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INHIBITION OF DUCK HEPATITIS B VIRUS REPLICATION IN VITRO BY 2', 3'-DIDEOXY-3'-AZIDOTHYMIDINE AND RELATED COMPOUNDS

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Summary. - We have adopted the *in vitro* hepatocyte culture system of the duck infected with duck hepatitis B virus (HDBV) to an antiviral assay system. Using this method, we found that 2', 3'-dideoxy-3'-azidothymidine (N_3dT) and 2', 3'-dideoxy-3'-O-methylthymidine (OMeT) had antiviral effects against DHBV replication in the concentrations of 20-50 μmol/l and 4-40 μmol/l, respectively. The N_3dT inhibited the single strand DNA formation (negative strand), which is an intermediate of virus replication. However, the inhibition of single strand DNA synthesis by OMeT was relatively weak. These two compounds may have different mechanisms of DHBV DNA replication inhibition. Two other 3'-substituted pyrimidine analogues tested were very weak inhibitors. Antiviral agents that inhibit the reverse transcriptase activity of the hepadnavirus DNA polymerase could be potential candidates for the chemotherapy of these viruses.

Key words: duck hepatitis B virus; azidothymidine analogues; antivirals

Introduction

Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae;* it causes acute and chronic liver disease and hepatocarcinoma in man. The introduction of vaccination against HBV is expected to decrease liver disease caused by this virus in the next generation (Paletti *et al.,* 1984). However, there are estimated 284, 000 000 carriers in the world that will not benefit from vaccination. Treatment with interferon (Greenberg *et al.,* 1976), or adenine arabinoside and its monophosphate derivative (Basendine *et al.,* 1980; Perriollo *et al.,* 1985) have

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been used to treat HBsAg positive chronic liver disease. Unfortunately, these therapies have not been highly effective and these drugs in question may be toxic. We recently reported on an *in vitro* screening system for antiviral agenst with anti-duck hepatitis B virus (DHBV) activity and found that the 2',3'-dideoxy purine analogues were very effective (Suzuki *et al.*, 1988, Lee *et al.*, 1989). The original method for the *in vitro* culture of duck hepatocytes was developed by Tuttleman *et al.* (1986*b*) and the detail of replication mechanism of DHBV was studied at a molecular level using this *in vitro* system (Tuttleman *et al.*, 1986*a*). We have adapted this system to study the activity of compounds against DHBV replication. In this paper we have determined the anti-DHBV activity of four 3'-subtituted thymidine analogues. 2', 3'-dideoxy-3'-azidothyminide (N_3dT) and 2',3'-dideoxy-3'-0-methylthyminide (OMeT) showed inhibitory activities for DHBV viral replication. Since the replication mechanism of DHBV is very similar to that of HBV (Will *et al.*, 1987), the duck hepatocyte culture system should be not only useful for the *in vitro* screening of candidate compounds, but also for the study of their anti-HBV activity and the mechanism of action.

Materials and Methods

Virus infection of cell cultures and animals. The pathogen free duck eggs were purchased from SPAFAS Ltd. (Norwich, CT) and incubated in our laboratory. Within 24 hr after hatching, ducklings were infected with DHBV-positive duck serum (20–50 μl, i.v.) which was kindly provided by Dr. W. Mason, Fox Chase Cancer Center, Philadelphia. Four days post-infection (p.i.) the sera from infected ducklings were tested for the establishment of persistent infection by dot blot hybridization (Mason *et al.*, 1982). By this method the infection efficiency was 100 %. Hepatocytes were obtained from 1–2 week old ducklings using a minor modification of the method described by Tuttleman *et al.* (1986*b*).The livers were perfused with Swimm's medium (Gibco, Chagrin Falls, OH) through heart to liver and exiting at open portal vein. Collagenase type 1V (Sigma, St. Louis) was added to the perfusion solution in contrast to Tuttleman *et al.* (1986*b*) who perfused the liver via the portal vein using collagenase type I. The medium on cultured hepatocytes was exchanged every 48 hr, whereas Tuttleman *et al.*(1986*b*) changed the media every 24 hr. The compounds that were tested for antiviral activity were added to the culture 2 days after plating and maintained at a constant level throughout the culture period.

Preparation and analysis of viral DNA. The total intracellular DNA was extracted from cells using the standard phenol extraction method (Maniatis *et al.*, 1982). The cells in a 6 cm diameter Petri dish (approximately 5×10^6 cells) were lysed (lysis buffer contained 0.2 % SDS, 150 mmol/l Tris-HCl pH 8.0, 10 mmol/l EDTA, 5 mmol/l EGTA and 150 mmol/l NaCl). The lysate was digested with 0.5 mg/mol of pronase E (Sigma at 37°C for 2 hr) and deproteinized with an equal volume of phenol saturated with 20 mmol/l Tris-HCl pH 7.5, 0.5 mmol/l EDTA and 0.1 % 8-hydroxyquinoline. Concentrated ammonium acetate pH 7.0 (2.5 mol/l) was added to the aqueous phase to yield a 0.25 mol/l ammonium acetate solution. The nucleic acids were precipitated with 2 vol of 100 % ethanol; the pellet was washed with ethanol and dried. The DNA was dissolved in a solution containing 12.5 mmol/l Tris-HCl pH 7.5, 10 mmol/l EDTA, 30 % glycerol and 0.01 % bromphenol blue. One-fifth of the DNA sample was loaded onto the agarose gel. Each lane had almost the same amount of DNA, which was checked by ethidium bromide stain after electrophoresis. A horizontal slab gel of 1.5 % agarose was used for DNA electrophoresis and 40 mmol/l Tris-acetate pH 8.0 containing 2 mmol/l EDTA was used as a running buffer. The gel was soaked on 0.1

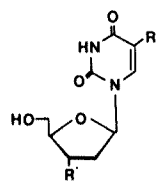
mol/l acetic acid for 30 min at 42°C, denatured with 0.2 mol/l NaOH-150 mmol/l NaCl, and neutralized. The DNA was transferred to nitrocellulose using the method of Southern (1975). Hybridization was performed at 42°C with a ^{32}P-labelled DNA probe prepared by labelling of the whole double strand DHBV genome (pDHO 10-DHBV) (kindly provided by Dr. J. Summers, Fox Chase Caner Center). Nick translation was performed according to the method of Rigby *et al.* (1977) using α-^{32}P dCTP (Amersham, Arlington Heights, Il, 3000 Ci/mmol). Autoradiography for 3-6 hr was done at -70°C with X-ray film (Eastman Co., Rochester, NY) with an intensifying screen. Extracellular virion DNA was extracted from the pooled culture media which were collected from 12-16 days after initial plating. The culture media were centrifuged at 16 000 xg for 10 min, and then layered onto a 10 to 20 % (w/v)sucrose gradient (4 ml) in 150 mmol/l NaCl-20 mmol/l Tris-HCl pH 7.5 and centrifugated at 113 000 xg for 16 hr at 4°C. The virus pellet was digested with 0.5 mg pronase E in the lysis buffer. The DNA samples were loaded directly onto the agrose gel and the DHBV DNA was examined by Southern blotting as described above.

Nucleoside analogues. The methods for synthesis of N_3dT, NH_2dT (Horwitz *et al.*, 1964), and N_3dU (Linn and Mancini, 1983) were reported previously. The synthesis of the OMeT will be described elsewhere. The nucleoside analogues were dissolved in 20 mmol/l Tris-HCl pH 7.5 and stock solutions were wrapped in tin foil to avoid light. Stock solutions were stored at -20°C used within 1 week of preparation. Chemical structures of these nucleoside analogues are shown in Fig.1.

Results

Effect of the nucleoside analogues on DHBV DNA replication

The effect of N_3dT on replication of viral DNA *in vitro* is shown in Fig. 2. Virus replication was evidenced by high viral DNA production from days 2 to 20 in absence of nucleoside analogues (lanes 1-4). In the presence of N_3dT (50 μmol/l), viral DNA replication was delayed and the amount of relaxed circular (RC) DNA, covalently closed circular (CCC) DNA, and single strand (SS) DNA was decreased (lanes 5 to 7, days 8, 14, and 20). At a concentration of 20 μmol/l, N_3dT the DHBV-DNA synthesis was inhibited less effectively (lanes 8-10, days 8, 14, and 20) although SS DNA was strongly inhibited at N_3dT concentrations above 20 μmol/l. Total viral DNA was measured by dot blot hybridization (data not shown). The dot spots on nitrocellulose filters were cut out and their radioactivity was counted. At 20 days after plating, 50 μmol/l of N_3dT inhibited the viral DNA synthesis by 88 % and 20 μmol/l of N_3dT inhibited the viral DNA synthesis by 75 %. Less than 10 % inhibition was observed when either N_3dU or



	N_3dT	N_3dU	NH_2dT	OMeT
R;	$-CH_3$	-H	$-CH_3$	$-CH_3$
R';	$-N_3$	$-N_3$	$-NH_2$	$-OCH_3$

Fig. 1
Structures and abbreviations of compounds tested for their ability to inhibit DHBV

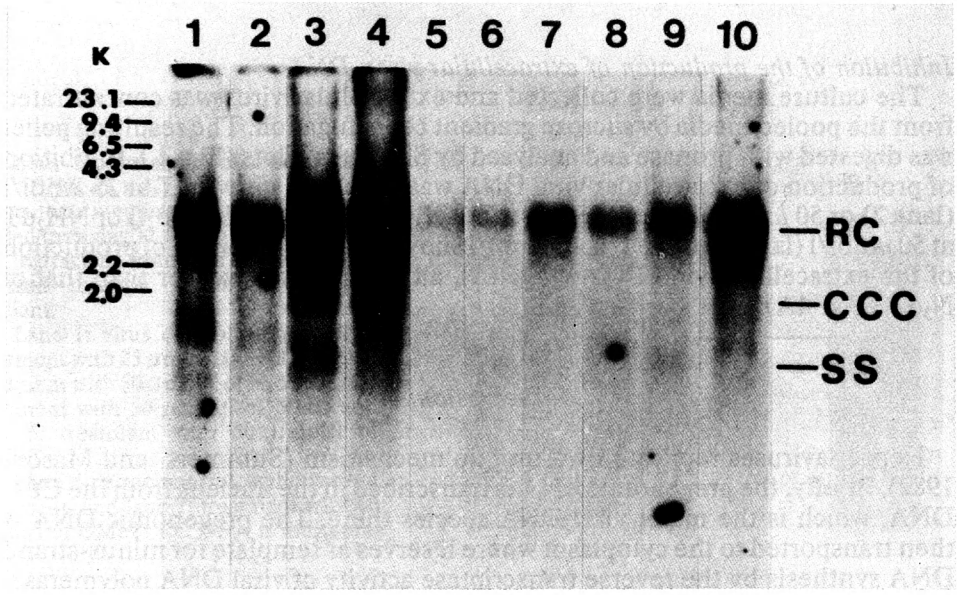


Fig. 2
Inhibition of rhe DHBV DNA replication by N_3dT. DNA was extracted at various times post-plating.
Drug was added to the culture at 2 days post-plating and the media containing the drug was changed every other day throughout culture period. DNA from one-fifth of a 6 cm-dish of cells was loaded onto 1.5 % agarose gel. Lanes 1 to 4: virus control (harvested at 2, 8, 14, and 20 days post-plating, lanes 1, 2, 3, and 4, respectively). Drug treated groups were harvested at 8, 14, and 20 days post-plating. Drug concentrations were 50 μmol/l (harvested at 8, 14, and 20 days post-plating, lanes 5, 6, and 7, respectively) and 20 μmol/l (harvested at 8, 14, and 20 days post-plating, lanes 8, 9, and 10, respectively). RC, CCC, and SS DNA of DHBV are indicated. Size markers were obtained from *Hind*-III digested λ DNA.

NH_2dT at 50 μmol/l was added. Southern blot analysis showed no difference between virus control group and N_3dU-nor NH_2dT-treated groups (data not shown). The effect of OMeT on the viral DNA replication is shown in Fig. 3. Lanes 1 to 5 represent uninhibited viral DNA production (days 2, 7, 12, and 16). Lanes 6 to 8 represent viral DNA production in the presence of 40 μmol/l OMeT (days 7, 12, and 16) and lanes 9 to 11 show viral DNA production in the presence of 4 μmol/l (days 7, 12, and 16). The antiviral effect of OMeT was weaker than that of N_3dT. It is noticeable, however, that the effect on the synthesis of SS DNA and CCC DNA was relatively weak as compared to the effect of N_3dT on these viral DNA intermediates. The relative amounts of RC to SS forms of viral DNA suggest that OMeT has similar effect in both RC and SS forms whereas N_3dT shows a stronger inhibitory effect on SS than RC forms. As judged by dot blot hybridization with the quantitation described above, OMeT inhibited the total viral DNA synthesis by 55 % at 40 μmol/l and 16 % at 4 μmol/l.

Inhibition of the production of extracellular viron DNA

The culture media were collected and extracellular virus was concentrated from the pooled media by sucrose gradient centrifugation. The resulting pellet was digested with pronase and analysed by Southern blots (Fig. 4.). Inhibition of production of extracellular viral DNA was observed with N_3dT at 25 μmol/l (lane 2) or 50 μmol/l (lane 3) but not with N_3dU at 50 μmol/l (lane 4) or NH_2dT at 50 μmol/l (lane 5). OMeT at 40 μmol/l showed some inhibition of production of the extracellular viral DNA (Fig. 4B), although it was weaker than that of N_3dT (Fig. 4A).

Discussion

Hepadnaviruses replicate by a unique mechanism (Summers and Mason, 1982). Briefly, the pregenomic RNA is transcribed in the nucleus from the CCC DNA, which is the major viral DNA species there. The pregenomic DNA is then transported to the cytoplasm where it serves as template for minus-strand DNA synthesis by the reverse transcriptase activity of viral DNA polymerase.

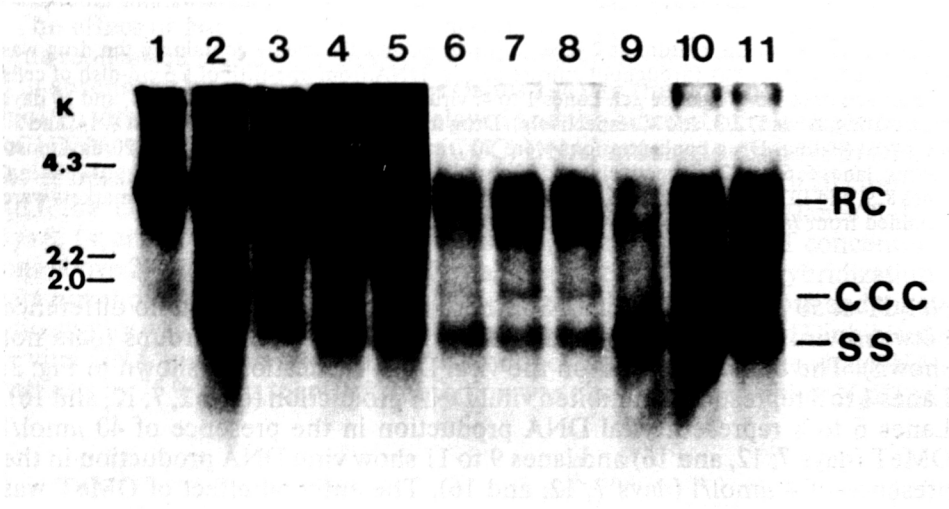


Fig. 3
Inhibition of DHBV DNA replication by OMeT
Drug treatment and gel conditions are the same as described in Fig. 2. Virus controls were harvested at 2, 7, 12, and 16 days post-plating (lanes 1, 2, 3, 4, and 5, respectively). Sample from the day 16 is duplicate (lanes 4 and 5). Drug treatment groups were harvested at 7, 12, and 16 days post-plating. Drug concentrations were 40 μmol/l (harvested at 7, 12, and 16 days, lanes 6, 7, and 8, respectively) and 4 μmol/l (harvested at 7, 12, and 16 days, lanes 9, 10, and 11, respectively). RC, CCC, and SS DHBV DNA size markers were obtained from *Hind*-III digested λ DNA.

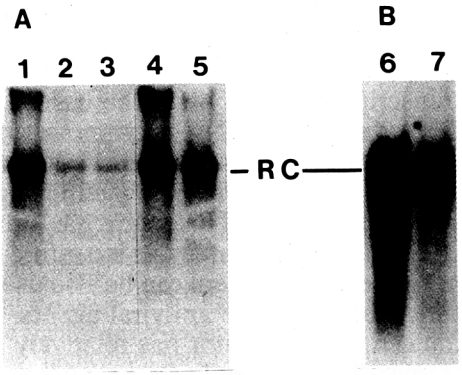


Fig. 4
Southern blot analysis of extracellular DNA on a 1.5 % agarose gel
The culture medium from each dish was pooled from day 12 to day 16. Viron DNA was extracted from the DHBV viron pelleted through a 10-20 % sucrose gradient.
(A) Lane 1: virus control without drug treatment with 25 μmol/l of N_3dT, lane 3: treatment with 50 μmol/l of N_3dT, lane 4: treatment with 50 μmol/l of N_3dU and lane 5: treatment with 50 μmol/l of NH_2dT.
(B) Lane 6: virus control without drug treatment, and lane 7: treatment with 40 μmol/l of OMeT. RC DNA is indicated.

The minus-strand DNA then acts as the template for the synthesis of plus-strand DNA. We have hypothesized that inhibitors of reverse transcriptase should inhibit the HBV DNA synthesis, using an *in vitro* system described here, we examined the ability of 3'-substituted thymidine analogues to inhibit DHBV replication. The N_3dT is a well known inhibitor of the reverse transcriptase of human immunodeficiency virus (HIV) (Mitsuya *et al.*, 1985). Analogues of N_3dT such as N_3dU, NH_2dT, and OMeT were synthesized and their ability to inhibit DHBV replication *in vitro* was examined. Several 3'-0-methyl nucleotide analogues have been reported to be chain terminators of the reverse transcriptase activity on avian myeloblastosis virus (Kutateladze *et al.*, 1986). These analogues failed to inhibit eukaryotic and DNA polymerases and terminal deoxynucleotidyl transferase (Kutateladze *et al.*, 1986). In the present study we found that N_3dT and OMeT showed some inhibitory effect on DHBV DNA replication. This finding was different from the result of Haritani *et al.* (1989), although the reason was still unknown. The concentration of N_3dT required to inhibit DHBV DNA was much higher than the concentration of N_3dT required to inhibit HIV replication (Mitsuya *et al.*, 1985). Since the activity of the N_3dT is dependet on the phosphorylation of the nucleoside to its 5'-triphosphate by cellular kinases (Furman *et al.*, 1986), low activity of thymidine kinase in liver cells compared to high thymidine kinase activity in T-lymphocyte may explain the relatively high concentrations of N_3dT required to inhibit DHBV replication. Another explanation is simply that N_3dT 5'-triphosphate has a lower binding affinity for DHBV DNA polymerase than HIV reverse transcriptase. The N_3dT effect on DHBV DNA synthesis was more noticeable on SS DNA which is synthesized by the reverse transcriptase activity of DHBV DNA polymerase. N_3dU and NH_2dT were not active in this system. It has been

reported that the triphosphate of NH_2dT was inactive against retroviral reverse transcriptase (Ono *et al.*, 1986). This suggests that DHBV DNA polymerase and reverse transcriptase might have similar recognition mechanisms for triphosphates of the N_3dT and NH_2dT.

It is noted that OMeT inhibited the RC DNA synthesis more effectively than SS DNA and CCC DNA synthesis. This result suggests that OMeT inhibited the viral DNA dependent DNA polymerase activity more than the reverse transcriptase activity. On the basis of these results, we believe that both the DNA dependent DNA polymerase and transcriptase activites of DHBV DNA polymerase have unique specificities for substrates and inhibitors. This observation may be important in attempting to design specific inhibitors of hepadnavirus replication. This study has shown that the *in vitro* culture system for DHBV in duck hepatocytes is useful for screening of antiviral agents for hepadnaviruses. The system can be also useful for determining of the mechanism of action of candidate antiviral agents. We found that N_3dT, a known reverse transcriptase inhibitor has relatively more selective activity against SS DNA synthesis than OMeT. Other inhibitors of reverse transcriptase may be effective inhibitors of hepadnaviruses replication.

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