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2′,3′-Dideoxy-β-L-5-Fluorocytidine Inhibits Duck Hepatitis B Virus Reverse Transcription and Suppresses Viral DNA Synthesis in Hepatocytes, both In Vitro and In Vivo

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β-L-Nucleoside analogs represent a new class of potent antiviral agents with low cytotoxicity which provide new hope in the therapy of chronic hepatitis B virus (HBV) infections. We evaluated the anti-HBV activity of 2′,3′-dideoxy-β-L-5-fluorocytidine (β-L-F-ddC), a β-L-nucleoside analog derived from 2′,3′-dideoxycytidine (ddC), in the duck HBV (DHBV) model. This compound was previously shown to inhibit HBV DNA synthesis in a stably transfected hepatoma cell line (F2215). Using a cell-free system for the expression of an enzymatically active DHBV polymerase, we could demonstrate that the triphosphate form of β-L-F-ddC does inhibit hepadnavirus reverse transcription. In primary duck hepatocyte culture, β-L-F-ddC showed a potent inhibitory effect on DHBV DNA synthesis which was concentration dependent. Although β-L-F-ddC was shown to be less active than ddC against the DHBV reverse transcriptase in vitro, β-L-F-ddC was a stronger inhibitor in hepatocytes. The oral administration of β-L-F-ddC in experimentally infected ducklings showed that β-L-F-ddC is a potent inhibitor of viral replication in vivo. Short-term therapy could not prevent a rebound of viral replication after the drug was withdrawn. Preventive therapy with β-L-F-ddC could delay the onset of viremia by only 1 day compared with the time to the onset of viremia in the control group. The in vivo inhibitory effect of β-L-F-ddC was much stronger than that of ddC and was not associated with signs of toxicity. Our data show that β-L-F-ddC inhibits hepadnavirus reverse transcription and is a strong inhibitor of viral replication both in vitro and in vivo.

Antiviral therapy of chronic hepatitis B virus (HBV) infection still remains a problem since several clinical trials have shown that a sustained response to interferon or to nucleoside analogs is observed in only 30 to 40% of the patients selected for study (15, 18, 29). This response rate is even lower in long-term HBV carriers and in immunocompromised patients. The design of new protocols for chemotherapy to eliminate HBV is needed since the majority of the patients will not clear the viral infection and will be at greatest risk of developing progressive liver disease and hepatocellular carcinoma (19).

The development of a new class of nucleoside analogs with an unusual L-configuration has provided new hope in the field of anti-HBV research (1, 21). For example, L-nucleoside analogs derived from 2′,3′-dideoxycytidine (ddC) such as (−)-2′,3′-dideoxy-3′-thiacytidine (3TC; Lamivudine), (−)-*cis*-5-fluoro-1 [(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (FTC), and 2′,3′-dideoxy-β-L-5-fluorocytidine (β-L-F-ddC) have been shown to be resistant to cytidine deaminase, to have a high affinity for deoxycytidine kinase, and to have low toxicity, especially toward mitochondrial DNA. It was shown that these different compounds have a potent and selective in vitro anti-HBV activity in hepatoma cell lines which permanently replicate HBV (1, 3, 4, 13, 14, 25). FTC was also shown to strongly inhibit viral replication in vivo in the duck HBV (DHBV) infection model (5). In view of the potent antiviral activity of β-L-F-ddC against HBV and its low cytotoxicity, and especially in view of its negligible inhibitory effect on host mitochondrial DNA synthesis (13, 14), β-L-F-ddC merits further development as a potential anti-HBV agent.

Recent advances in the knowledge of the hepadnavirus reverse transcription machinery have been made and may also be of help in the development of new anti-HBV strategies (20). It was indeed demonstrated that the polymerase of the DHBV synthesized in an in vitro translation reaction exhibits reverse transcriptase activity and that the viral polymerase acts as a primer for DNA synthesis (27, 28). Viral DNA synthesis is initiated by the formation of a covalent bond between the polymerase and nascent viral minus-strand DNA, which is extended in a template-dependent manner (27, 28). This in vitro model for the detailed study of reverse transcription in hepadnaviruses provides a new tool for the characterization of the mechanism of action of new anti-HBV polymerase inhibitors (28, 30). In addition, the primary hepatocyte culture system (6, 23) provides another study model that is complementary to permanently transfected cell lines and that can be used both to evaluate the antiviral activity of new compounds and to gain new insights into the mechanisms of action of these drugs. Finally, studies in the in vivo animal models of HBV infection, i.e., the woodchuck and the duck (7, 16, 17), are useful for confirming the anti-HBV activities of drugs which are active in vitro and testing their toxicity.

These new findings led us to examine in detail the anti-HBV activity of β-L-F-ddC in comparison with that of ddC in the DHBV infection model. Using a new in vitro assay for the expression of an enzymatically active DHBV polymerase, we could demonstrate that β-L-F-ddC is indeed an inhibitor of the RNA-directed DNA synthesis. We also provide evidence that β-L-F-ddC is a strong inhibitor of viral DNA synthesis both in

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primary duck hepatocytes and in vivo in experimentally infected ducklings.

MATERIALS AND METHODS

Drugs. β-L-F-ddC and its triphosphate form (β-L-F-ddC-TP) were synthesized at the Department of Pharmacology, Yale University School of Medicine (13, 14). ddC was purchased from Sigma, and the triphosphate form of ddC (ddC-TP) was obtained from Boehringer Mannheim. 3TC-triphosphate (3TC-TP) was a generous gift from J. Cameron and J. Kitson (Glaxo Research, Stevenage, United Kingdom).

An in vitro assay for the expression of enzymatically active DHBV reverse transcriptase. Plasmid pHP contains the DHBV polymerase gene under the control of the Sp6 promoter and the sequence coding for the RNA template of reverse transcription, as described previously (28). In addition, the polymerase gene was expressed together with a plasmid (pMscI-SL) which contains important sequences for reverse transcription, i.e., the hairpin structure of the encapsidation signal, DR1, and the template for the synthesis of the first 162 nucleotides of minus-strand DNA. When provided in *trans* during polymerase gene translation, this RNA sequence stimulates the enzymatic activity of the DHBV reverse transcriptase (26, 31). The polymerase gene was transcribed and translated in a coupled transcription-translation rabbit reticulocyte lysate system (TNT coupled reticulocyte system; Promega) according to the manufacturer's instructions. The reverse transcriptase reaction was performed as described previously (27, 28) after incubation of the translation mixture at 30°C for 30 min with an equal volume of a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 30 mM NaCl, 20 mM $MgCl_2$, deoxynucleoside triphosphate (dNTP)-C (200 μM), and [α-^{32}P]dCTP (3,000 Ci/mmol, 0.30 μM). Inhibitors were added at the indicated concentrations. Radiolabelled viral DNA covalently attached to polymerase was analyzed through 0.1% sodium dodecyl sulfate (SDS)–10% polyacrylamide gels as described previously (28). This provided a qualitative assay for the analysis of viral DNA. A quantitative dot assay was performed after spotting the reverse transcriptase assay reaction mixture onto a DE-81 filter (Whatman). The paper was then washed at room temperature three times in 300 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then twice in 250 ml of 95% ethanol as described earlier for the Moloney murine leukemia virus reverse transcriptase (9). Radioactivity was measured in a Beckman LS 6000SC scintillation counter.

Primary hepatocyte cultures. Primary hepatocyte cultures were prepared from 4-week-old Pekin ducks chronically infected with DHBV. The procedures of liver perfusion and hepatocyte isolation and the culture conditions were described previously (8, 23). Hepatocytes were seeded at confluence onto 35-mm-diameter petri dishes, and the serum-free growth medium was changed daily. The addition of drugs to the culture medium was carried out from day 3 to day 8 postseeding.

Experimental inoculations of ducklings. Five-day-old ducklings were inoculated intravenously with a DHBV-positive serum sample known to be infectious, and each duckling received 1.5×10^7 viral genome equivalent by a protocol described previously (11). The ducklings were administered nucleoside analogs orally either before the experimental inoculation (preventive therapy) or 3 days postinoculation (therapeutic regimen) according to the protocols described in Results.

Analysis of viral DNA. DHBV DNAs from experimentally infected ducklings and from hepatocyte culture supernatants were detected by a DNA spot hybridization assay at different time points, as indicated in the figure legends. Fifty microliters of serum or 800 μl of culture supernatant was spotted directly onto nitrocellulose filters. After denaturation and neutralization, the filters were hybridized with a full-length DHBV genomic DNA probe labelled with ^{32}P. The filters were autoradiographed, and the spots were counted in a scintillation counter.

Hepatocytes were rinsed with phosphate-buffered saline and were stored at −80°C for DNA isolation. Intracellular viral covalently closed circular (CCC) DNA (non-protein-bound DNA) and replicative intermediates (protein-bound DNA) were isolated as described by Summers et al. (22). Viral DNA (corresponding to 1/10 of a plate, i.e., 0.5 μg of cellular DNA) was analyzed by electrophoresis through 1% agarose gels, transferred by blotting to nylon membranes (Hybond N$^+$; Amersham), and hybridized with a full-length DHBV genomic DNA probe labelled with ^{32}P.

RESULTS

β-L-F-ddC-TP inhibits viral minus-strand DNA synthesis. Using an in vitro assay for the expression of an enzymatically active DHBV reverse transcriptase, we have analyzed the inhibitory effects of the triphosphate forms of ddC derivatives on the synthesis of viral minus-strand DNA. Viral DNA synthesis was analyzed by the incorporation of dNTPs (dGTP, dATP, and TTP) and radiolabelled [α-^{32}P]dCTP. Nascent viral DNA covalently linked to the DHBV polymerase was analyzed qualitatively through 0.1% SDS–10% polyacrylamide gels and quantitatively by a dot assay on DE-81 filters. The incorporation of [α-^{32}P]dCTP in the presence of increasing concentrations of ddC-TP, β-L-F-ddC-TP, and 3TC-TP was reproducibly inhibited. At concentrations higher than 50 μM ddC-TP, β-L-F-ddC-TP, or 3TC-TP, the inhibition was almost complete (>75%). The 50% inhibitory concentrations of ddC-TP, β-L-F-ddC-TP, and 3TC-TP were 4.2 ± 2.1 μM (standard deviation), 21.7 ± 5.5 μM, and 6.2 ± 2.5 μM, respectively. The results of a typical experiment are shown in Fig. 1.

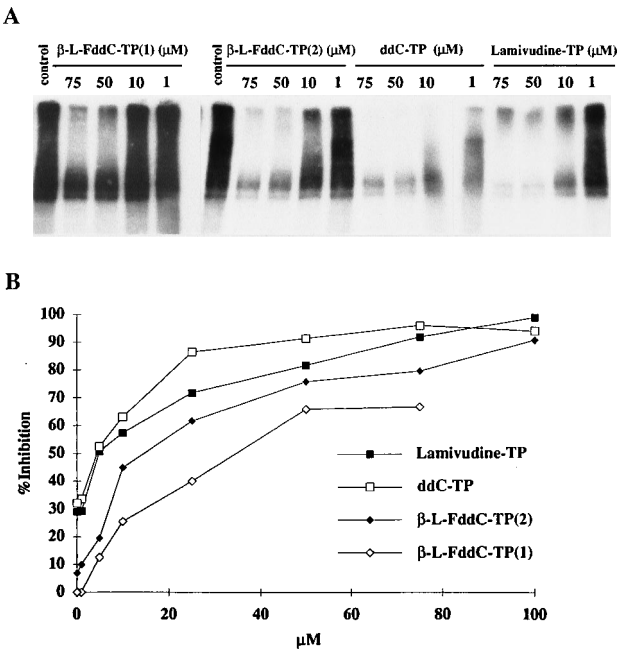

FIG. 1. β-L-F-ddC-TP inhibits viral minus-strand DNA synthesis by the DHBV polymerase. The DHBV polymerase was incubated in the presence of radiolabelled [α-^{32}P]dCTP and cold deoxynucleotides together with nucleotide analogs at the indicated concentrations, as described in Materials and Methods. Viral minus-strand DNA synthesis was analyzed through 0.1% SDS-10% polyacrylamide gels (A) and quantitatively by a dot assay (B). The inhibitory effects of ddC-TP and 3TC-TP (Lamivudine-TP) were assessed in the presence of 0.15 μM [α-^{32}P]dCTP (final concentration), and that of β-L-F-ddC-TP was assessed in the presence of 0.15 [β-L-F-ddC-TP (2)] or 0.75 [β-L-F-ddC-TP (1)] μM [α-^{32}P]dCTP (final concentrations), respectively.

Then, we examined whether β-L-F-ddC-TP is a competitive inhibitor of dCTP incorporation. The same experiment described above was performed with different concentrations of radiolabelled [α-^{32}P]dCTP (final concentrations, 0.15 and 0.75 μM). When the concentration of [α-^{32}P]dCTP was increased from 0.15 to 0.75 μM, the 50% inhibitory concentration of β-L-F-ddC-TP shifted from 14.6 to 34.7 μM (Fig. 1).

Moreover, we could show that β-L-F-ddC-TP does not inhibit the incorporation of the first nucleotide of viral minus-strand DNA, dGMP (priming reaction), but does inhibit the incorporation of the following dGMPs (3′ from the short viral DNA primer) (data not shown).

β-L-F-ddC inhibits viral DNA synthesis in primary duck hepatocyte cultures. The inhibitory effect of β-L-F-ddC was then studied in primary hepatocyte cultures from chronically infected ducklings. Different concentrations of ddC and β-L-F-ddC were studied in parallel. Three days after plating, hepatocytes were incubated with nucleoside analogs which were renewed on a daily basis for 6 consecutive days. Quantification of the virion DNA released into the culture supernatant showed a daily increase in the control culture (Fig. 2A), as

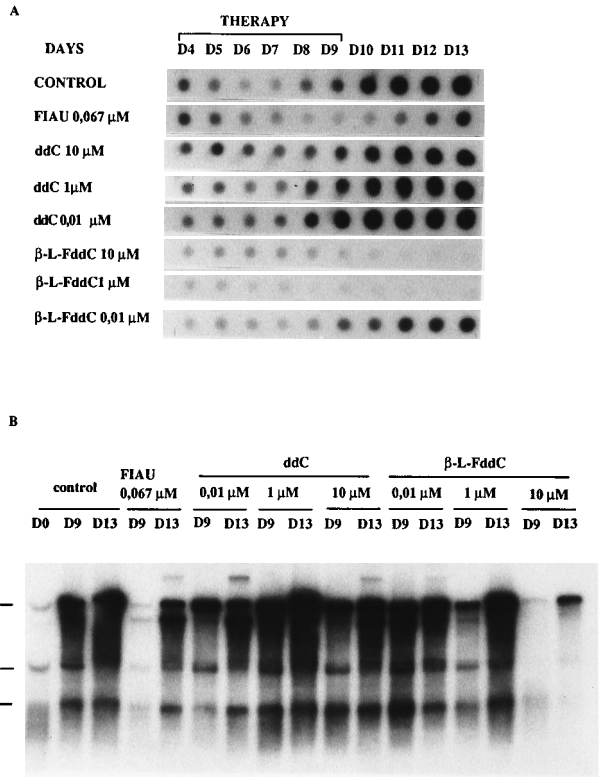

FIG. 2. β-L-F-ddC inhibits DHBV DNA synthesis in DHBV-infected primary duck hepatocytes. β-L-F-ddC and ddC were added at the indicated concentrations 3 days after plating for 6 days. Supernatants of tissue culture were analyzed daily for virion DNA by a dot blot assay (A). Cells were harvested at the end of therapy (day 9) and 4 days after drug release (day 13). Total DNA was extracted and subjected to Southern blot analysis (B). The positions of relaxed circular (RC), CCC, and SS DNAs are indicated. The results of a typical experiment are shown. FIAU, fialuridine.

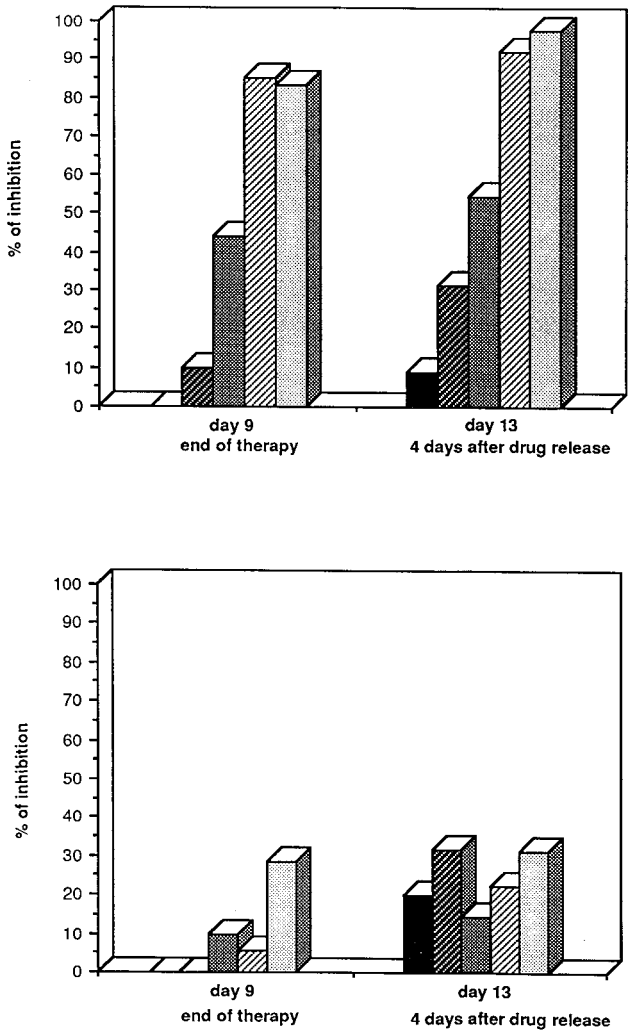

FIG. 3. Inhibitory effect of β-L-F-ddC on virion release in primary duck hepatocytes. Cultures were treated 3 days after plating for 6 days with β-L-F-ddC (top panel) and ddC (bottom panel) at the indicated concentrations. The viral DNA released in the supernatant of primary hepatocyte culture was analyzed by a dot blot assay. Results are expressed as a mean percentage of inhibition compared with that in the control culture. Numbers represent mean values for six individual plates in two independent experiments. ■, 0.01 μM; ▨, 0.1 μM; ▩, 1 μM; ▨, 10 μM; ▩, 100 μM.

described previously (6, 11). We reproducibly observed a significant inhibitory effect of β-L-F-ddC which was concentration dependent. At a concentration of 1 μM, the release of virion DNA was significantly inhibited (>80% inhibition). This inhibitory effect reached a plateau for concentrations of 10 μM (Fig. 3). This level of inhibition could not be reached by ddC even at high concentrations (100 μM). The overall results are summarized in Fig. 3, and data from one typical experiment are shown in Fig. 2A.

Southern blot analysis of intracellular viral DNA showed at the end of the treatment schedule a decrease in the amount of replicative intermediates induced by β-L-F-ddC (Fig. 2B). At a concentration of 10 μM or more, β-L-F-ddC induced a dramatic inhibition of viral DNA synthesis, as shown by the dramatic decrease in the amount of viral single-stranded (SS) DNA (Fig. 2B). However, viral CCC DNA was still detected at the end of therapy as a faint band (Fig. 2B). ddC treatment at concentrations up to 10 μM did not induce any significant reduction of viral SS DNA synthesis.

No significant signs of cytotoxicity were observed on the daily microscopic examination of cultured cells, supernatant testing for transaminase, and cellular DNA examination on agarose gels.

β-L-F-ddC has a long-lasting inhibitory effect in hepatocytes. Results of analysis of extracellular viral DNA after drug release are depicted in Fig. 2 and 3. In the control cultures, the release of virion DNA increased daily, as shown in Fig. 2. In ddC-treated cultures, a very low level of inhibition could be observed after drug withdrawal (Fig. 3). In contrast, after β-L-F-ddC was withdrawn, we observed an inefficient rebound of viral replication compared with that in the control cultures, and the strong inhibition of viral replication (>80% inhibition) lasted even after drug release (Fig. 3). This particular pattern was observed when β-L-F-ddC was administered at concentrations of 10 and 100 μM. Southern blot analysis of intracellular viral DNA showed an increase in replicative intermediates after drug withdrawal. When β-L-F-ddC was previously given at a concentration of 10 μM or more, no rebound of viral DNA synthesis (i.e., reappearance of viral SS DNA) could be detected 4 days after drug withdrawal (Fig. 2).

β-L-F-ddC is a potent inhibitor of DHBV replication in vivo. Ducklings at 5 days of age were inoculated following a protocol which has been described previously (11). DHBV viremia was reproducibly detectable at days 3 to 4 postinoculation and reached a peak at days 5 to 6. This experimental model allowed

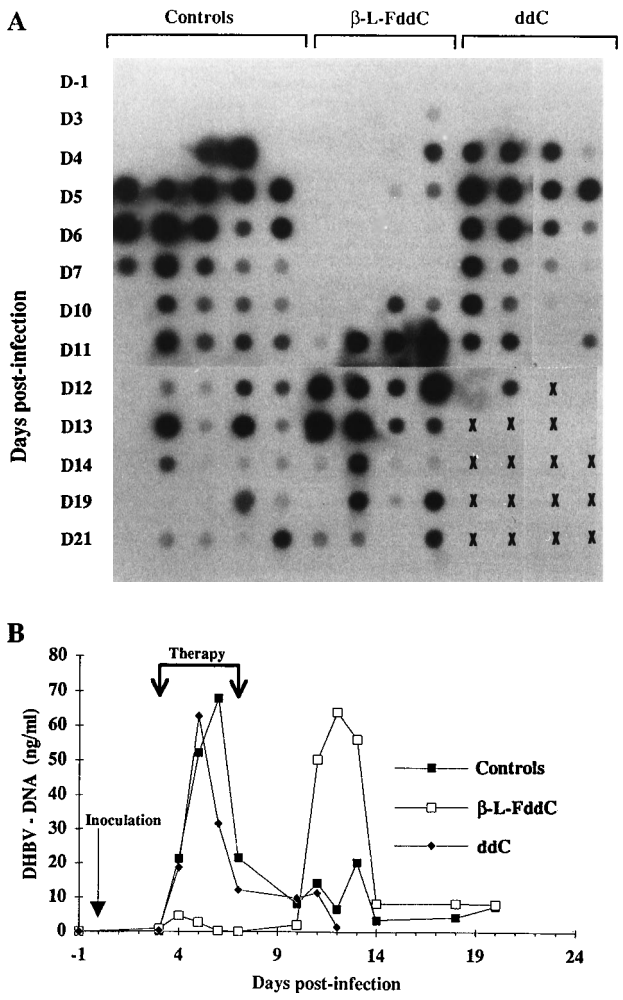

FIG. 4. Inhibition of DHBV replication in experimentally infected ducklings by β-L-F-ddC. Ducklings at 5 days of age were inoculated with a DHBV-positive serum sample. Drugs were given orally at day 3 postinoculation for 5 days at a dosage of 50 mg/kg twice a day. Five animals were not treated and served as a control group. Four animals received β-L-F-ddC, and 4 animals received ddC. Viral DNA levels in serum were analyzed by a dot blot assay (A). The mean level of viral DNA in each group of animals is plotted in panel B.

us to study the inhibitory effects of ddC and β-L-F-ddC on DHBV replication in vivo.

In the first experiment, drugs were given orally at day 4 postinoculation for 4 days at a dosage of 25 mg/kg of body weight twice a day. Four ducklings were untreated and served as a control group, three animals received ddC, and four animals were given β-L-F-ddC. In the group of ddC-treated animals, we did not observe any decrease in viremia compared with the level of viremia in the control group. In the β-L-F-ddC-treated group, the mean peak of viremia was decreased by 62% (data not shown).

In a second experiment, drugs were given orally at day 3 postinoculation for 5 days at a dosage of 50 mg/kg twice a day (Fig. 4A and B). This dosage was chosen since previous experiments showed that administration of β-L-F-ddC at a dosage of 100 mg/kg/day for 30 days is not toxic in mice (2a). This experiment confirmed that β-L-F-ddC is a very potent inhibitor of viral replication in vivo, since a dramatic and almost complete inhibition of viremia (97% inhibition) was observed in each of the four treated animals. However, a relapse of viral replication was observed in all animals 4 days after β-L-F-ddC withdrawal. In the group of four ddC-treated ducklings, viral replication was not significantly inhibited. Signs of toxicity were observed in all ddC-treated animals, since all of them died during the week after the drug was withdrawn (at days 12, 13, and 14 postinoculation).

Preventive treatment with β-L-F-ddC in vivo. We then examined whether a pulse therapy before the experimental inoculation could prevent or delay the appearance of viremia in vivo. Preventive therapy with β-L-F-ddC or ddC could delay the onset of viremia by only 1 or 2 days, respectively, compared with the time of onset in the control group (data not shown).

DISCUSSION

Antiviral therapy of chronic HBV infection remains an important clinical problem because of the lack of efficacy of the currently available treatments. New nucleoside analogs which belong to the L-nucleoside family appear to be promising compounds for the therapy of these viral infections. It was recently shown that β-L-F-ddC has an inhibitory effect on HBV DNA synthesis in a hepatoma cell line carrying the HBV genome (F2215) (13, 14). However it remains to be established which stage of the viral replication cycle is inhibited by β-L-F-ddC. Using a cell-free system for the expression of an enzymatically active DHBV polymerase (26–28, 30), we now provide direct evidence that β-L-F-ddC-TP does inhibit hepadnavirus reverse transcription, i.e., elongation of viral minus-strand DNA. Our data also suggest that β-L-F-ddC-TP is likely to be a competitive inhibitor of dCTP incorporation into the nascent viral minus-strand DNA (Fig. 1). These observations are in agreement with the inhibitory effect of β-L-F-ddC on the human immunodeficiency virus reverse transcriptase observed in tissue culture cells (13, 14, 25). Interestingly, the inhibitory effect of β-L-F-ddC-TP on the DHBV reverse transcriptase was weaker than that of ddC-TP and 3TC-TP, two other potent inhibitors of the human immunodeficiency virus reverse transcriptase (Fig. 1). Although the precise mechanism of action of β-L-F-ddC-TP could not be determined definitely, the inhibition of dGMP incorporation in viral minus-strand DNA suggests that it acts as a terminator of DNA synthesis (data not shown).

The problem with short-term treatments with most nucleoside analogs, either in vitro or in vivo, is that they induce an inhibition of viral replication which is rapidly followed by a rebound of viral DNA synthesis after the drug is withdrawn (7, 8, 10, 24). In a primary duck hepatocyte culture chronically infected with DHBV, β-L-F-ddC showed a potent inhibitory effect on DHBV DNA synthesis which was concentration dependent. Although β-L-F-ddC-TP was shown to have a lower level of activity against the DHBV reverse transcriptase in vitro compared with that of ddC-TP, β-L-F-ddC showed a much stronger inhibition than ddC in infected hepatocytes (Fig. 3). These results suggest that β-L-F-ddC, an L-nucleoside analog derived from ddC, has a different intracellular metabolism. β-L-F-ddC may be more resistant to cytidine deaminase or may have a higher affinity for deoxycytidine kinase, which in turn may explain the better inhibitory effect of β-L-F-ddC in hepatocytes compared with that of ddC. A specific study of the intracellular metabolism of both compounds is required to confirm this hypothesis. Interestingly, we observed no rebound of DHBV replication after 4 days of drug release when β-L-F-ddC was used at a concentration of 10 or 100 μM (Fig. 2). This observation suggests that once it is phosphorylated, β-L-F-ddC may have a long intracellular half-life. Furthermore, no significant signs of cytotoxicity were observed in β-L-F-ddC-treated

primary hepatocytes, in agreement with the lack of an inhibitory effect of this compound on cell growth and mitochondrial DNA synthesis in dividing cell lines (13, 14).

The in vivo administration of β-L-F-ddC in experimentally infected ducklings confirmed our in vitro results and showed that β-L-F-ddC is a very potent inhibitor of viral replication. The level of inhibition was dose dependent, and short-term therapy could not prevent a rebound of viral replication after the drug was withdrawn (Fig. 4). This rebound appeared early (4 days) after the end of therapy and may suggest that the half-life of β-L-F-ddC in vivo is shorter than that in vitro (in hepatocyte culture). This could explain why our attempt at preventive therapy was not successful. Indeed, preventive therapy with β-L-F-ddC could delay the onset of viremia by only 1 day compared with the time of onset in the control group. The in vivo inhibitory effect of β-L-F-ddC was much stronger than that of ddC, confirming data obtained from the study of primary hepatocyte cultures. Furthermore, the administration of ddC at high dosages (100 mg/kg/day) did not induce a significant decrease in the level of viremia, as already reported by others (12), but signs of toxicity were observed, since all animals died during the week following the end of therapy. It will be interesting to see whether liver toxicity and mitochondrial dysfunction were associated with this high dose of ddC (2, 21). Another interesting issue will be to compare, in vivo, the antiviral activity of β-L-F-ddC with that of 3TC, another L-nucleoside analog derived from ddC.

A major difficulty in treating chronic HBV infection comes from the particular mechanism by which HBV maintains the infection at the level of the individual hepatocyte. Studies that have used the in vitro and in vivo model systems of hepadnavirus replication have shown that chronic infection of hepatocytes is maintained by the presence in the nucleus of 30 to 40 copies of CCC DNA (20, 22). Viral CCC DNA is the template for viral transcription, and its synthesis can be blocked by a reverse transcriptase inhibitor. However, short-term therapy cannot deplete the CCC DNA pool, which is the source of viral production after the therapy is stopped (20). Accordingly, short-term therapy with β-L-F-ddC in primary hepatocyte culture could not eliminate viral CCC DNA (Fig. 2), and this could be the reason for the rapid rebound of viral replication which was observed in vivo after drug withdrawal (Fig. 4). Long-term therapy with a potent and nontoxic drug such as β-L-F-ddC may deplete the pool of intracellular viral CCC DNA and subsequently decrease the viral load and eliminate HBV infection, as already suggested by Mason and colleagues (5, 8, 16, 20). Such experiments in chronically infected animals are required to confirm this hypothesis.

In conclusion, we have provided evidence that β-L-F-ddC inhibits hepadnavirus reverse transcription and is a strong inhibitor of viral replication both in vitro and in vivo.

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