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The Anti-Hepatitis B Virus Activities, Cytotoxicities, and Anabolic Profiles of the (−) and (+) Enantiomers of *cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolan-5-yl]Cytosine

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The anti-hepatitis B (anti-HBV) activities of the (−) and (+) enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (2′-deoxy-3′-thia-5-fluorocytosine [FTC]) were studied by using an HBV-transfected cell line (HepG2 derivative 2.2.15, subclone P5A). The (−) isomer was found to be a potent inhibitor of viral replication, with an apparent 50% inhibitory concentration of 10 nM, while the (+) isomer was found to be considerably less active. Both isomers showed minimal toxicity to HepG2 cells (50% inhibitory concentration, >200 μM) and showed minimal toxicity in the human bone marrow progenitor cell assay. In accord with the cellular antiviral activity data, the 5′-triphosphate of (−)-FTC inhibited viral DNA synthesis in an endogenous HBV DNA polymerase assay, while the 5′-triphosphate of the (+) isomer was inactive. Unphosphorylated (−)-FTC did not inhibit product formation in the endogenous assay, suggesting that the antiviral activity of the compound is dependent on anabolism to the 5′-triphosphate. Both (−)- and (+)-FTC were anabolized to the corresponding 5′-triphosphates in chronically HBV-infected HepG2 cells. The rate of accumulation and the steady-state concentration of the 5′-triphosphate of (−)-FTC were greater. Also, (−)-FTC was not a substrate for cytidine deaminase and, therefore, is not subject to deamination and conversion to an inactive uridine analog. The (+) isomer is, however, a good substrate for cytidine deaminase.

Hepatitis B virus (HBV), the causative agent of acute and chronic hepatitis, directly affects about 5% of the world's population. Chronic carriers of HBV are at an increased risk of liver damage that, in the worst cases, can lead to cirrhosis of the liver and/or to hepatocellular carcinoma. Vaccination against HBV is one way to effectively prevent HBV infection. However, vaccination is not an effective therapy for the estimated 200 million chronic carriers. Although several antiviral agents such as alpha interferon, adenine arabinoside monophosphate, and acyclovir have been tested as therapeutic agents, only alpha interferon has demonstrated some promise (7, 8, 9, 17, 23, 25).

The replication cycle of hepadnaviruses includes the reverse transcription of an RNA template (6). This process is catalyzed by a polymerase that shares significant sequence homology with the reverse transcriptase from retroviruses (17). As a consequence, it has been demonstrated that a number of compounds that inhibit human immunodeficiency virus (HIV) replication in vitro (for example, 2′,3′-dideoxycytidine) also inhibit HBV replication in vitro (4, 14, 15, 18, 24). These agents await further study to determine their usefulness as therapeutic agents for the treatment of HBV infections.

Here we report the anti-HBV activities, cytotoxicities, and anabolism of the resolved enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (FTC) (Fig. 1). The anti-HBV activity of the racemic material has been reported previously (4). Our results show that the antiviral activity of the racemic mixture can be attributed to the 5′-triphosphate of the (−) isomer.

MATERIALS AND METHODS

Compounds. (±)-FTC and racemic 2′-deoxy-3′-thiacytidine (BCH-189; Fig. 1) were synthesized by D. Liotta, J. Wurster, and L. Wilson. Separation of the enantiomers was carried out by formation of the 5′-O-butyl ester of the racemic mixture and selective hydrolysis of the (+) enantiomer by using pig liver esterase to give 95% enantiomerically pure (+)-FTC or (+)-BCH-189. The 5′-O-butyl esters of (−)-FTC and (−)-BCH-189, which are not substrates for pig liver esterase, were hydrolyzed chemically to give >99% enantiomerically pure compound. The 5′-triphosphates of (−)- and (+)-FTC were synthesized by S. Hopkins and J. Wilson as described previously (26). The purity of the 5′-triphosphate was determined by ^{31}P nuclear magnetic resonance and high-pressure liquid chromatographic (HPLC) analyses. The carbocyclic analog of deoxyguanosine, 2′-CDG, was synthesized by S. Daluge of the Organic Chemistry Division, Burroughs Wellcome Co.

Hybridization of DNA. ^{32}P-labeled riboprobes, which were used for the detection of plus- or minus-strand HBV DNA, were synthesized by using the plasmid pGEMEX-1 of HBV (prepared at Burroughs Wellcome Co. by T. Powdrill) as the template. Hybridization and washing conditions were as described previously (11, 20). The radioactivity associated with the hybridized filters was measured by using a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). The amount of viral DNA was deter-

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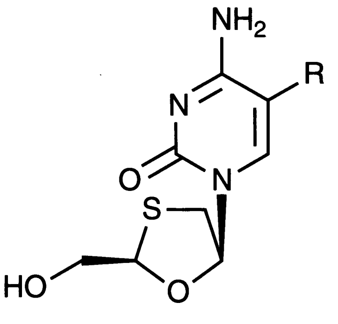

FIG. 1. Structures of FTC (R = F) and BCH-189 (R = H). The absolute stereochemistries shown are those of (−)-FTC (24a) and (−)-BCH-189 (3). Both compounds have a 1-β-L configuration.

mined by comparing the hybridization intensities of the viral DNA samples with those of cloned plasmid DNA of known concentrations.

Assay for antiviral activity. (i) Extracellular HBV DNA. The HBV producer cell line HepG2 derivative 2.2.15 (19), subclone P5A, was cultured as described by Korba and Milman (11). Confluent resting cells were treated with the indicated compounds in culture medium for 10 days; medium was changed daily. Culture fluids were collected on days 0, 4, 7, and 10 and clarified by centrifugation at 2,500 × *g* for 5 min. Aliquots (200 μl) of the supernatants were frozen at −80°C, thawed at 37°C, and mixed with an equal volume of 2 M NaOH. After 30 min at room temperature, 100 μl of 5 M ammonium acetate was added and samples were applied to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) by using a minifold slot apparatus (Schleicher & Schuell). Filters were neutralized by washing with a solution containing 1 M Tris-HCl (pH 7.5) and 2 M NaCl; this was followed by washing with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were dried at 80°C for 2 h under vacuum and were then hybridized (20).

(ii) Intracellular HBV DNA. After 10 days of incubation with drug, total intracellular DNA was isolated (4, 24). Cells were lysed with 5× TE (1× TE is 10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) containing 1% sodium dodecyl sulfate (SDS). DNA was purified by incubating the lysates with 1 mg of proteinase K (GIBCO Bethesda Research Laboratories, Grand Island, N.Y.) per ml at 37°C overnight; this was followed by extraction with phenol-chloroform. The DNA was precipitated with isopropanol, resuspended in 0.3 M NaOH, and precipitated with ethanol. Samples were resuspended in water, digested with pancreatic RNase (Boehringer Mannheim, Indianapolis, Ind.) and *Hin*dIII restriction endonuclease (GIBCO Bethesda Research Laboratories), separated by electrophoresis on a 1% agarose gel, and blotted onto nitrocellulose filters. Filters were dried at 80°C for 2 h under vacuum and hybridized (20).

Cytotoxicity assay. The cytotoxicity of FTC was evaluated by growth inhibition assays by using P5A cells and normal human bone marrow progenitor cells. Toxicity towards P5A cells was determined by seeding cells at a density of 2,500 cells per well into 96-well microtiter plates containing GIBCO RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum–2 mM L-glutamine. After 48 h of incubation at 37°C, the medium was replaced with medium containing concentrations of either the (−) or the (+) enantiomer of FTC ranging from 0.02 to 200 μM. Cell cultures were incubated at 37°C for 9 days; the medium was replaced on alternate days with fresh medium containing FTC. Cell growth was determined every other day by staining cells with the DNA-specific fluorochrome bisbenzimide H33342 (Calbiochem, San Diego, Calif.). Human marrow progenitor cell colony-forming assays were performed as described previously (5).

Anabolism of (±)-FTC, (+)-FTC, and (−)-FTC. Anabolism studies were performed by using HepG2-derivative 2.2.15, subclone P5A, cells. Cells were seeded into 75-cm^2 flasks containing GIBCO RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, so that the cell culture was 30% confluent (10^7 total cell number). Twenty-four hours later, 20 μM (±), (+), or (−) enantiomer of FTC was added and the cultures were incubated for an additional 24 or 48 h at 37°C. Following the incubation, cells were washed twice with 5 ml of ice-cold phosphate-buffered saline and extracted in situ with 3 ml of ice-cold 80% acetonitrile. After incubating on ice for 5 min, the extracts were centrifuged at 2,000 × *g* for 10 min at 4°C to remove cellular debris. All extracts were then dried with a Savant Speed-Vac. The dried extracts were resuspended in 500 μl of deionized water. The extracts were analyzed by ion-exchange HPLC by using an Alltech Partisil (10 μ) SAX column (4.6 by 250 mm). The column was eluted for 1,200 s with 0.2 M phosphoric acid–0.8 M KCl–0.05 M $MgCl_2$–5% acetonitrile (pH 3.0) at a rate of 1 ml/min. The eluent profile was monitored at 270 and 200 nm. Peak areas were integrated electronically, and concentrations were calculated by comparison with an internal standard [(±)- or (−)-FTC 5′-triphosphate] that was added to replicate samples at the time of extraction. Intracellular FTC 5′-triphosphate was compared with the chemically synthesized compound for retention time and UV absorbance ratios.

Deoxycytidine kinase assays. Calf thymus deoxycytidine kinase (EC 2.7.1.40) was purified from frozen calf thymus (Pel-Freez Biologicals, Rogers, Ark.) as described previously (13) by using streptomycin sulfate, protamine sulfate, and ammonium sulfate fractionations; this was followed by chromatography on Sephacryl S-200 and Whatman P-11 cellulose phosphate. The resulting preparation was concentrated by precipitation with ammonium sulfate (0.516 g/ml), and the precipitate was dissolved in 0.1 M Tris-HCl buffer (pH 7.6) containing 5 mM dithiothreitol. The concentrated enzyme solution was stored at −70°C and had a specific activity of 18 nmol/min/mg of protein. Deoxycytidine kinase was assayed spectrophotometrically at 25°C as described previously (13). Kinetic constants were determined from initial velocity analysis (2).

Deoxycytidylate kinase assays. Calf thymus deoxycytidylate kinase (EC 2.7.4.14) was partially purified from frozen calf thymus (Pel-Freez Biologicals) following the procedure of Seagrave and Reyes (22), with the following modifications. Thawed calf thymus was blended in buffer A (25 mM potassium phosphate [pH 7.5], 10 mM dithiothreitol, 1 mM $MgCl_2$, 10% glycerol), and crude extract was recovered as the supernatant of a 27,000 × *g* centrifugation. The protamine sulfate step was replaced with a streptomycin sulfate precipitation step (crude extract was made to 10% streptomycin sulfate and dCMP kinase remained in the resulting supernatant). Subsequently, dCMP kinase was precipitated by a 55 to 85% ammonium sulfate fractionation. The dialyzed redissolved pellet was applied to Affi-Gel Blue gel resin (Bio-Rad). The resin was washed with buffer A, and then dCMP kinase was eluted with a gradient of 0 to 1 M KCl in buffer A. Finally, the dialyzed pool was reapplied to a smaller Affi-Gel Blue gel column, the resin was washed with 1 mM ATP in buffer A, and dCMP kinase was eluted with 1 mM ATP–1 mM dCMP in buffer A. The final preparation was purified 670-fold compared with the purity of the crude extract. Nucleoside monophosphate kinase activities were assayed spectrophotometrically as described previously (16), except that 50 mM potassium PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.8] was used as the buffer.

CD assays. (−)-FTC and (+)-FTC were incubated with either human blood or cynomolgus monkey plasma as a source of mammalian cytidine deaminase (CD; EC 3.5.4.5). Human blood collected into 0.1% EDTA was incubated for 3 h at 37°C with 100 μM (−)- and (+)-FTC. Following the incubation, the samples were centrifuged for 5 min by using a microcentrifuge and were then deproteinated by using Centrifree ultra filters (5,000 × *g*, 45 min, 4°C; Amicon, Beverly, Mass.). The extent of deamination of the compounds was determined by reversed-phase HPLC by using a Ranin Microsorb 5 μ C_{18} column (4.3 by 250 mm) equilibrated with 25 mM ammonium phosphate (pH 3.0). Both (−)- and (+)-FTC and their deaminated products were eluted with a gradient of acetonitrile (0 to 12%) over 20 min in the same buffer. The same procedure was used for incubations with cynomolgus monkey plasma, except that the incubations were carried out for 1 h and the concentration of each compound was 500 μM.

Mammalian CD was purified from HeLa cell cytosol by using two successive anion-exchange columns. Forty milligrams of protein was first chromatographed through a column (1 by 10 cm) of Trisacryl M DEAE (2205-300; Pharmacia LKB, Piscataway, N.J.) equilibrated with 50 mM Tris-HCl (pH 8.3)–10% glycerol–1 mM dithiothreitol (equilibration buffer). CD was eluted in 200 mM NaCl with a linear gradient at 2 ml/min. Pooled fractions containing CD (20 mg of total protein) from this column were dialyzed against equilibration buffer and chromatographed through a Mono Q column (0.5 by 5 cm; Pharmacia LKB) at 1 ml/min. Active fractions contained about 180 mM NaCl. Glycerol and dithiothreitol were added to the pooled CD (0.2 μmol of cytidine deaminated per min mg of protein; 60% recovery of activity) to a final concentration of 40% and 2 mM, respectively, and the mixture was stored at −70°C. Assays with purified HeLa cell CD were carried out by incubating cytidine, (−)-FTC, or (+)-FTC at 37°C in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5) with enzyme.

Deamination of (−)- and (+)-FTC by CD was measured by reversed-phase HPLC as described above by using 25 mM ammonium phosphate (pH 3.0) and 9% acetonitrile. The isosbestic wavelength of 277 nm was monitored to determine the ratio of product to substrate. A colorimetric method (1) to determine product formation (ammonia) was used with the purified *Escherichia coli* CD (12).

Endogenous HBV DNA polymerase assay. HBV particles in culture supernatants incorporate nucleoside-5′-triphosphates into plus-strand HBV DNA. Using agarose gel electrophoresis, we monitored the incorporation of [α-^{32}P]deoxynucleoside-5′-triphosphates into the 3.2-kb DNA product in the presence and absence of the 5′-triphosphate of either (−)- or (+)-FTC. HBV particles were collected and concentrated by polyethylene glycol precipitation from culture fluid collected from HepG2 2.2.15, subclone P5A, cells. Clarified culture fluid was mixed with one-fourth volume of a solution containing 50% polyethylene glycol 8000 and 0.6 M NaCl. The virus particles were pelleted by centrifugation at 2,500 × *g* for 15 min. Pellets were resuspended in 2 ml of buffer containing 0.05 M Tris-HCl (pH 7.5) and were dialyzed against the same buffer containing 100 mM KCl. Samples were frozen at −80°C. Each reaction (100 μl) contained HBV particles; 50 mM Tris-HCl (pH 7.5); 300 mM KCl; 50 mM $MgCl_2$; 0.1% Nonidet P-40; 10 μM each dATP, dGTP, and dTTP; and 10 μCi of [^{32}P]dCTP (3,000 Ci/mmol; final concentration, 33 nM; Dupont NEN, Boston, Mass.). Reactions were incubated at 37°C for 1 h and were stopped by adding 50 mM EDTA. SDS was added to a final concentration of 1%, and proteinase K was added to a final concentration of 1 mg/ml. After incubation at 37°C for 1 h, samples were extracted with phenol-chloroform and precipitated with ethanol. DNA was resuspended in gel buffer (0.04 M Tris-acetate, 0.001 M EDTA) and separated by electrophoresis through a 1.5% agarose gel. The gel was dried and exposed to a phosphorimaging screen (10). The image that was obtained by using a Molecular Dynamics Phosphorimager (10) is shown in Fig. 3.

RESULTS

Anti-HBV activity. The anti-HBV activities of (±)-FTC, (+)-FTC, and (−)-FTC in the HBV producer cell line HepG2 2.2.15, subclone P5A, are given in Table 1. Each compound decreased the amount of extra- and intracellular HBV DNA in a dose-dependent fashion. A 50% inhibitory concentration (IC_{50}) of 60 nM was obtained for (±)-FTC on the basis of a reduction in the levels of extracellular virus. This value is comparable to that previously reported by Doong et al. (4). The IC_{50} calculated from the inhibition of intracellular viral DNA was 400 nM. In comparisons of IC_{50}s determined from inhibition of intracellular viral DNA, (−)-FTC was found to be 40-fold more active than (+)-FTC. Both (+)- and (−)-BCH-189 and 2′-CDG were used as positive controls in these studies (Table 1) (4, 18). As with (−)-FTC, (−)-BCH-189 was the more potent enantiomer, with an IC_{50} of 10 nM compared with an IC_{50} of 1,200 nM for (+)-BCH-189. Although 2′-CDG (IC_{50}, 1 nM) was somewhat more active in this assay than was either (−)-FTC or (−)-BCH-189, it was also much more toxic toward uninfected cells (see below) (Table 2).

Comparative cytotoxicities of (±)-FTC, (+)-FTC, and (−)-FTC. The cytotoxicities of (−)-FTC, (+)-FTC, and (±)-FTC were compared in a cell growth assay by using HepG2 2.2.15, subclone P5A, cells and an in vitro human bone marrow progenitor cell assay. Little or no toxicity was exhibited by these compounds in the P5A growth inhibition assay. All IC_{50}s were greater than 200 μM. Only in the human in vitro bone marrow assay were toxicity differences observed (Table 2). (+)-FTC showed more toxicity toward erythroid progenitor cells than did (−)-FTC. The relatively small difference in toxicity between the (−) and (+) enantiomers of FTC was in contrast to observations for the enantiomers of BCH-189. In this case, the (+) enantiomer of BCH-189 showed significant cytotoxicity, while the (−) enantiomer was relatively nontoxic (Table 2). Both BCH-189 and FTC and the respective enantiomers were significantly less toxic in this system than was Retrovir or 2′-CDG.

Anabolism of racemic and resolved FTC. Because (−)- and (+)-FTC are analogs of deoxycytidine, and therefore, the 5′-triphosphate derivatives are potential substrates for viral DNA polymerase, it was important to determine whether these compounds are phosphorylated in cell culture. The nucleotide profile of human hepatocellular carcinoma cells (HepG2) incubated with 20 μM (±)-FTC revealed the presence of the 5′-triphosphate derivative of the compound. Following a 24-h incubation with the compound, the level of the 5′-triphosphate derivative was 22.1 pmol/10^6 cells (15.8 μM). Similar levels of (±)-FTC 5′-triphosphate were formed

TABLE 1. Inhibition of HBV replication in cultures of the HBV producer cell line HepG2 2.2.15, subclone P5A, by increasing concentrations of racemic and resolved FTC

Compound	Concn (μM)[a]	HBV replication (% of control) in [a,b]:		IC_{50} ± SDOM for supernatant virus (μM)[c]
		Supernatant on day 7	Intracellular viral DNA on day 10	
(±)-FTC	0.045	96	55	0.083 ± 0.05
	0.137	43	70	
	0.4	15	55	
	1.2	6	31	
	3.7	0.6	32	
	11.0	0	37	
	33.0	4	42	
(+)-FTC	0.137	142	85	0.96 ± 0.5
	0.4	68	79	
	1.2	76	34	
	3.7	48	19	
(−)-FTC	0.001	89	120	0.01 ± 0.005
	0.006	67	100	
	0.036	25	73	
	0.216	36	28	
	1.2	13	20	
	7.8	0	30	
	46.0	0	20	
(+)-BCH-189[d]	0.045	111	75	0.86 ± 0.03
	0.137	106	88	
	0.4	88	42	
	1.2	50	15	
3TC[d]	0.001	83	108	0.008 ± 0.003
	0.006	57	125	
	0.036	31	110	
	0.216	3	63	
	1.2	7	20	
	7.8	3	19	
	46	0	20	

[a] Values are from a single determination, but are representative for all the experiments that were run.

[b] Untreated controls averaged 120 pg of minus-strand HBV DNA per ml of culture medium and 500 pg of minus-strand HBV DNA per extract in intracellular extracts.

[c] IC_{50}s were calculated by fitting the data by using the Probit computer program (SAS Probit procedure, version 82.4; SAS Institute, Cary, N.C.). Each (IC_{50}) is the mean of three independent determinations ± the standard deviation of measurement (SDOM).

[d] (+)-BCH-189 and (−)-BCH-189 (3TC) were included for comparison.

TABLE 2. Cytotoxicity assay with human bone marrow progenitor cells

Compound	IC_{50} ± SE (μM)[a]		No. of expt
	CFU-GM	BFU-E	
(±)-FTC	250 ± 30	100 ± 5	8
(+)-FTC	200 ± 10	70 ± 8	6
(−)-FTC	300 ± 40	220 ± 8	6
(±)-BCH-189	90 ± 10	5 ± 1	8
(+)-BCH-189	10 ± 2	4 ± 1	6
(−)-BCH-189 (3TC)	250 ± 8	180 ± 2	6
Zidovudine	10 ± 3	0.3 ± 0.06	55
2′-CDG[b]	0.4 ± 0.3	4 ± 2	3

[a] CFU-GM, CFU-granulocyte macrophage; BFU-E, burst-forming unit–erythroidal.

in the P5A cells (Fig. 2). The ability of P5A cells to phosphorylate both (−)- and (+)-FTC to the corresponding 5′-triphosphate forms was also investigated (Fig. 2). Comparison of the rate of formation and the steady-state levels of the 5′-triphosphate formed in cells incubated with identical concentrations of each of the three compounds showed that the amount of 5′-triphosphate decreased in the following order: (−)-FTC > (±)-FTC > (+)-FTC.

Inhibition of HBV DNA synthesis. The 5′-triphosphate of (−)-FTC but not that of (+)-FTC inhibited HBV DNA synthesis in an endogenous DNA polymerase reaction (Fig. 3). While (+)-FTC did not affect product formation, (−)-FTC inhibited product formation in a dose-dependent manner. In order to determine whether (−)-FTC competes only with dCTP for binding to the enzyme or with other 2′-deoxynucleoside 5′-triphosphate substrates as well, competition studies were performed. In those studies, the ability of increased concentrations of dCTP, dTTP, or dGTP to reverse the ability of (−)-FTC 5′-triphosphate to block the incorporation of [^{32}P]dATP into product was examined. The reaction conditions were the same as those outlined in Materials and Methods, except that the concentration of a given substrate was increased. A 10-fold excess (330 nM) of dCTP blocked inhibition by (−)-FTC 5′-triphosphate (data not shown). It could not be definitively determined from these experiments whether (−)-FTC 5′-triphosphate was an alternate substrate for the HBV polymerase. Control experiments, in which unphosphorylated (−)-FTC was used at concentrations up to 50 μM, showed no inhibition of product formation catalyzed by the HBV DNA polymerase.

Phosphorylation of (−)- and (+)-FTC by deoxycytidine kinase and deoxycytidylate kinase. The structural similarity of (−)-FTC and deoxycytidine suggested that deoxycytidine kinase might catalyze the phosphorylation of this analog to the monophosphate derivative. When (−)-FTC and (+)-FTC were incubated with deoxycytidine kinase purified from calf thymus, both analogs served as substrates for the enzyme and had similar apparent K_m (K_m′) values (Table 3). However, the apparent V_{max} (V_{max}′) for (−)-FTC was approximately fourfold greater than that for (+)-FTC. Both of the 5′-monophosphate forms of (−)-FTC and (+)-FTC were substrates for deoxycytidylate kinase (Table 3). The K_m′

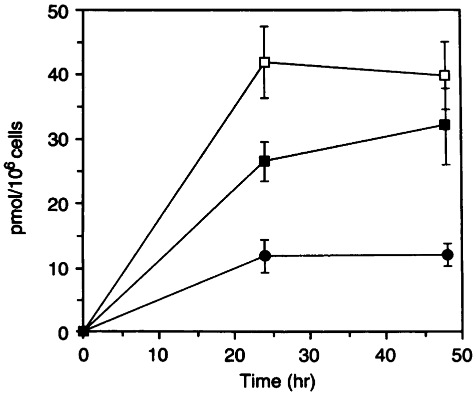

FIG. 2. Time course for the anabolism of (+)-FTC (●), (−)-FTC (□), and (±)-FTC (■) in HepG2 2.2.15, subclone P5A, cells to their respective 5′-triphosphates. Cells were incubated with (+)-, (−)-, or (±)-FTC at a final concentration of 20 μM for up to 48 h. Cell extracts were analyzed by SAX HPLC.

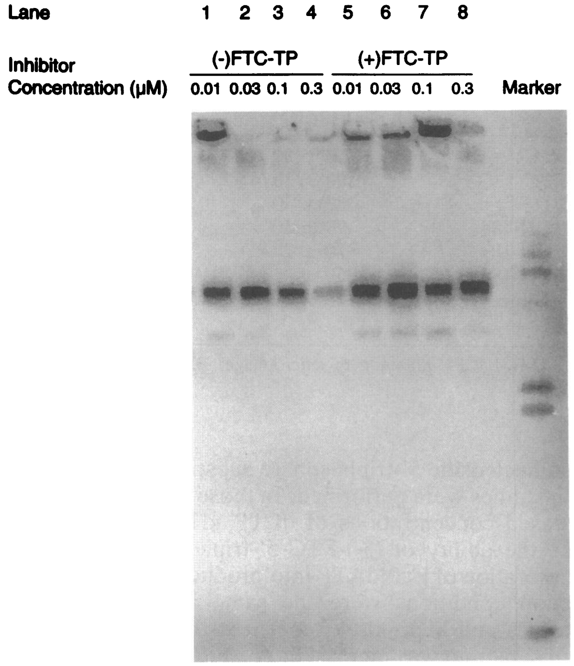

FIG. 3. Inhibition of dCTP incorporation into HBV DNA by the 5′-triphosphate of (+)-FTC or (−)-FTC. The marker lane contains radiolabeled bacteriophage lambda DNA digested with *Hin*dIII restriction nuclease. Lanes 1 to 8 contain the products of dCTP incorporation into HBV DNA in the presence of (+)-FTC and (−)-FTC.

value for (−)-FTC 5′-monophosphate was 2- and 2.8-fold lower than the K_m' values for dCMP and (+) FTC 5′-monophosphate, respectively. Furthermore, the V_{max}' for (−)-FTC 5′-monophosphate was approximately 2.9-fold greater than the V_{max}' for (+)-FTC 5′-monophosphate.

(−)- and (+)-FTC as substrates for CD. One concern for the cytidine series of analogs was that deamination would lead to the formation of the inactive uridine analogs. In addition, the 5-fluoro-2′-deoxy-3′-thiauridine analog of FTC is a potential precursor for the generation of 5-fluorouracil via cleavage of the glycosidic linkage. (−)- and (+)-FTC were incubated either with human or monkey plasma as a source of enzyme or with purified enzyme from *E. coli* or HeLa cells. No deamination of (−)-FTC was observed when the compound was incubated with either human blood or monkey plasma. However, 7 and 65% of (+)-FTC was deaminated with human blood and monkey plasma, respectively. The results of incubating (−)- and (+)-FTC with purified CD are given in Table 3. (+)-FTC was a significantly better substrate for both purified enzymes.

DISCUSSION

Racemic FTC is a potent inhibitor of HBV replication in vitro, as shown here and as reported previously (4). Testing of the resolved enantiomers has revealed (−)-FTC to be approximately 40-fold more potent than (+)-FTC. It is possible that the (+) isomer has no intrinsic activity and that the apparent activity is due to the 4% contamination with the (−) isomer.

With the exception of 2′-CDG, which is highly toxic, (−)-FTC is one of the most potent anti-HBV compounds identified to date in cell culture. The intrinsic lack of activity suggested for (+)-FTC in cell culture is supported by the results of the endogenous DNA polymerase assay in which the 5′-triphosphate of (−)-FTC, but not that of (+)-FTC, is an inhibitor of DNA polymerase activity. Diminished activity of the (+) isomer has also been seen against HIV type 1 in cellular assay systems (21). However, the difference in the anti-HIV activities of the two isomers is only 10-fold. In addition, the 5′-triphosphates of (−)- and (+)-FTC have comparable K_i values (2.8 and 8.6 µM, respectively) for purified HIV type 1 reverse transcriptase (21). Therefore, the differential activities of the two isomers cannot be attributed to differential recognition by the HIV reverse transcriptase. One possible explanation suggested by the anabolism studies in HepG2 cells is that the (−) isomer is more extensively phosphorylated. Although HBV and HIV replication both involve reverse transcription of an RNA template and HBV DNA polymerase and HIV type 1 reverse transcriptase share sequence homology (18), the enantioselectivity of the HBV DNA polymerase suggests that there may be significant differences in the topologies of the substrate binding sites of the two enzymes.

Neither (−)- nor (+)-FTC showed toxicity toward the parental human hepatoma cell line HepG2 (IC_{50}s, >200 µM). In the human bone marrow progenitor cell assay, (−)-FTC again displayed minimal toxicity, while (+)-FTC showed slightly increased toxicity toward erythroid progenitor cells. It is interesting that the isomers of BCH-189 have strikingly different toxicity profiles. While (−)-BCH-189 has only minimal toxicity against HepG2 cells (3) and human bone marrow progenitor cells (Table 2), (+)-BCH-189 showed significant toxicity in both assays. At present, we cannot

TABLE 3. Substrate properties of (+)-FTC and (−)-FTC

Substrate[a]	Phosphorylation by:				Deamination by HeLa cell and *E. coli* CDs	
	CTDK[b]		CTDMK[c]			
	K_m' (µM) (±SE)	V_{max}' ± SE[d]	K_m' (µM)	V_{max}'[e]	V_{max}', HeLa CD[f]	V_{max}', *E. coli* CD[f]
dCMP			0.98	100		
(+)-FTC	18 ± 3.7	3.2 ± 0.13	1.4	5.8	8.6	1.1
(−)-FTC	23 ± 1.0	12.0 ± 0.17	0.5	16.8	<0.0017	0.03

[a] A minimum of nine initial velocity measurements were made at substrate concentrations ranging from 2 to 1,000 µM in the calf thymus deoxycytidine kinase assay. The concentration of substrate used in the CD assay with the HeLa cell enzyme was 0.5 mM, and with the *E. coli* enzyme it was 1 mM.

[b] CTDK, calf thymus deoxycytidine kinase.

[c] CTDMK, calf thymus deoxycytidine-5′-monophosphate kinase.

[d] Percent relative to deoxycytidine.

[e] Percent relative to deoxycytidine-5′-monophosphate.

[f] Percent relative to cytidine.

explain the difference in the toxicity profiles resulting from substitution of F for H at the 5′ position.

Since the unphosphorylated (−)-FTC did not inhibit HBV DNA polymerase, the antiviral activity of the compound is probably dependent on conversion to the corresponding 5′-triphosphate. Anabolism experiments carried out in P5A cells showed that both (−)- and (+)-FTC are converted to the corresponding 5′-triphosphate derivatives. However, (−)-FTC is anabolized more readily than is (+)-FTC. These results suggest that the differences in activities against HIV type 1 seen in cellular assay systems may be explained, in part, by the difference in substrate efficiency for deoxycytidine kinase and deoxycytidylate kinase that exists between (−)- and (+)-FTC (Table 3).

One concern for these cytidine analogs was the possibility of catabolism by CD to give the corresponding uridine analog. The uridine analogs of FTC have been shown to be inactive against HBV in cellular assays (4). In addition, the uridine analog of FTC is a potential precursor of 5-fluorouracil via cleavage of the glycosidic linkage. Studies of the action of CD on FTC (Table 3) indicated that (+)-FTC but not (−)-FTC is a substrate for mammalian CD. These results are in contrast to an earlier report in which inhibitors of CD and deoxycytidine deaminase were found not to increase the antiviral efficacy of (±)-FTC in human hepatoma cells (4). The conclusion was that no deamination occurred. However, our data suggest that deamination of (+)-FTC could have occurred, but because (+)-FTC has little or no anti-HBV activity, there was no effect on the apparent potency of (±)-FTC.

In summary, (−)-FTC is a potent and selective anti-HBV compound. The site of action appears to be the virally encoded DNA polymerase. Activity at the polymerase requires the compound to be phosphorylated at the 5′ position. This phosphorylation occurs in HBV-infected HepG2 cells where substantial levels of the 5′-triphosphate are formed. Although there is a possibility that (−)-FTC is deaminated by CD to the inactive uridine derivative, results of the present study show that the compound is a poor substrate for the enzyme. Catabolic enzymes preferentially recognize the 1-β-D configuration of the (+) isomer, while the anabolic enzymes and the polymerases prefer the 1-β-L configuration of the (−) isomer. The selective activity and favorable enzymatic profile of (−)-FTC indicate that this compound is worthy of additional investigation for the treatment of HBV infections.

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