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Inhibition of Duck Hepatitis B Virus Replication by 2′,3′-Dideoxy-3′-Fluoroguanosine In Vitro and In Vivo†

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The antiviral activity of 2′,3′-dideoxy-3′-fluoroguanosine (FdG) or its triphosphate was evaluated in the duck hepatitis B virus (DHBV) system in vitro and in vivo. In primary DHBV-infected hepatocytes FdG results in a dose-dependent inhibition of viral replication with a nearly complete inhibition at a concentration of 1 μM. Also in vivo, FdG treatment of DHBV-infected ducklings reduces DHBV DNA replication by more than 90%. These data demonstrate that FdG is a strong inhibitor of DHBV replication in vitro and in vivo.

Hepatitis B virus (HBV) causes acute and chronic hepatitis and is associated with a wide spectrum of clinical presentations, including an asymptomatic carrier state and the development of hepatocellular carcinoma (3). HBV belongs to a group of hepatotropic DNA viruses which further includes the hepatitis viruses of the woodchuck, ground squirrel, duck, and heron. The functional similarity of the HBV DNA polymerase to the retroviral reverse transcriptase has led to the examination of inhibitors of human immunodeficiency virus reverse transcriptase as inhibitors of HBV and duck HBV (DHBV) DNA polymerase. Most nucleoside analogs are directed preferentially against the viral polymerase as the key enzyme in the viral replication cycle. HBV and DHBV DNA polymerase can be differentially inhibited, e.g., by 3′-azido-2′,3′-ddTTP (14, 25, 34). Pyrimidine derivatives, such as TTP analogs (29) and sugar-modified 5-methyldeoxycytidines (22), were shown to inhibit HBV DNA polymerase completely in a cell-free system and partially in cell culture. 2′,3′-Dideoxycytidine has a potent inhibitory effect on viral replication in chronically DHBV-infected ducks (15). Purine 2′-3′-dideoxynucleosides inhibit with high selectivity the synthesis of DNA during replication of HBV and oncogenic RNA viruses (30). On the basis of the inhibition of HBV DNA polymerase activity in a cell-free system by 2′,3′-dideoxy-3′-fluoroguanosine (FdG) triphosphate (22) we investigated the antiviral activity of FdG in vitro and in vivo.

Primary duck hepatocytes and ducklings were used as in vitro and in vivo models of DHBV infection. FdG and FdG triphosphate were synthesized and purified as described elsewhere (16). Purity of FdG was more than 98% as determined by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

Primary duck hepatocytes were isolated from 10-day-old DHBV-infected ducks (27, 32). The effect of FdG on DHBV replication was analyzed in vitro by incubation of DHBV-infected primary duck hepatocytes with different concentrations of FdG. Culture media containing no or 0.01, 0.1, 1, or 10 μM FdG were changed daily. Incubation for 7 days with 0.1 μM FdG results in a 90% reduction of viral replication in hepatocytes (i.e., the 90% inhibitory concentration of FdG is 0.1 μM), whereas a 50% reduction was achieved with 0.05 μM (the IC_{50}) (Fig. 1A). By comparison, concentrations of 2′,3′-dideoxyguanosine (ddG) or 2′,3′-dideoxy-3′-thiacytidine (SddC) about 10 times higher (Fig. 1B) are required to achieve a 50 and 90% reduction of viral replication, as assessed by densitometric scanning of the autoradiograms (Pharmacia Image Master DTS). The effective concentration which caused a 50% reduction in cell number was more than 200 μM for FdG, ddG, and SddC, as could be demonstrated by viability testing

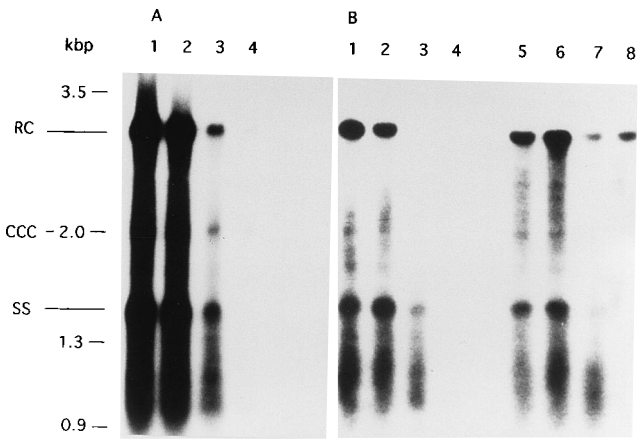

FIG. 1. (A) Effect of FdG on DHBV replication in vitro. Southern blot analysis of DNA isolated from primary duck hepatocytes after 9 days in culture was carried out as described elsewhere (25), using a ^{32}P-labelled, full-length DHBV DNA probe. Autoradiographic exposure time was 2 days at −80°C. Lane 1, primary hepatocytes isolated from DHBV DNA-positive ducks (control); lanes 2 to 4, primary hepatocytes isolated from DHBV DNA-positive ducks incubated with 0.01, 0.1, or 1.0 μM FdG, respectively. RC, relaxed circular DNA; CCC, covalently closed circular DNA; SS, full-length single-stranded DNA. (B) Effects of ddG and SddC on DHBV replication in vitro. Southern blot analysis was carried out as described for panel A. Lanes 1 and 5, primary hepatocytes isolated from DHBV DNA-positive ducks (control); lanes 2 to 4, primary hepatocytes isolated from DHBV DNA-positive ducks incubated with 0.1, 1.0, or 10 μM ddG, respectively; lanes 6 to 8, primary hepatocytes isolated from DHBV DNA-positive ducks incubated with 0.1, 1.0, or 10 μM SddC, respectively.

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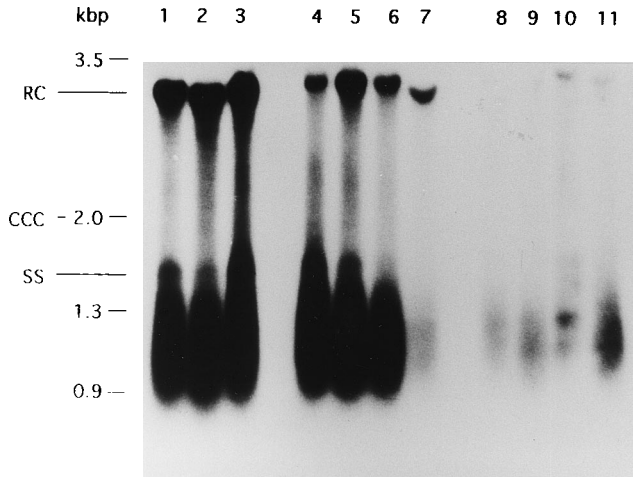

FIG. 2. Effect of FdG on DHBV replication in vivo after 9 days of treatment. Southern blot analysis was carried out as described in the legend to Fig. 1A. Lanes 1 to 3, three DHBV-positive ducklings (control); lanes 4 to 7, four DHBV-positive ducklings treated with 1 mg of FdG per kg of body weight; lanes 8 to 11, four DHBV-positive ducklings treated with 5 mg of FdG per kg of body weight. Autoradiographic exposure time was 2 days at −80°C. RC, relaxed circular DNA; CCC, covalently closed circular DNA; SS, full-length single-stranded DNA.

using the MTT assay (7) (Table 1) for the nonreplicating primary duck hepatocytes. In replicating cells as shown in B- and T-lymphocyte cell cultures, cellular DNA synthesis, as monitored by the incorporation of [*methyl*-^{3}H]deoxyribosylthymidine into DNA, was also not markedly affected by FdG at a concentration of 200 μM (2). Moreover, the concentration of the compound which caused a 50% reduction in mitochondrial DNA synthesis for FdG triphosphate was determined as described previously (5) and was about 2.0 μM, compared to 13 μM for ddG (31) and 47 μM for SddC (4, 6). Thus, at concentrations that completely inhibit DHBV replication, FdG had no effect on cell viability or mitochondrial DNA synthesis. Moreover, the antiviral effect of FdG was further analyzed in vivo in DHBV-infected ducks. One day after hatching, DHBV DNA-negative ducklings were infected by intravenous injection of 200 μl of DHBV-positive serum (~10^9 virions per ml), as described previously (26). The animals were given 1 or 5 mg of FdG per kg of body weight daily by intravenous injection. After 9 days the animals were sacrificed. DNA was isolated from liver and serum and analyzed by Southern blot hybridization. As shown in Fig. 2, the in vivo administration of FdG results in a dose-dependent inhibition of viral replication. At a dose of 1 mg of FdG per kg no significant reduction of DHBV DNA in liver could be observed (Fig. 2, lanes 4 to 7). A daily dose of 5 mg of FdG per kg, however, results in a 90% reduction of DHBV DNA replication in the liver (Fig. 2, lanes 8 to 11) and the sera of the ducklings (Fig. 3). No reduction in body weight, growth, and general health was observed in the treated animals. From the macroscopic aspect the duck livers revealed no alterations. Liver histologies were not performed, however, because of the short treatment period. At present, there are some promising compounds for chronic hepadnaviral infections. In clinical trials arabinoside-AMP, alpha interferon, 2′,3′-dideoxyinosine (12), and ribavirin (8), among others, were evaluated. Promising results were shown with alpha interferon, as demonstrated in 15 controlled studies (35). About 40 to 50% of the patients lost the HBV e antigen and HBV DNA within 12 months. SddC inhibits human immunodeficiency virus type 1 replication (13) and inhibits HBV replication in stably transfected Hep-G2.2.15 cells (10) by the (−) stereoisomeric form (4, 5). Moreover, in a randomized placebo-controlled multicenter trial, lamivudine suppressed HBV DNA in a dose-dependent fashion (9, 33). In Hep-G2.2.15 cells 2′,3′-dideoxyinosine, 3′-azido-2′,3′-dideoxythymidine, and ddG reduce viral replication, ddG being the most potent agent (1). The carbocyclic analog of 2′-deoxyguanosine was shown to be a strong inhibitor of HBV and DHBV replication (11, 28), with a long-lasting efficacy in vitro and in vivo (20). TTP analogs very effectively inhibit HBV DNA polymerase in vitro (24, 29). The modified pyrimidine nucleoside triphosphates 2′,3′-dideoxy-3′-fluorothymidine and 2′,3′-dideoxy-3′-fluoro-5-methylcytidine were shown to be very effective against hepadnaviral replication in Hep-G2.2.15 cells and ducks (23). Further, 2′,3′-dideoxycytidine inhibits DHBV replication in vivo (15). It is likely that these compounds act in their triphosphate

TABLE 1. Comparative potencies of FdG, ddG, and SddC as monitored by inhibition of DHBV replication and cytotoxicity

Compound	IC_{50} (μM)	EC_{50} (μM)[a]	SI[b]
FdG	0.05	>200	>4,000
ddG	0.5	>200	>400
SddC	0.5	>200	>400

[a] EC_{50}, drug concentration that inhibits cell growth by 50%.
[b] SI, selectivity index (EC_{50}/IC_{50}).

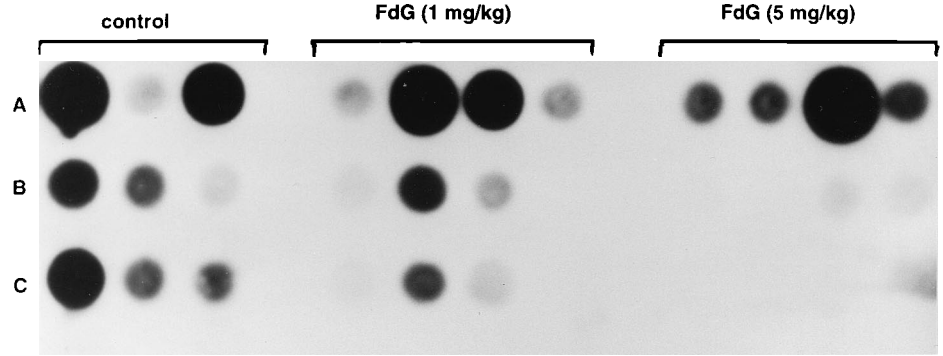

FIG. 3. Dot blot hybridization of duck sera before and after treatment with 1 and 5 mg of FdG per kg of body weight. Hybridization analysis was carried out as described in the legend to Fig. 1A. (A) Before treatment; (B) after 6 days of treatment; (C) after 9 days of treatment. The order of sera corresponds to the order of animals in Fig. 2.

forms as specific competitive inhibitors of HBV or DHBV polymerase (21, 24). In addition, purine 2′,3′-dideoxynucleosides, e.g., 2,6-diaminopurine 2′,3′-dideoxyriboside and 2′,3′-dideoxyadenosine, are also potent inhibitors of DHBV replication in vitro and in vivo (17, 30). In our analysis FdG was almost 10 times more potent than ddG and SddC in vitro and as effective as 2,6-diaminopurine 2′,3′-dideoxyriboside, 2′,3′-dideoxyadenosine, and 2′,3′-dideoxyinosine in vivo compared with published data (17–19, 30). In conclusion, FdG has a high selectivity index for inhibition of DHBV replication in vitro (>4,000). The concentration at which it causes a 50% reduction in mitochondrial DNA synthesis is about 40 times higher than the DHBV IC_{50}. Moreover, this novel compound demonstrates also an inhibitory effect in vivo. Therefore, FdG is a new compound with anti-DHBV activities comparable to those of other established nucleoside analogs.

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