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In Vitro and In Vivo Comparison of the Abilities of Purine and Pyrimidine 2′,3′-Dideoxynucleosides To Inhibit Duck Hepadnavirus

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Four purine and two pyrimidine 2′,3′-dideoxynucleosides were studied for their ability to inhibit duck hepadnavirus replication. The purine 2′,3′-dideoxynucleosides were more potent antiviral agents than the pyrimidine 2′,3′-dideoxynucleosides. The concentration for 50% inhibition of viral replication (IC_{50}) was determined for each of the effective agents. Two drugs with low IC_{50}s, 2,6-diaminopurine 2′,3′-dideoxyriboside and 2′,3′-dideoxyadenosine, were chosen for in vivo studies. Animals received 10 mg/kg intramuscularly twice daily. Rapid clearance of hepadnavirus DNA from the sera of the animals was seen as a result of treatment with 2,6-diaminopurine 2′,3′-dideoxyriboside; however, treatment with 2′,3′-dideoxyadenosine did not clear the virus.

Hepatitis B virus (HBV) is an extremely important human pathogen. Infection with HBV can result in severe forms of acute or chronic liver diseases. There are now an estimated 285×10^6 HBV carriers worldwide. Chronic infection with HBV has been associated with a high risk for the development of primary hepatocellular carcinoma (2, 4).

HBV is a member of the *Hepadnaviridae* family, of which several animal hepadnaviruses have been identified. These include woodchuck hepatitis virus (17), ground squirrel hepatitis virus (9), and duck hepatitis B virus (DHBV) (11). Human and animal hepadnaviruses share common biological features including the virion ultrastructure, genomic structure, and a unique mechanism of replication.

Effective antiviral therapy against HBV infection has not been developed. Therapeutic trials with adenine arabinoside or interferon have shown these agents to be relatively ineffective, and their use has been accompanied by considerable toxicity (1, 3, 16, 20, 21). However, prednisone withdrawal followed by treatment with recombinant alpha interferon has shown more promise in the treatment of chronic type B hepatitis (13). We chose the primary hepatocyte culture of DHBV (19) to screen for antiviral agents effective against hepadnaviruses. The antiviral activities of many nucleoside analogs were assayed. In this report, we compare the antiviral activity of the purine 2′,3′-dideoxynucleosides with that of the pyrimidine 2′,3′-dideoxynucleosides. We show that the purine 2′,3′-dideoxynucleosides block viral replication at very low concentrations, whereas the pyrimidine 2′,3′-dideoxynucleosides have little effect. One of these compounds, 2,6-diaminopurine 2′,3′-dideoxyriboside (ddDAPR), was very effective in vivo. These results suggest that some of the purine 2′,3′-dideoxynucleosides are promising chemotherapeutic agents against hepadnaviruses, including HBV.

MATERIALS AND METHODS

Nucleoside analogs. 2′,3′-Dideoxythymidine (ddThd) 2′,3′-dideoxyadenosine (ddAdo), 2′,3′-dideoxycytidine (ddCyd), 2′,3′-dideoxyguanosine (ddGuo), and 2′,3′-dideoxyinosine (ddIno) were purchased from Pharmacia, Inc. (Dorval, Quebec, Canada) or synthesized by Raylo Chemicals (Edmonton, Alberta, Canada). ddDAPR was synthesized as previously described (14). ddDAPR was provided in sufficient quantities for in vivo studies by W. Muhs of Raylo Chemicals. All nucleoside analogs were dissolved in 20 mM Tris hydrochloride buffer (pH 7.5).

Ducks. Fertilized Peking duck eggs were purchased from Macey Foods Ltd. (Brandon, Manitoba, Canada) or Sunshine Chick Hatchery and Supplies Ltd. (Edmonton, Alberta, Canada). Newly hatched ducklings were infected with a 50-μl intravenous injection of serum containing DHBV. Persistently infected animals were identified by detection of the presence of DHBV DNA in sera by dot hybridization.

Cell culture and drug treatment. Primary cultures of duck hepatocytes were prepared as previously described (19). Cells were cultured in 60-mm cell culture dishes in L-15 medium containing 5% fetal calf serum, penicillin G sodium (50 IU/ml), streptomycin sulfate (10 μg/ml), and nystatin (25 units/ml). Nucleoside analogs were added to the hepatocyte cultures on day 2 and maintained in culture with medium changes every second day.

In vivo drug treatment. Persistent DHBV infection was confirmed in 6-week-old ducklings by dot hybridization prior to treatment. Four ducklings in each treatment group were treated with ddAdo (10 mg/kg), ddDAPR (10 mg/kg), or placebo (20 mM Tris hydrochloride [pH 7.5], 1 ml/kg) twice daily by intramuscular injection. Sera from ducks were collected weekly and tested for the presence of DHBV DNA by dot hybridization.

Preparation of DNA from hepatocyte cultures. Preparation of cellular DNA was performed as previously described by Tuttleman et al. (18). In brief, cells were lysed with a buffer containing 0.2% sodium dodecyl sulfate, 100 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 5 mM EGTA, and 150 mM NaCl. The cell lysate was digested with 0.5 mg of pronase E (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 1.5 to 2 h and deproteinized by extraction with an equal volume of phenol saturated with 20 mM Tris hydrochloride (pH 7.5)–0.5 mM EDTA and 0.1% 8-hydroxyquinoline. Concentrated ammonium acetate (2 M, pH 7.0) was added to the aqueous phase to yield a final 0.2 M ammonium acetate solution, and the nucleic acids were precipitated with

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2 volumes of ethanol. The pellet of nucleic acid was washed with ethanol and dried. The DNA was dissolved in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE buffer).

Dot hybridization. Serum or DNA samples were loaded onto nylon (Hybond-N; Amersham Corp., Arlington Heights, Ill.) filters with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). DNA on the filter was denatured with 0.5 M NaOH–1.5 M NaCl at room temperature for 30 min and neutralized in 1 M Tris-hydrochloride–1.5 M NaCl (pH 8.0). The filters were exposed to UV light for 2 to 5 min prior to hybridization. Hybridization was performed as previously described (8). The filters were prehybridized for 4 to 16 h at 42°C in 50% formamide–5× SSPE (20× SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 [pH 7.4], 20 mM EDTA)–5× Denhardt solution (50× Denhardt solution is 10 g of Ficoll, 10 g of polyvinylpyrrolidone, 10 g of bovine serum albumin dissolved in water to a final volume of 1 liter)–0.1% sodium dodecyl sulfate–100 μg of denatured salmon sperm DNA per ml. Hybridization was initiated by adding DHBV [^{32}P]DNA probe at 10^6 cpm/ml under the same prehybridization conditions overnight. Filters were washed in 1× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate, pH 7.0)–0.1% sodium dodecyl sulfate at 65°C for 2 h and 1× SSC at room temperature for 30 min. Filters were dried and autoradiographied at −70°C with Kodak XAR film with an enhancer screen.

Radioactive probe. *Escherichia coli* containing plasmid pDH010-DHBV was a generous gift from J. W. Summers. The plasmid was purified from *E. coli* cultures with CsCl gradients as previously described (8). This is a full-length genomic probe. The ^{32}P-labeled DHBV probe was prepared by the nick-translation method with an Amersham nick translation kit.

Dose-response curves. DNA from hepatocyte cultures was extracted, and the amount of DHBV DNA present was determined by dot hybridization. The dots were cut out from the nylon filters, and the radioactivity of each was counted in a scintillation counter. Antiviral effect was expressed as a percentage of DHBV DNA in the treated cultures relative to that in the untreated control. The amount of DHBV DNA in untreated infected hepatocytes was considered to be 100%. Each point represents the mean of three determinations, and this experiment was repeated twice.

LDH and protein assay. The lactate dehydrogenase (LDH) assay was done as previously described (15) with minor modifications. Cells in a 60-mm plate were washed twice with a solution containing 0.25 M sucrose in 0.20 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Cells were then collected in 1 ml of the same solution containing 0.5 mM dithiothreitol. The samples were sonicated to obtain a homogeneous solution. Cellular extraction was diluted 100 times into 1 ml of cocktail solution of 1.4 mM sodium pyuvate, 0.34 mM NADH, and 0.18 M Tris hydrochloride (pH 7.4). The change in A_{340} over 1 to 2 min was recorded. The protein content of the cell was determined by the Lowry method (10).

RESULTS

In vitro antiviral activity assay. Our studies indicated that the purine 2′,3′-dideoxynucleosides inhibit DHBV replication in hepatocyte cultures, whereas the pyrmidine 2′,3′-dideoxynucleosides have little or no effect on viral replication. The results shown in Fig. 1A to C indicate that ddDAPR, ddGuo, and ddAdo were effective inhibitors of DHBV in hepatocyte cultures. ddIno was the least effective

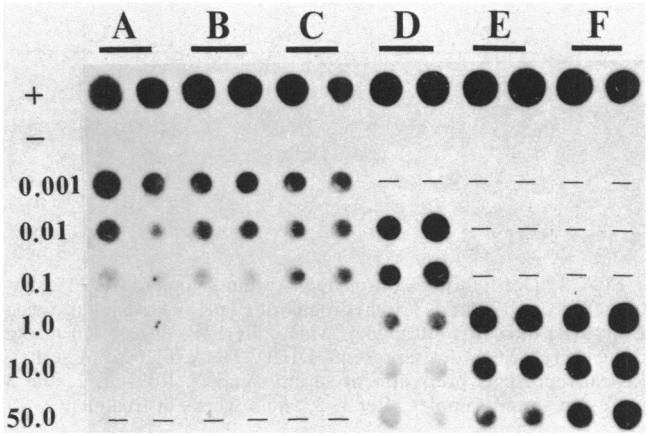

FIG. 1. Dot hybridization of DNA extracts from DHBV-infected hepatocyte cultures treated in duplicate with different concentrations of six types of 2′,3′-dideoxynucleosides. (A) ddDAPR; (B) ddGuo; (C) ddAdo; (D) ddIno; (E) ddCyd; (F) ddThd. Positive controls (+) and negative controls (−) were DNA extracts from DHBV-infected hepatocyte cultures and uninfected hepatocyte cultures, respectively. Drug concentrations shown in the left side were in micrograms per milliliter. All DNA extracts were taken from hepatocytes after 18 days in culture.

of the purine 2′,3′-dideoxynucleosides and failed to inhibit DHBV replication completely (Fig. 1D). The pyrmidine 2′,3′-dideoxynucleosides were relatively ineffective, failing to inhibit DHBV replication at 50 μg/ml (Fig. 1E and F). The inhibitory concentration for a 50% decrease in viral replication (IC_{50}) by 2′,3′-dideoxynucleosides was determined from the dose-response curves (Fig. 2). ddGuo and ddDAPR were the strongest inhibitors of DHBV replication, and both had an IC_{50} of 0.07 μg/ml. ddAdo also inhibited viral replication at low concentrations with an IC_{50} of 0.12 μg/ml. The IC_{50} for ddIno was 1.5 μg/ml. There was no inhibition of viral replication by ddThd or ddCyd at 10 μg/ml. ddCyd at 30 μg/ml showed some inhibition of DHBV DNA.

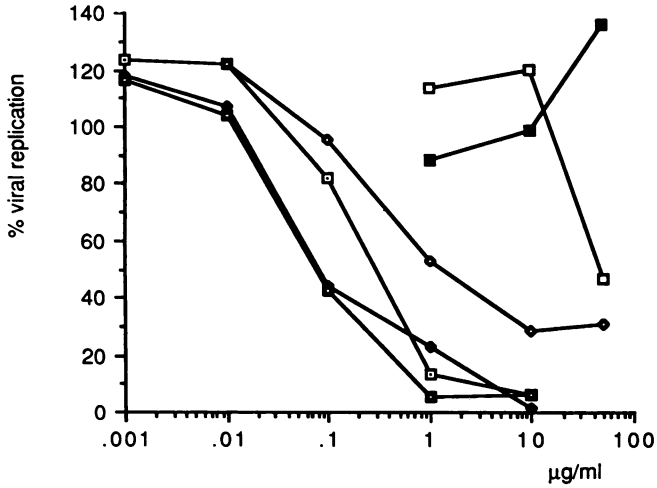

FIG. 2. Dose-response curves for dideoxynucleosides in hepatocyte cultures treated for 18 days. Each datum point represents the mean of duplicate samples. The viral replication is expressed as the amount of DHBV DNA in treated cells relative to that in untreated cells (100%). Symbols: ⊡, ddAdo; ◆, ddGuo; ■, ddDAPR; ◆, ddIno; ■, ddThd; □, ddCyd.

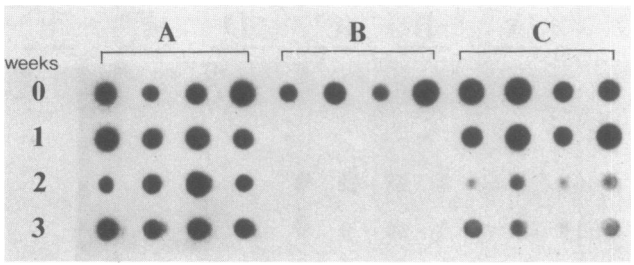

FIG. 3. Dot hybridizaton of duck serum samples. (A) Placebo-treated group (20 mM Tris hydrochloride [pH 7.5], 1 ml/kg twice daily); (B) ddDAPR (10 mg/kg twice daily); (C) ddAdo (10 mg/kg twice daily). Each dot represents DHBV DNA in 10 μl of serum. Row 0 represents pretreatment serum samples. Rows 1, 2, and 3 represent serum samples after 1, 2, and 3 weeks of treatment.

Effects of ddDAPR and ddAdo in vivo. Twelve 6-week old ducks were divided into three groups and were treated with ddDAPR (10 mg/kg twice a day), ddAdo (10 mg/kg twice a day), or placebo (20 mM Tris hydrochloride [pH 7.5], 1 ml/kg). Dot hybridization of the weekly serum samples is shown in Fig. 3. Persistent infection of the ducks is evident from the high level of DHBV DNA in the sera of all ducks prior to treatment. This remained unchanged in the placebo-treated group (Fig. 3A). Treatment with ddDAPR, the most effective anti-DHBV drug in vitro, resulted in a rapid clearance of the DHBV DNA from the sera of the treated animals. A small amount of DHBV DNA was present after 1 week of treatment; however, none was detected after week 2 of treatment (Fig. 3B). Treatment with ddAdo at the same concentration as ddDAPR was much less effective in clearing the sera of DHBV DNA. A slight decrease in the amount of DHBV DNA was observed after 2 weeks of treatment but remained unchanged after further treatment (Fig. 3C).

Cellular toxicity. Intracellular LDH activities were used as an indication of cell viability in cultures treated with drugs. The protein content from individual plates of treated and untreated cultures was similar. LDH activity (micromoles of NADH converted per minute per plate) per milligram of protein in the treated and untreated cells was compared. We were unable to detect significant cellular toxicity from treatment with 2′,3′-dideoxynucleosides at concentrations of 0.001 to 50 μg/ml. Cells were exposed to these concentrations for as long as 18 days in culture.

DISCUSSION

We studied 2′,3′-dideoxynucleoside analogs as potential antiviral agents against HBV. In the in vitro hepatocyte culture system, the purine 2′,3′-dideoxynucleosides, ddDAPR, ddGuo, ddAdo, and ddIno, showed much more potent inhibition of DHBV replication than did the pyrimidine 2′,3′-dideoxynucleosides. ddDAPR and ddGuo were the most effective (IC_{50} = 0.07 μg/ml), and ddIno was the least effective with an IC_{50} about 20 times higher than that of ddDAPR and ddGuo. Viral replication was still apparent with ddIno at a concentration of 50 μg/ml. The pyrimidine 2′,3′-dideoxynucleosides were 100- to 1,000-fold less active than the purine 2′,3′-dideoxynucleosides.

ddAdo and ddDAPR were chosen for the in vivo study based on the in vitro data and the limited availability of ddGuo. ddDAPR was shown to be a very effective anti DHBV agent in vivo. After 1 week of treatment with ddDAPR, the DHBV DNA in duck sera was dramatically decreased and was totally eliminated by week 2. ddDAPR is rapidly deaminated to ddGuo (12), which is a strong inhibitor of DHBV replication. In contrast, there was only a small decrease in DHBV DNA content in the sera of ducks after 1 week of treatment with ddAdo, and after 3 weeks of treatment with ddAdo, a low level of DHBV DNA persisted in the sera. The ineffectiveness of ddAdo in eliminating DHBV from the sera in the in vivo study is probably related to its rapid deamination to ddIno (12). Our in vitro studies show that ddIno is much less effective than ddAdo. ddAdo may be more effective in vitro than in vivo based on its slower deamination in cell cultures than in animals.

Intracellular LDH activity has been used as an index for cell viability (6). We used LDH activity as a means to detect cellular toxicity of the 2′,3′-dideoxynucleosides. Using this technique, we were unable to detect significant hepatocyte toxicity as a result of treatment with the different 2′,3′-dideoxynucleosides in the range of 0.001 to 50.0 μg/ml for as long as 18 days.

This study revealed an unexpected difference between purine and pyrimidine 2′,3′-dideoxynucleosides in their ability to inhibit DHBV replication. The mode of selective sensitivity of DHBV to the purine analogs is unknown. There are several theoretical possibilities for this selective inhibition. The cellular uptake and phosphorylation of these compounds may differ. However, this would be unlikely to produce the dramatic differences observed. The negative strand of DHBV-DNA is protein primed, and dGuo is the initial nucleotide attached to the primer protein (5, 7). The purine 2′,3′-dideoxynucleosides may substitute for this dGuo and block DNA synthesis. It is also possible that the purine 2′,3′-dideoxynucleosides interfere with assembly of the replicating core particles. With either of these latter two possibilities, the inhibitor would be specific for the virus and cause little interference with cellular DNA replication. Since HBV replicates by a similar mechanism (22), we believe that the purine 2′,3′-dideoxynucleosides have significant potential as antiviral agents against HBV.

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