC-TP. By ignoring the points below 0.06 μM dCTP in the calculations, we obtained kinetics that more closely approached the Michaelis-Menten model, and the competitive K_i for 3TC-TP was calculated at 0.78 ± 0.10 μM. Under these conditions, the inactivation of the polymerase by 3TC-TP is negligible and the calculated K_i represents the approximate value of the K_d of 3TC-TP for the nucleotide triphosphate binding site of the polymerase.

We demonstrated irreversible inactivation of RT activity by 3TC-TP by incubating replicating core particles with 3TC-TP for a short time and by measuring the residual RT activity after removal of free 3TC-TP by filtration. The rate of inhibition was dependent on the 3TC-TP concentration, and the kinetics could be described by a Michaelis-Menten equation with a K_m of 0.71 ± 0.05 μM. Although this is not a true Michaelis-

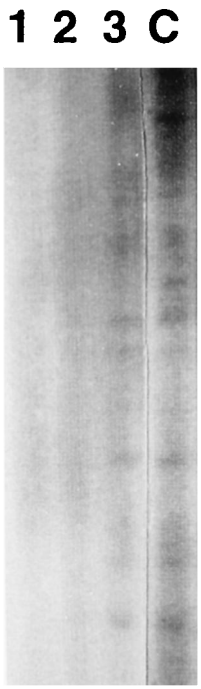

FIG. 4. Sequencing gel electrophoresis of endogenously labeled DNA from liver core. Lanes 1 to 3, DHBV DNA was labeled in the presence of about 0.015 μM [α-^{32}P]3TC-TP and 50 μM (each) dGTP, dATP, and dTTP for 0 (on ice), 15, and 120 min. Lane C, liver core preparations were labeled in the presence of 0.015 μM [α-^{32}P]dCTP, 50 μM (each) dGTP, dATP, and dTTP and 10 μM ddCTP as chain terminator. The X-ray film for lanes 1 to 3 was exposed for 10 days at −70°C with an intensifying screen. For lane C, the exposure time was 24 h.

Menten kinetics, the following arguments indicate that the calculated K_m is a good approximation of the K_d of 3TC-TP. In fact, in the reaction

$$P_{DNA} + \text{3TC-TP} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} P_{DNA}\cdot\text{3TC-TP} \xrightarrow{k_{cat}} P_{DNA\text{-}3TC\text{-}MP}\ \text{(inactive)}$$

$P_{DNA} \cdot$ 3TC-TP reaches a rapid equilibrium because for the DNA polymerases k_1 is much greater than k_{cat}. For the Klenow polymerase, k_1 is 10^8 M^{-1} s^{-1} and k_{cat} is 13 s^{-1} (4, 9). k_{cat} for RTs is probably several orders of magnitude lower (35). Therefore, the initial rate of the inactivation reaction is equivalent to the initial rate of a Michaelis-Menten equilibrium. K_m is defined as $K_d + k_{cat}/k_1$. Since k_{cat}/k_1 is a small number compared with K_d, the K_m calculated in our experiments should approach the K_d of 3TC-TP binding to the polymerase. Therefore, it is not surprising that the K_m of inactivation is not significantly different from the competitive K_i calculated from the inhibition kinetics discussed above.

Competitive kinetics of inhibition and irreversible activation were also observed when DNA-directed DNA polymerase activity was measured in virions purified from serum. This indicates that 3TC-TP can also act as a chain terminator during the synthesis of the second DNA strand, and previous work showed that 3TC-TP inhibited DNA polymerase activity in hepatitis B virus mature extracellular virions (5).

Further evidence that the same chain termination mechanism may apply in vivo is offered by the results of experiments in which hepatocytes from congenitally infected ducks were treated with 3TC for a short time (3 h). The core particles extracted from these cells were irreversibly inhibited, and the inhibition was dependent on the concentration of 3TC present during the incubation. The amount of DHBV-specific DNA was not changed. This shows that 3TC can cause complete chain termination of DHBV in vivo. Endogenous sequencing of the replicating DHBV core shows that 3TC-TP blocks the growing DNA chain at the C positions, producing a sequencing pattern which is almost identical to that obtained with ddCTP as chain terminator. Physical incorporation of α-^{32}P-labeled 3TC-TP in the nascent DNA chain also produced a ladder of radioactive bands on a sequencing gel that corresponded to those formed by chain termination with ddCTP.

The apparent K_i of 3TC-TP for the DHBV polymerase is much higher than the K_m of the normal substrate dCTP (0.8 versus 0.1 μM). Given these values, it may be difficult to see how 3TC is such a potent antiviral agent. However, effective inhibition by 3TC-TP depends on the probability that a chain termination event will occur during a cycle of virus replication. In vivo, there will be approximately 750 opportunities for incorporation of 3TC during reverse transcription and almost as many chances for termination of DNA synthesis by 3TC during the second-strand synthesis. One chain incorporation event is sufficient to stop the replication. If we assume that in vivo the probability of 3TC-TP being incorporated in place of dCTP is 1:100, it can be calculated that 3TC-TP will block the maturation of 84% of viral genomes during the RT step and 94% of viral genomes if the synthesis of the second DNA strand is included. These calculations were based on formulas published by Goody et al. (12). This is similar to the inhibition of bacterial growth by dideoxynucleotides. Although *Escherichia coli* polymerase discriminates 1:1,000 against dideoxynucleotides (1), these molecules are very powerful inhibitors of DNA replication (22) because of many chances for incorporation offered by the large size of the bacterial genome. Similar arguments can be used to reconcile our data with the very low 50% inhibitory dose (0.05 μM) reported by Chang et al. (5) for the human hepatitis B virus. In their work, the polymerase reaction was measured as the amount of viral genome that reached full length, as opposed to the initial rate of dCTP incorporation as reported in our study.

In conclusion, three lines of evidence demonstrated that 3TC-TP acts as a chain terminator of DHBV DNA replication. (i) 3TC-TP competes with dCTP for the nucleotide triphosphate site of the polymerase. (ii) 3TC-TP causes an irreversible, concentration-dependent inhibition of the endogenous RT and polymerase activities (i.e., the activities are still inhibited after the free 3TC-TP is removed from the reaction mixtures). (iii) 3TC-TP is incorporated into the DHBV DNA, generating a ladder of bands that are similar to those generated by chain termination with ddCTP. The results in this study are consistent with the potent inhibition of hepadnavirus replication by 3TC, despite relatively unimpressive K_i values for 3TC-TP (0.8 μM) compared with the K_m for dCTP of 0.1 μM.

ACKNOWLEDGMENTS

We thank Karl Fischer for help with the endogenous sequencing. This research was supported by a grant from Glaxo Canada.

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