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In Vivo Antiviral Activity and Pharmacokinetics of (−)-*cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolan-5-yl]Cytosine in Woodchuck Hepatitis Virus-Infected Woodchucks

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The (−) enantiomer of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine [(−)-FTC)], a substituted oxathiolane compound with anti-hepatitis B virus activity in vitro, was assessed for its efficacy in woodchucks with naturally acquired woodchuck hepatitis virus (WHV) infection. Pharmacokinetics and in vitro anabolism were also determined. (−)-FTC was anabolized to the 5′-triphosphate in a dose-related fashion, reaching a maximum concentration at about 24 h in cultured woodchuck hepatocytes. Following administration of a dose of 10 mg/kg of body weight intraperitoneally (i.p.), the clearance of (−)-FTC from plasma was monoexponential, the terminal half-life was 3.76 ± 1.4 h, and the systemic clearance was 0.12 ± 0.06 liters/h/kg. The antiviral efficacy of (−)-FTC in the woodchuck model was assessed by quantitation of serum WHV DNA levels and by WHV particle-associated DNA polymerase activity at two dosages, 30 and 20 mg/kg given i.p. twice daily (b.i.d.), respectively. The level of WHV DNA in serum was reduced 20- to 150-fold (average, 56-fold) in the 30-mg/kg-b.i.d. treatment group and 6- to 49-fold (average, 27-fold) in the 20-mg/kg-b.i.d. treatment group. Viral DNA polymerase levels diminished accordingly. One week after treatment was discontinued, WHV levels returned to pretreatment levels in both studies. These animals were biopsied before and following treatment with 30 mg of (−)-FTC per kg. Their livers were characterized by a mild increase in cytoplasmic lipid levels, but this change was not associated with altered liver enzyme levels. Serum chemistry and hematology results were within the normal ranges for all treated animals. We conclude that (−)-FTC is a potent antihepadnaviral agent and that it has no detectable toxic effects in woodchucks when given for up to 25 days. Further development of (−)-FTC as an anti-hepatitis B virus therapy for patients is warranted.

Chronic hepatitis B virus (HBV) infection is a significant cause of life-threatening liver disease, including cirrhosis and hepatocellular carcinoma (3, 60). HBV infection is a particularly important health concern in view of the fact that more than 300 million individuals, approximately 5% of the world's population, are infected with the virus (31). Although a safe and effective vaccine is available, drug therapies for antiviral intervention in those people already infected are limited. Interferon therapy and nucleoside analogs alone and in combination have proven to have some efficacy, but not all patients respond and toxic side effects often limit their use (2, 4, 19, 32, 37, 42, 48, 51, 63).

HBV is a member of the hepadnavirus family that includes three well-characterized animal hepadnaviruses, duck hepatitis B virus (DHBV), ground squirrel hepatitis virus, and woodchuck hepatitis virus (WHV) (20). Each of these models has been used to test antihepadnaviral agents (29, 43, 54). Although most trials with antihepadnaviral agents have been conducted with DHBV-infected ducks, WHV-infected woodchucks are a particularly well-suited model for several reasons (25, 29, 30, 52, 53, 57, 59). WHV is more similar to HBV in genetic composition than are the other two viruses, sharing about 70% nucleotide sequence homology with HBV (18), and the range of hepatic injury produced by chronic infections in woodchucks more closely mimics that seen in HBV-infected patients than that seen in the other two models (8, 9, 40, 44). Also, DHBV DNA polymerase appears to differ from those of WHV and HBV (15, 23, 28).

We selected the (−) enantiomer of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine [(−)-FTC] for in vivo assessment because it was less toxic to cultured cells than the (+) enantiomer, which corresponds to the natural (D) form of the molecule (58) and because the (−) form has potent in vitro activity against HBV (12, 17) and human immunodeficiency virus (HIV) (49). This report describes the efficacy and safety of the (−) enantiomer of (−)-FTC in woodchucks naturally infected with WHV.

MATERIALS AND METHODS

The woodchucks used in this study were obtained from Cocalico Farms, Cocalico, Pa. The woodchucks had been caught in the wild, had been naturally infected with and carried WHV, and were at least 1 year old. All animals were housed in the North Carolina State University College of Veterinary Medicine in accord with the North Carolina State University Institutional Animal Care and Use Committee guidelines. Woodchucks were fed a commercially available herbivore diet (Leaf Eater Diet; Marion Zoological, Inc., Marion, Kans.) and were offered water ad libitum. Animals were housed individually or in groups of two if both animals received the same treatment. Woodchucks were determined to be carriers of WHV by serial testing of serum for viral DNA, WHV surface antigen (WHsAg), and antibodies to WHsAg and WHV core antigen. Determination of serum WHV DNA levels is described below. Viral antigen and antibody levels were determined by radioimmunoassay by Paul Cote of the Georgetown School of Medicine (7, 39).

Blood samples for hematology and clinical chemistry studies were collected 10 to 11 days prior to the studies, weekly during the study period, and for 4 weeks

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following the cessation of treatment. Blood was obtained from the femoral or tarsal vein following sedation with Innovar-Vet (fentanyl citrate [0.4 mg/ml] and droperidol [20 mg/ml]; Pittman-Moore, Mundelein, Ill.). The narcotic component (fentanyl) was routinely reversed with an appropriate dose of naloxone, a narcotic antagonist (Astra Pharmaceuticals, Westborough, Mass.). Whole blood was collected for routine hematology studies. Serum was analyzed with a multichannel analyzer (Monarch 2000; Instrumentation Laboratories, Lexington, Mass.) for a variety of enzyme and biochemical parameters to monitor organ function and to screen for toxic effects of the test compound. Serum samples collected for assessment of WHV DNA levels and antibodies to WHV were stored at −80°C until analyzed. Prior to the start of the study, serum was analyzed for levels of gamma glutamyl transpeptidase (GGT) as a marker for hepatocellular carcinoma. All drug-treated animals in the study had GGT levels of 10 U/liter or lower, indicative of a low likelihood of hepatic neoplasia. In study 1 [in which the animals received 30 mg of (−)-FTC per kg of body weight twice daily (b.i.d.)], two of seven control animals were included, although they had elevated serum GGT levels (for animal HC18, 58 U/liter; for animal HC73, 67 U/liter). The other five animals had GGT levels of less than 10 U/liter. In study 2 [in which the animals received 20 mg of (−)-FTC per kg b.i.d.], all animals had GGT levels of less than 10 U/liter. The results of all other biochemical tests were within the normal range.

Liver wedge biopsy specimens were obtained from all of the woodchucks in the 30-mg/kg dose group 10 to 11 days prior to treatment and on the last day of treatment. Animals were anesthetized with isoflurane, and laparotomy was performed in accordance with the protocol of the North Carolina State University Institutional Animal Care and Use Committee. Portions of liver samples were immediately placed into liquid nitrogen and stored at −80°C. Additional liver sections were obtained from three of the (−)-FTC-treated woodchucks (animals HC100, HC102, and HC103) pre- and posttreatment and were fixed in formaldehyde for histologic examination. Paraffin-embedded liver tissue sections of 6 μm in thickness were stained with hematoxylin and eosin and periodic acid-Schiff stain with and without diastase for histologic review. Hepatic lipid was assessed with oil red-stained, 8-μm-thick frozen sections of liver.

Compounds. (−)-FTC was synthesized and purified essentially as described by Burroughs-Wellcome Compound Registry (5) through which it was obtained. It was dissolved in sterile saline at a concentration of 10 or 15 mg/ml, stored at 4°C until shortly before use, and then warmed to 37°C. Control animals received intraperitoneal (i.p.) injections of sterile saline. Animals were weighed weekly during the course of treatment, and drug doses were adjusted accordingly.

Anabolism. Primary WHV-infected woodchuck hepatocytes were seeded into 100-mm dishes with Dulbecco modified Eagle F12 culture medium [^{3}H](−)-FTC (Moravck Biochemicals) at concentrations of 0.01, 0.1, 1.0, or 10 μM was added to each dish, and the dishes were allowed to incubate for 24 h at 37°C in 5% CO_2. Following incubation, a 500-μl aliquot of the medium was removed, frozen at −20°C, and saved for further analysis. Cell counts were made by trypsinizing an extra flask and counting the number of cells with a hemocytometer. [^{3}H](−)-FTC was extracted as described previously (35). Intracellular FTC nucleoside and (−)-FTC nucleotides were analyzed and quantitated by high-performance liquid chromatography as described previously (35).

Pharmacokinetics of (−)-FTC in woodchucks. For initial determination of the levels of (−)-FTC in blood, four drug-naive, WHV-infected woodchucks were anesthetized with the fentanyl citrate and droperidol before i.p. injection of 10 mg of (−)-FTC per kg. Plasma samples were collected at 0 h (predosing) and 0.17, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24 h following administration of the dose. Analyses of (−)-FTC concentrations and metabolite concentrations were performed as described previously (16). In addition, on the last day of drug treatment in the first antiviral efficacy study (treatment with (−)-FTC at [30 mg/kg b.i.d.], all six (−)-FTC-treated animals were anesthetized and bled at 0, 1, 2, 4, 8, and 12 h following administration of the last dose.

Extraction and analysis of serum samples containing WHV DNA. Full-length (3.2-kb) WHV DNA from WHV strain 1 in pBR322, a gift from John E. Newbold, University of North Carolina School of Medicine, Chapel Hill, was prepared in *Escherichia coli* (Sure supercompetent cells; Stratagene, La Jolla, Calif.) by the transformation procedure recommended by the supplier.

Serum samples were dispensed into aliquots and were placed at −80°C within 2 h of blood collection. For WHV DNA analysis, serum was digested overnight at 37°C with proteinase K at 1 mg/ml and 10 μg of salmon testis DNA per ml in 2% sodium dodecyl sulfate (SDS)–25 mM EDTA–100 mM HEPES (pH 7.4).

DNA was extracted from serum once with phenol and then $CHCl_3$, followed by precipitation with ammonium acetate and isopropyl alcohol (0.75 M and 50%, respectively). The precipitate was collected by centrifugation and was dissolved in 10 mM Tris-HCl–1 mM EDTA (pH 8.0). DNA was denatured as described previously (26), adhered to nitrocellulose with a slot blot apparatus (Schleicher & Schuell, Keene, N.H.), and treated as described above for the detection of WHV DNA.

Treatment of liver biopsy specimens. Approximately 50 mg of frozen liver from each animal was homogenized in cold 100 mM Tris-HCl–5 mM EDTA–1% SDS (pH 8) and lysed at 42°C for 3 h in the presence of 1 mg of proteinase K per ml. DNA was extracted, precipitated by the addition of NaCl, and ethanol dissolved in distilled water, and cellular DNA was cut with the *Hin*dIII restriction endonuclease in the appropriate digestion buffer. A sample representing 2% of the original material was applied to 1% agarose and electrophoretically separated in TAE (Tris-acetate-EDTA) buffer. The agarose gel was stained with ethidium bromide for detection of total DNA, and then the DNA was denatured, neutralized, and transferred to nitrocellulose by a pressure blotter (Stratagene). The adhered DNA was treated as described previously (26, 27), except that 20 mM $NaPO_4$ (pH 6.5) and 25 g of purified salmon testis DNA were added. Nitrocellulose blots were exposed to a PhosphorImager screen (Molecular Dynamics, Inc., Sunnyvale, Calif.) for 4 or 20 h and scanned by recommended methods. A grid or series of rectangles was used to integrate the pixel volume for samples and a background. The pixel volume was determined for each unknown sample from the slot blot and for a known concentration of purified plasmid WHV DNA (1,000 to 3 pg/slot). Values for duplicate samples were averaged, and the values for pre- and posttreatment samples were compared. A linear regression equation of a standard curve of pixel volume values for WHV DNA (3 to 1,000 pg) by using a weighting factor of 1 was developed. Picogram values were calculated for each unknown sample. Purified salmon testis DNA was used in separate slots to determine the actual background values.

Assay for particle-associated DNA polymerase activity. WHV DNA polymerase activity was assayed as described by Kaplan et al. (24), with modifications (23). Each reaction mixture consisted of 10 μl of woodchuck serum, [^{32}P]dCTP (3,000 Ci/mmol) (0.2 μM), dGTP, dATP, and dTTP (10 μM [each]), $MgCl_2$ (50 mM), KCl (400 mM), Nonidet P-40 (0.1%), 2-mercaptoethanol (0.2%; vol/vol), and EGTA (5 mM). The reaction mixtures were incubated for 2 h at 37°C, and the reaction was then stopped by the addition of proteinase K (1 mg/ml), 1% SDS, and 50 mM EDTA. Samples were extracted with phenol-chloroform, precipitated, dissolved in 5× TAE buffer, and applied to a 1.5% agarose gel in TAE buffer. After electrophoresis, the gel was rinsed with 10% trichloroacetic acid, rinsed briefly with water, and then dried and exposed to a PhosphorImager Screen. Pixel volumes from duplicate samples were averaged, the background volume was subtracted, and the pixel volumes for the samples from different experiments were normalized to the pixel volume from a reference serum sample included in each gel.

DNA sequencing. The DNA sequence of WHV in serum samples from animal HC102 was determined by PCR amplification of the region of the polymerase open reading frame from nucleotides 710 and 1221, followed by dye termination fluorescence sequencing by the protocols recommended by the supplier (Applied Biosystems Division, Perkin-Elmer Corporation, Norwalk, Conn.). Serum samples were prepared by mixing 5 μl of serum with 50 μl of a solution containing 1.5 mM $MgCl_2$, 0.45% (wt/vol) Tween 20, 0.45% (wt/vol) Nonidet P-40, and 0.2 mg of proteinase K per ml. Samples were heated at 55°C for 1 h and then at 95°C for 15 min. Five microliters of each sample was added to PCR mixtures containing primers 5′-ACGGCAGGAAATTGCACTTG-3′ (nucleotides 710 to 729) and 5′-GAAAGCCATACGGGAAGCAATAG-3′ (nucleotides 1221 to 1199), 10% dimethyl sulfoxide, *Taq* Extender buffer (Stratagene, La Jolla, Calif.) *Taq* polymerase, and *Taq* extender (Stratagene) polymerase. DNA was amplified for 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 30 s in a Perkin-Elmer 9600 GeneAmp PCR system. Following amplification, the samples were concentrated with Microcon 100 microconcentrators (Amicon, Inc., Beverly, Mass.). The same primers were used for sequence analysis by the protocols and with the products recommended by the supplier (Applied Biosystems Division, Perkin-Elmer Corporation). Analysis of the sequence was performed with Sequencher, version 3.0, software.

Analysis of data. Statistical analysis of data related to viral suppression in serum was performed by a general linear models procedure with repeated measures of analysis of variance by using weeks as the repeat factor, and the data were compared with those for saline-treated animals (47, 61).

The results of clinical chemistry studies for the treated and control groups were compared by a multivariate one-way analysis of variance by using the statistical software system procedure PROC MULTITEST (45, 46). *P* values for individual rows in tables were adjusted for multiple comparisons by using the bootstrap option to approximate the minimum *P* value for all tests.

Clinical chemistry parameters were measured weekly. Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), GGT, and sorbitol dehydrogenase (SDH) were chosen for statistical analysis. For ALP, ALT, and GGT, a square-root transformation described by Pollard (38) was performed to stabilize the variance. For each parameter, a one-way repeated measures layout (62) was analyzed by bootstrap resampling (61) to assess differences between the control and the treated groups. The bootstrap *P* values were adjusted for the repeated measurement for each parameter. For this study, for each animal, there were 10 measurements for each parameter. Areas under the plasma drug concentration-time curve (AUCs) were obtained by the linear trapezoidal rule, with extrapolation to infinity.

RESULTS

Anabolism. Primary woodchuck hepatocytes were incubated for 24 h with [^{3}H](−)-FTC at initial concentrations of 0.01, 0.1, 1.0, and 10 μM (Table 1). The levels of (−)-FTC mono-, di-, and triphosphates, as well as those of the putative diphosphocholine and diphosphoethanolamine metabolites, increased with increasing extracellular concentrations. The diphospho-

TABLE 1. Concentrations of intracellular metabolites from primary woodchuck hepatocytes incubated for 24 h with various concentrations of [^{3}H](−)-FTC[a]

FTC concn (μM)	Metabolite concn (pmol/10^6 cells)					
	(−)-FTC	(−)-FTC-diphosphoethanolamine	(−)-FTC-5′-monophosphate	(−)-FTC-diphosphocholine	(−)-FTC-5′-diphosphate	(−)-FTC-5′-triphosphate
0.01	0.10 ± 0.02	ND[b]	0.08 ± 0.04	ND	0.06 ± 0.05	0.06 ± 0.03
0.1	0.59 ± 0.18	ND	0.07 ± 0.02	ND	0.24 ± 0.06	0.14 ± 0.04
1.0	8.95 ± 1.18	0.10 ± 0.02	0.73 ± 0.09	0.17 ± 0.05	1.70 ± 0.25	1.01 ± 0.06
10	88.7 ± 8.51	0.38 ± 0.07	2.14 ± 0.90	0.34 ± 0.02	7.66 ± 1.13	2.44 ± 0.64

[a] Cells were extracted and intracellular nucleosides and nucleotides were quantitated by high-performance liquid chromatography. Data are means ± standard deviations (n = 3). The metabolites are only tentatively identified.
[b] ND, level of metabolite was below the limit of detection (0.05 pmol/10^6 cells).

choline and diphosphoethanolamine metabolites were only tentatively identified by comparison of their respective retention times with the retention times from anabolite profiles of more thoroughly defined (−)-FTC-treated cellular extracts (35). The intracellular levels of these metabolites were not large enought to allow for further characterization. The 5′-triphosphate concentrations were much lower than those seen with the human-derived cell lines Hep-G2 (2.2.15) (3.6 pmol/10^6 cells) (35) or CEM (14.2 pmol/10^6 cells) (data not shown). However, other rodent-derived cell lines incubated under similar conditions gave comparable results. BNL CL.2 (mouse hepatocyte) and H-4-II-E (rat hepatocyte) cultures incubated with 1 μM [^{3}H](−)-FTC for 24 h gave (−)-FTC-5′-triphosphate levels of 0.99 and 0.31 pmol/10^6 cells, respectively (data not shown). This difference in (−)-FTC anabolism among different species may be due to alterations in the specificity of the anabolic enzyme deoxycytidine kinase. Such differences in specificity between rodent- and human-derived deoxycytidine kinase have been reported in the literature (21), although woodchuck deoxycytidine kinase was not examined directly.

Pharmacokinetics. The plasma concentration-time curves following administration of a 10-mg/kg dose of (−)-FTC i.p. for four animals are presented in Fig. 1. Table 2 summarizes the results of the pharmacokinetic analysis of these data. The decline in plasma (−)-FTC levels appeared to be monoexponential. The AUC was 384 ± 152 μM/h, the maximum concentration of drug in serum (C_{max}) was 45 ± 11 μM, the time to C_{max} (T_{max}) was 90 min, the half-life ($t_{1/2}$) was 3.76 ± 1.40 h, and the systemic clearance (CL) was 0.12 ± 0.06 liters/h/kg. There was little or no protein binding (data not shown). At the end of the 25-day dosing regimen of 30 mg/kg b.i.d. i.p., the terminal elimination $t_{1/2}$ was 4.2 ± 1.5 h, the AUC from 0 to 12 h was 694 ± 266 μM/h, C_{max} was 126 ± 26.8 μM, and T_{max} was at 1 h for the six treated animals. On the basis of the results of these studies, the plasma of woodchucks was exposed to levels of (−)-FTC that ranged between 100 and 10 μM during the course of the study.

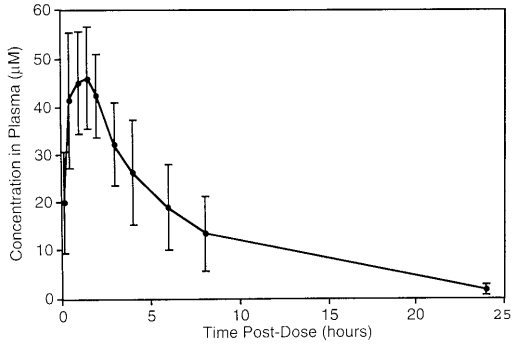

FIG. 1. Plasma drug concentration-time curve following i.p. administration of 10 mg of (−)-FTC per kg to woodchucks.

In vivo activity. The results of the study with the dosage of 30-mg/kg b.i.d. i.p. are presented first. A significant reduction in WHV DNA levels in serum (Fig. 2) was seen for all six treated woodchucks within 1 week of the start of treatment. The serum WHV DNA level was reduced 20- to 150-fold (average, 56-fold) from the pretreatment levels. There was no suppression of viral DNA in saline-treated woodchucks (Fig. 2). Serum DNA polymerase activity measured by the incorporation of [32P]dCTP into WHV DNA was similarly reduced during treatment in woodchucks treated with (−)-FTC (Fig. 2) but not in controls (Fig. 2). Suppression of the levels of WHV DNA polymerase in serum continued during the treatment period. Within 2 weeks following the cessation of treatment, the levels of WHV DNA and DNA polymerase in serum returned to near normal.

Administration of a lower dosage of (−)-FTC (20 mg/kg b.i.d. i.p.) had a similar but not as pronounced an effect on serum WHV DNA levels and polymerase activity as the higher dosage (Fig. 3). There was a 6- to 49-fold (average, 27-fold) drop in WHV DNA levels by the end of treatment. A moderate increase in serum WHV DNA level but not in DNA polymerase activity occurred in animal HC102 during the fourth week of treatment. Sustained reductions in the DNA polymerase levels of all treated woodchucks were seen. The DNA sequence of WHV in samples obtained from week −2 through week 4 postdosing were analyzed to determine whether the increased amount of DNA was due to resistant virus or a burst of chain-terminated viruses.

No changes in the nucleotide sequence of the region from nucleotides 710 to 1221 were observed for samples collected during the second study from treated animal HC102. The consensus reverse transcriptase amino acid sequence YMDD, which is conserved among hepadnaviruses and HIVs, was not altered. Alterations in this sequence are associated with resistance to (−)-FTC in laboratory strains of HIV as well as

TABLE 2. Pharmacokinetics of (−)-FTC (10 mg/kg i.p.) in woodchucks

Parameter	Mean ± SD
AUC (μM · h)	384 ± 152
CL (liters/kg/h)	0.12 ± 0.06
$t_{1/2}$ (h)	3.7 ± 1.4
C_{max} (μM)	45 ± 11
T_{max} (h)	1.5

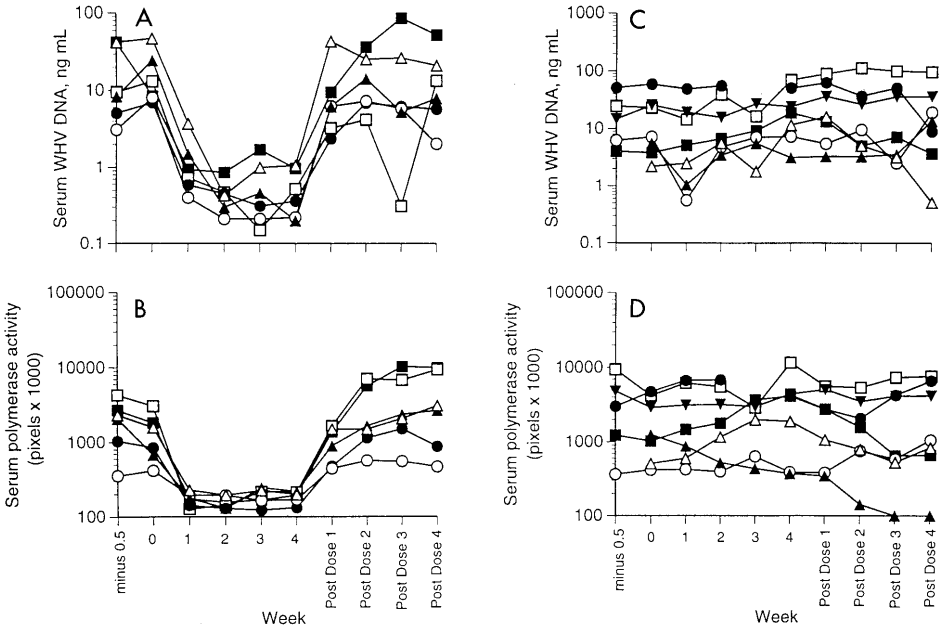

FIG. 2. Effects of administration of (−)-FTC (30 mg/kg b.i.d. i.p.) on serum WHV DNA levels and serum polymerase activities in infected woodchucks. (A and B) Treated animals HC042 (■), HC072 (●), HC102 (▲), HC070 (□), HC100 (○), and HC103 (△). (C and D) Control animals HC108 (■), HC066 (○), HC074 (△), HC026 (□), HC073 (▲), HC075 (▼), and HC060 (●).

resistance to lamivudine in clinical samples from patients infected with HIV and HBV (1, 50, 58). We conclude that the WHV DNA detected in the serum of animal HC102 during (−)-FTC therapy was not the result of development of resistant virus, and since polymerase activity was not detected in serum, the DNA was probably associated with chain-terminated particles (33). Chain-terminated HBV particles are also detected in culture supernatants of (−)-FTC-treated chronic producer cells (10).

WHV DNA levels in the liver biopsy specimens were also reduced in all six treated animals by 1 month of (−)-FTC (30 mg/kg) treatment (Table 3). Reductions ranged from 68 to 98% compared to pretreatment virus levels. Specifically, the WHV genome length was reduced; the quantity of single-

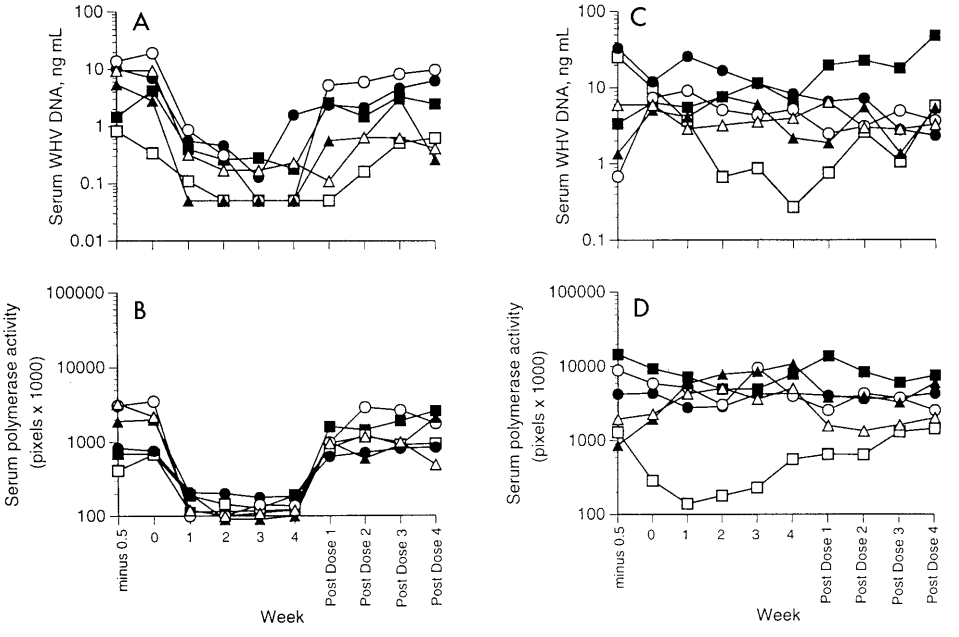

FIG. 3. Effects of (−)-FTC treatment (20 mg/kg b.i.d. i.p.) on serum WHV DNA levels and serum polymerase activities in infected woodchucks. (A and B) Treated animals HC070 (■), HC102 (●), HC125 (▲), HC100 (□), HC118 (○), and HC126 (△). (C and D) Control animals HC103 (■), HC117 (●), HC121 (▲), HC116 (□), HC119 (○), and HC127 (△).

TABLE 3. WHV DNA in liver of naturally WHV-infected woodchucks following treatment with (−)-FTC for 25 days

Woodchuck	Assay no.[a]	No. of pixels/mg of liver (corrected for cell concn)		% Reduction of WHV DNA (avg)
		Liver biopsy specimen Pretreatment	Liver biopsy specimen 24 h after last dose	
HC042	1	1,031,397	125,379	91
	2	1,021,535	59,565	
HC070	1	394,477	139,495	78
	2	426,613	43,566	
HC072	1	2,970,157	722,774	82
	2	3,039,023	356,704	
HC100	1	1,110,761	155,002	68
	2	823,693	456,315	
HC102	1	2,570,703	55,159	98
	2	2,611,796	45,294	
HC103	1	2,770,837	533,340	81
	2	1,884,066	330,128	

[a] Assays 1 and 2 were replicates following treatment with 30 mg of (−)-FTC per kg, respectively.

stranded, replicative intermediates remained close to the same level as the quantity before treatment, but it was shifted to smaller-size DNA fragments (Fig. 4).

Levels of WHsAg or antibodies against WHsAg or WHV core antigen in treated or control animals did not change. There were no systematic treatment-related differences in the level of AST, ALT, ALP, SDH, or GGT. Hematology test values did not differ between treated and control groups. No clinical signs of toxicity were seen during the treatment or recovery periods in treated animals, and the food and water consumption of the treated animals was comparable to that of the control animals. Neither saline-treated (control) nor (−)-FTC-treated animals showed a significant weight change during or after dosing.

Liver biopsy specimens were obtained from three (−)-FTC-dosed woodchucks (animals HC100, HC102, and HC103) prior to dosing and at the conclusion of the study. The pretreatment biopsy specimens were characterized by scant to moderate lymphocytic and plasmacytic infiltrates of the portal tracts and minor foci of mononuclear inflammatory cells in the parenchyma, which are typical of chronic WHV infection. In focal areas, hepatocytes in pretreatment biopsy specimens were slightly swollen by vacuoles. Similar histopathological findings were noted following a month of treatment. Hepatocytes from all three animals were characterized by a light diffuse microvesicular vacuolization. These vacuoles contained lipid and small amounts of glycogen. Mild variations in the size of hepatocyte nuclei were also apparent following treatment.

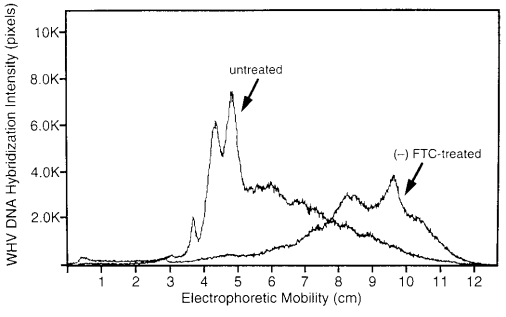

FIG. 4. Profile of WHV hybridization intensity from liver biopsy samples (from animal HC042) before and after treatment with (−)-FTC.

DISCUSSION

In this study we have demonstrated that (−)-FTC is a potent suppressor of WHV replication in woodchucks that are naturally infected with and chronic carriers of WHV. Serum WHV DNA levels and polymerase activity were reduced by the end of the first week of treatment and continued for as long as the animals were treated.

These results support in vitro studies of the anti-HBV efficacy of (−)-FTC in cultured human hepatocytes (17). Previously, the in vivo anti-HBV efficacy of (−)-FTC has been demonstrated in mice engrafted with human chronic producer cells (2.2.15 cells), and ducks congenitally infected with DHBV have also responded to (−)-FTC (6, 13).

Safety, an important consideration when potential antiviral drugs are evaluated, can be monitored more accurately in animal models than by in vitro assays. For example, some nucleoside analogs such as 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (FEAU), and dideoxycytosine were effective in vitro against HBV, but they proved to be toxic when given to patients or woodchucks (14, 22, 32, 56, 63). (−)-FTC-treated human hepatocytes did not show signs of toxicity (17). Liver specimens from ducks treated for up to 12 weeks with (−)-FTC had a normal histologic appearance and no increase in hepatocyte turnover, while liver specimens from ducks treated with 2′-carbodeoxyguanosine had significant histologic abnormalities within 3 weeks of treatment and increased hepatocyte replication (13, 30). Toxicity was not observed in mice orally dosed with up to 400 mg of (−)-FTC per kg per day for 30 days (data not shown). In our study, representative biopsy specimens from three of the six (−)-FTC-treated woodchucks (30 mg/kg b.i.d.) had minor increases in cytoplasmic lipid vacuole levels following 25 days of treatment, but this change was not associated with any biochemical evidence of liver injury.

Several animals (animals HC70, HC100, and HC102) that were treated in the first study were included in the second

study as treated animals. It is significant that repeated exposure to (−)-FTC did not produce toxic effects. In contrast, woodchucks reexposed to FEAU showed marked anorexia, despite a lack of signs of toxicity during the first exposure (14).

Recent reports indicate that HBV can overcome initial repression during extended treatment of orthotopic liver transplant patients with lamivudine (1) due to the emergence of resistance mutations in the HBV DNA polymerase gene. One woodchuck (animal HC102) in the second study showed an elevated serum WHV DNA level during the fourth week of therapy. The significance of this result for one sample is difficult to interpret since serum WHV DNA polymerase activity was reduced and because therapy was discontinued at that time. The possibility of a resistance-inducing mutation in the polymerase gene between nucleotides 710 and 1221 was eliminated by sequencing the virus in the serum samples. Two additional woodchucks (animals HC70 and HC100) that were treated with (−)-FTC in the earlier study responded to the second treatment with reductions in viral DNA levels similar to those for previously untreated animals.

Woodchuck hepatocytes are able to anabolize (−)-FTC in a fashion similar to that in which other rodents anabolize it (36). The levels of each nucleotide metabolite of (−)-FTC increased in a dose-dependent and a time-dependent fashion. The anabolism of (−)-FTC to its active triphosphate form was detected even with low concentrations of (−)-FTC (0.1 μM) in the medium. The levels obtained were very similar to those seen when other rodent cell lines (BNL CL.2 [mouse hepatocyte] and H-4-II-E [rat hepatocyte]) were incubated with (−)-FTC. Although less (−)-FTC-5′-triphosphate was measured in woodchuck primary hepatocytes than those derived from human cell lines, the level of active metabolite was obviously high enough in the group treated with (−)-FTC at a dosage of 30 mg/kg b.i.d. i.p. to show efficacy in this model (35).

On the basis of the results of the pharmacokinetic studies, at the end of 25 days of treatment with (−)-FTC at 30 mg/kg b.i.d., the woodchucks attained significant levels of (−)-FTC in plasma (between 100 and 10 μM). One explanation for the less pronounced efficacy of (−)-FTC at 20 mg/kg b.i.d. in the second study may be that the levels in plasma were not always effective throughout the day, although it should be noted that the drop in serum WHV DNA polymerase activity in animals dosed with 30 mg/kg b.i.d. was not correlated with exposure to (−)-FTC.

The CL for (−)-FTC in woodchucks is unusually low, suggesting that woodchucks do not have a tubular secreting system for nucleotides, as do rats and mice (the CL for mice is 2 to 4 liters/kg/h). Alternatively, their kidneys may reabsorb (−)-FTC to a greater degree than rats and mice. A related compound (lamivudine) is also cleared more slowly from woodchucks than from other animals (41). Still, the $t_{1/2}$ of FTC in the woodchuck is sufficiently short that there is little chance that it would accumulate in the animals upon multiple dosing.

In woodchucks, a weak in vitro inhibitor, acyclovir, was efficacious only when the drug was given twice daily at a dose of 15 to 25 mg/kg (55). Zidovudine (AZT; Retrovir), was found to have no activity in vitro or in vivo, despite the use of several dosing regimens (34). The lack of activity of AZT is presumed to be due to a lack of phosphorylation in vivo since AZT-triphosphate is an effective inhibitor of WHV polymerase (23). (−)-FTC is phosphated by woodchuck hepatocytes. (−)-FTC-triphosphate was found in (−)-FTC-treated woodchuck hepatocytes, and it is also an inhibitor of WHV polymerase (11).

(−)-FTC has been demonstrated to be a safe and potent antihepadnaviral agent in woodchucks naturally infected with WHV under the conditions used for this study. In view of these data and the fact that (−)-FTC may be more active against HBV than WHV and since cultured human hepatocytes anabolize (−)-FTC more actively than woodchuck hepatocytes, (−)-FTC should be further evaluated for the treatment of HBV-infected humans.

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