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Intracellular Metabolism of (−)- and (+)-*cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolan-5-yl]Cytosine in HepG2 Derivative 2.2.15 (Subclone P5A) Cells

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The (−) and (+) enantiomers of the nucleoside analog *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (2′,3′-dideoxy-5-fluoro-3′-thiacytidine; FTC) have been shown to inhibit hepatitis B virus replication in vitro in HepG2 derivative 2.2.15 (subclone P5A) cells. (−)-FTC and (+)-FTC were anabolized to 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate in this cell line. (−)-FTC was more efficiently phosphorylated to the 5′-triphosphate than (+)-FTC, and levels of 3.6 and 0.2 pmol/10^6 cells, respectively, were detected after incubation with 1 μM compound for 24 h. A time course study showed that nucleotides were formed rapidly in a dose-dependent manner and reached a steady-state intracellular concentration by 3 to 6 h. The intracellular half-life of (−)-FTC 5′-triphosphate was 2.4 h. Both (−)- and (+)-FTC were converted to diphosphocholine derivatives, analogous to CDP-choline, but only (+)-FTC was converted to the diphosphoethanolamine derivative, analogous to CDP-ethanolamine. (−)-FTC was not detectably deaminated at either the nucleoside or nucleotide level. (+)-FTC was partially deaminated by these cells. The transport of (−)- and (+)-FTC was examined in HepG2 cells. (+)-FTC enters these cells by way of the nitrobenzylthioinosine-susceptible, equilibrative nucleoside transporter. In contrast, the influx of (−)-FTC was only partially susceptible to inhibitors of nucleoside transport, indicating that (−)-FTC may have multiple transport mechanisms. These metabolic results are consistent with the conclusion that (−)-FTC 5′-triphosphate mediates the anti-hepatitis B virus activity of (−)-FTC.

Hepatitis B virus (HBV) is estimated to chronically infect 200 million to 300 million people worldwide. These chronically infected individuals have a 100-fold elevated risk for the development of hepatocellular carcinoma (5). Current therapy for chronic carriers is limited to interferon, which benefits a minority of the infected patient population (25, 28). In recent years, the propagation of hepadnaviruses in human hepatocellular carcinoma cell lines has made it possible to examine the in vitro efficacies of potential antiviral drugs and to study their intracellular metabolisms (30).

The replication of hepadnaviruses requires a reverse transcriptase activity (32). Zalcitabine (2′,3′-dideoxycytidine; ddC), which has been shown to be a potent inhibitor of human immunodeficiency virus replication, has also been demonstrated to have in vitro efficacy against human and duck HBVs (23, 24). Related analogs have recently been reported to have potent anti-HBV and anti-human immunodeficiency virus activities. Several laboratories have shown that (±)-2′,3′-dideoxy-3′-thiacytidine (BCH-189) and (−)-*cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (2′,3′-dideoxy-5-fluoro-3′-thiacytidine; FTC) (Fig. 1) exhibit potent anti-human immunodeficiency virus (12, 29, 38) and anti-HBV activities in HepG2 (derivative 2.2.15) and HepG2 derivative 2.2.15 (subclone P5A) cells (9, 14, 15). Furman et al. (15) showed that (−)-FTC has a 10-fold lower 50% inhibitory concentration (IC_{50}) for the inhibition of HBV replication in HepG2 derivative 2.2.15 (subclone P5A) than (+)-FTC. The potent antiviral activity and very low toxicity of this compound make (−)-FTC a promising candidate for antiviral therapy. This report describes a study of the intracellular anabolism of (−)-FTC in HepG2 derivative 2.2.15 (subclone P5A) cells.

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MATERIALS AND METHODS

Purity of FTC. FTC was prepared at Wellcome Research Laboratories (Research Triangle Park, N.C.) by published methods (10). FTC exists as (−) and (+) enantiomers (Fig. 1) which can be separated both enzymatically and by chiral chromatography. In order to determine the relative contamination of (−)-FTC with (+)-FTC and vice versa, chiral high-performance liquid chromatography (HPLC) with a 4.6-by-250-mm acetylated β-cyclodextrin column (Cyclobond I; Astec, Inc., Whippany, N.J.) was used. The column was eluted at a rate of 1 ml/min in an isocratic fashion with 0.1% triethylamine in water containing 0.1% acetonitrile at pH 7.3. The (−)-FTC and (+)-FTC were eluted with retention times of 492 and 538 s, respectively. The contamination of (−)-FTC by (+)-FTC was found to be less than 1%. The contamination of (+)-FTC by (−)-FTC was found to be less than 2%.

Purities of [6-^{3}H](−)- and [6-^{3}H](+)-FTC. Tritiated (−)-FTC and (+)-FTC were commercially obtained from Moravek Biochemicals (Brea, Calif.). Analysis of the tritiated products by the methods described above showed a 2.7% contamination by [^{3}H](+)-FTC in the [^{3}H](−)-FTC preparation and a 2.5% contamination by [^{3}H](−)-FTC in the [^{3}H](+)-FTC preparation. Digestion of [^{3}H](−)-FTC with 160 U of *Escherichia coli* cytidine deaminase (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M K_2HPO_4 (pH 7.4) at 37°C for 75 min resulted in a product that contained less than 0.1% [^{3}H](+)-FTC. The digested product was then further purified via chromatography on a 1.5-by-4-cm column containing QAE Sephadex A-25 (Sigma)

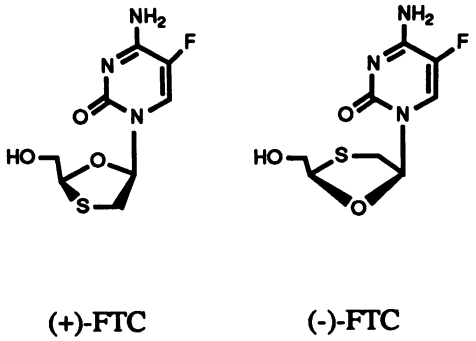

FIG. 1. Structures of the (+) and (−) enantiomers of FTC.

equilibrated in 300 mM ammonium hydroxide and then solid-phase extraction on a Sep-Pak C18 cartridge (Millipore, Milford, Mass.) equilibrated in deionized water and eluted with 100% methanol. The purified [^{3}H](−)-FTC product had a specific activity of 9 Ci/mmol. [^{3}H](+)-FTC was purified by chiral chromatography as described above. The purified [^{3}H](+)-FTC product had a specific activity of 9 Ci/mmol and contained less than 0.1% [^{3}H](−)-FTC.

Transport of (−)-FTC and (+)-FTC into HepG2 cells. HepG2 cells for transport assays were obtained from the American Type Culture Collection and were cultured in minimum essential medium with Earle's salts (GIBCO, Grand Island, N.Y.) containing 2 mM glutamine and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Transport assays were performed on confluent HepG2 monolayers at room temperature according to modifications of a rapid, cold buffer stop assay (34). Monolayers (2.5×10^6 to 3.5×10^6 cells) in six-well plates were washed with serum-free Hanks' balanced salt solution (BSS) containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and were incubated in 1.5 ml of fresh serum-free Hanks' BSS for 10 min at room temperature on a rocker platform. Assays were initiated by the addition of 0.5 ml of [^{3}H](−)-FTC (0.2 to 1.1 μCi) or [^{3}H](+)-FTC (0.1 to 0.3 μCi) in serum-free Hanks' BSS to a total volume of 2.0 ml. After the appropriate incubation time, the medium was aspirated and the monolayers were rapidly washed twice with 5 ml of ice-cold serum-free Hanks' BSS. A simplified medium (Na^+-transport buffer) consisting of 120 mM NaCl, 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose (pH 7.4) (36) was used for the experiments measuring Na^+ dependence. When appropriate, NaCl was replaced with 120 mM KCl in the medium. Cells were digested in situ with 1 ml of 0.5 N NaOH and were neutralized with 60 μl of dilute H_3PO_4. Radioactivity was quantitated by liquid scintillation counting in 5 ml of Scinti-Verse BD (Fisher Scientific, Fair Lawn, N.J.). The initial velocities of FTC influx were determined by linear regression analysis of the slopes of cell-associated [^{3}H]FTC versus time for duplicate assays. Kinetic constants were determined by directly fitting the data to a hyperbola by the method of Wilkinson (37) and using the computer program of Cleland (11). The intracellular water volume was determined for HepG2 cells in suspension by subtracting the extracellular space, measured by using [^{14}C]sucrose, from the total water volume, measured by using ^3H_2O. Separation of extracellular label from the cell-associated label was accomplished by centrifugation through silicone-paraffin oil (84:16).

Cell culture. The HepG2-derived cell line 2.2.15 (subclone P5A) was obtained from B. Korba of Georgetown University (21). Cells were cultured in GIBCO RPMI 1640 medium containing 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (Hyclone) at 37°C in a humidified incubator with 5% CO_2 (21). Cells were seeded into triplicate T-75 flasks at approximately 30% confluency (10^7 cells total). Twenty-four hours later, either FTC or [^{3}H]FTC (75 mCi/mmol) was added at the appropriate final concentration. For the double-label experiments, cells were incubated with either 10 μM [^{3}H](+)-FTC or 10 μM [^{3}H](−)-FTC (100 mCi/mmol) and either 40 μM [^{14}C]choline chloride (28 mCi/mmol; Amersham, Arlington Heights, Ill.) or 40 μM [^{14}C]ethanolamine (27 mCi/mmol; Amersham). The cultures were incubated as described above for the time interval indicated in each experiment. The cell number was determined by trypsinizing cells from replicate flasks and counting the cells by using a hemocytometer. For samples used in the investigation of the half-life of (−)-FTC 5′-triphosphate, the cells were washed twice with 5 ml of phosphate-buffered saline (PBS) at 37°C before reincubation in medium containing no drug for 2, 4, 6, 12, 24, 36, or 48 h.

Extraction for HPLC analysis. At the conclusion of an incubation, aliquots of the medium from each sample were deproteinized by microcentrifugation in Ultrafree cartridges (molecular weight cutoff, 10,000; Millipore, Milford, Mass.) for 10 min at ambient temperature prior to HPLC analysis. Cells were washed twice with 5 ml of ice-cold PBS, and the cell monolayers were extracted in situ with 3 ml of ice-cold 80% acetonitrile. After incubating on ice for 5 min, the extracts were centrifuged at $2,000 \times g$ for 10 min at 4°C to remove cellular debris. Extracts were then dried by using a Savant Speed-Vac. The dried extracts were reconstituted in 500 μl of deionized water and were stored at −20°C until further analysis. Recovery of nucleoside 5′-triphosphates was greater than 95%, as determined by the addition of recovery standards to replicate cell extracts.

HPLC. The cellular extracts were analyzed by ion-exchange HPLC by using a 4.6-by-250-mm, 10-μm, Partisil SAX column (Alltech, Deerfield, Ill.). The mobile phase was formed by mixing buffer A (2 mM KH_2PO_4, 8 mM KCl, 5.5 mM $MgCl_2$, 0.05% acetonitrile [pH 3.0]) and buffer B (0.2 M KH_2PO_4, 0.8 M KCl, 0.05 M $MgCl_2$, 5% acetonitrile [pH 3.0]) in proportion such that the total flow rate was 1.0 ml/min. The elution gradient profile consisted of four segments, an initial isocratic step for 10 min in 100% buffer A that was followed by a linear gradient of 0 to 52% buffer B over 32 min and a linear gradient of 52 to 100% buffer B over 16 min and that ended with an isocratic elution in 100% buffer B for an additional 30 min. The UV absorbance of the eluent was monitored at 270 and 300 nm by using a Milton Roy SM4000 detector (Laboratory Data Control, Riviera Beach, Fla.). Intracellular metabolites were compared with authentic standards for retention times and UV absorbance ratios when appropriate. Authentic standards of (−)-FTC 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate were provided by Jeanne Wilson of our laboratories (15).

Aliquots of media and isolated metabolites were analyzed by reversed-phase HPLC on a 4.6-by-250-mm octadecylsilica column (Microsorb; Rainin, Inc., Woburn, Mass.) at 1 ml/min in 0.1% trifluoroacetic acid and 0.05% triethylamine (pH 2.2) with a linear gradient of from 2 to 16.5% acetonitrile over 20 min. Fractions were collected at 1-min intervals, and the radioactivity was quantitated by liquid scintillation counting in 5 ml of Ready Safe scintillation fluid (Beckman, Fullerton, Calif.).

Selected fractions from the ion-exchange HPLC analysis of the ^{3}H and ^{14}C double-label experiment were desalted by

mixing the fractions with 0.1 volume of concentrated ammonium hydroxide and 3 volumes of 100% methanol. The supernatants were then dried in a Savant Speed-Vac. The residue was resuspended in 10 mM $MgCl_2$, and the mixture was split into three aliquots. Two of the aliquots were digested with either alkaline phosphatase or phosphodiesterase I, as described below. The ultrafiltrates were then analyzed by reversed-phase HPLC on a 4.7-by-235 mm octadecylsilica column (Partisphere; Whatman, Maidstone, England) at 1 ml/min in 20 mM ammonium acetate (pH 5.0) with a linear gradient of from 0.6 to 3.6% acetonitrile over 50 min. Fractions of the column effluent were collected at 1-min intervals, and the radioactivity was quantitated by liquid scintillation counting in 5 ml of Ready Safe (Beckman).

Digestion of cellular extracts with phosphodiesterase I and alkaline phosphatase. Cell extracts corresponding to approximately 10^7 cells were incubated overnight at 37°C with 90 U of alkaline phosphatase (Boehringer, Mannheim, Germany) and 5 U of snake venom phosphodiesterase I (Pharmacia, Uppsala, Sweden) in 100 μl of 100 mM K_2HPO_4 (pH 8). Samples were deproteinated by microcentrifugation in Ultrafree cartridges (molecular weight cutoff, 10,000; Millipore) for 10 min at ambient temperature. The ultrafiltrate was then analyzed by ion-exchange HPLC on a SAX column as indicated above, and the amount of radioactivity in each fraction obtained over 1 min was determined by liquid scintillation counting.

(−)-FTC 5′-monophosphate synthesis. The nucleoside [(−)-FTC, 0.12 g, 0.49 mmol] was dissolved in 2.4 ml of 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (Aldrich, Milwaukee, Wis.). After cooling this solution to −10°C in an ice-methanol bath, 0.18 ml of phosphorus oxychloride (1.7 mmol) was added with vigorous stirring. The reaction was stopped after 1 min by adding 10 ml of cold water. Following incubation on ice for 2 h, the pH was adjusted to 1 with sodium hydroxide. The reaction was approximately 90% complete, as judged by thin-layer chromatography on cellulose in *n*-propanol–15 M NH_4OH–H_2O (6/3/1), monophosphate (R_f = 0.27).

The reaction mixture was diluted to 700 ml with water, and the solution was applied to a 2.5-by-10-cm chromatography column containing 50 ml of AG50W-X8 (hydrogen form; Bio-Rad, Hercules, Calif.) which had previously been equilibrated with water. The column was eluted with water. The (−)-FTC 5′-monophosphate eluted between 750 and 1,200 ml of eluent. Fractions containing nucleotide were pooled and dried in vacuo.

Choline phosphate preparation. Choline phosphate calcium chloride (5.1 g, 15 mmol; Aldrich) dissolved in 50 ml of water was added to 100 ml of 1 M ammonium bicarbonate. The resulting calcium carbonate precipitate was removed by centrifugation. The choline phosphate was separated from chloride by chromatography on Dow 1 ion-exchange resin (Sigma) in 50 mM ammonium bicarbonate and was then converted to the free acid with Dow 50 ion-exchange resin (acid form; Sigma).

(−)-FTC diphosphocholine synthesis. Reaction mixtures contained 0.02 mmol of (−)-FTC 5′-monophosphate, 0.36 mmol of choline phosphate, 1.2 mmol of 1,3-dicyclohexylcarbodiimide, 0.4 ml of water, and 3 ml of pyridine. After stirring at 60°C for 2 h, the reaction was stopped by adding 5 ml of deionized water. Several reactions were done on this scale, with conversions to (−)-FTC diphosphocholine ranging from 30 to 70%. The combined reaction mixtures [0.17 mmol of (−)-FTC 5′-monophosphate as total starting material] were diluted to 170 ml with deionized water and were applied to a chromatography column containing 50 ml of DEAE Sephadex A-25 (Pharmacia, Piscataway, N.J.) which had previously been equilibrated in 50 mM ammonium bicarbonate. The column was washed with 50 mM ammonium bicarbonate to elute the (−)-FTC diphosphocholine. This chromatography did not completely resolve the excess choline phosphate from the (−)-FTC diphosphocholine, so the mixture was treated with 1,700 U of alkaline phosphatase in 30 ml of 50 mM ammonium bicarbonate for 100 min at 50°C. The chromatography step was repeated to yield 0.068 mmol (40%) of (−)-FTC diphosphocholine.

(−)-FTC diphosphocholine spectra. In 0.1 M HCl, λ_{max} was at 291 nm and λ_{min} was at 247 nm. In 50 mM potassium phosphate (pH 7.0), λ_{max} was at 237 and 279 nm and λ_{min} was at 226 and 259 nm. In 0.1 M NaOH, λ_{max} was at 236 and 279 nm and λ_{min} was at 228 nm and 259 nm. These spectra are consistent with those of the parent nucleoside.

^1H nuclear magnetic resonance in D_2O showed δ 8.0 (d, 1H, H-6), 6.1 (t, 1H, 1′), 5.3 (t, 1H, 4′), 4.2 (m, 3H, OCH_2 overlapping with H-5′), 4.1 (m, 1H, H-5′), 3.5 (m, 2H, NCH_2), 3.4 (m, 1H, H-2′), 3.1 (m, 1H, H-2′), 3.0 (m, 9H, NCH_3).

^{31}P nuclear magnetic resonance (D_2O) gave two doublets at δ −11.5 and −12.1.

Ion-spray negative-ion mass spectrometry yielded peaks at *m/e* of 493 ($M\text{-}H^+$) and *m/e* of 515 $(M\text{-}H + Na)^+$.

The (−)-FTC diphosphocholine was cleaved by snake venom phosphodiesterase I to yield a single peak with an HPLC retention time identical to that of (−)-FTC 5′-monophosphate.

The base/phosphate ratio was 1.0/1.9. The concentration of total phosphate was determined by the method of Ames (3). The concentration of aglycon was determined by using the UV extinction coefficient of the nucleoside (280 nm, pH 7, ε = 7,700 M^{-1} cm^{-1}).

RESULTS

Transport of (−)-FTC into HepG2 cells. The influx of (−)-FTC into HepG2 cells was nonconcentrative and did not depend on the presence of a Na^+ gradient (Fig. 2A). Influx proceeded slowly, and at 20 min, 89% of the intracellular radioactivity was unmetabolized [^3H](−)-FTC. Therefore, initial rates were determined at time points not exceeding 10 min. A K_m of 4 mM and a maximum rate of influx (V_{max}) of 338 pmol/min/10^6 cells were obtained. Influx was only partially inhibited by nucleosides and by inhibitors of nucleoside transport, while nucleoside aglycons were ineffective in inhibiting influx (Table 1).

Transport of (+)-FTC into HepG2 cells. The influx of (+)-FTC into HepG2 cells was slow, reaching equilibrium at approximately 20 min, whereupon the intracellular concentration of (+)-FTC was equal to the extracellular concentration (Fig. 2B). Na^+-dependent transport was not detected, since rates identical to the control rate were obtained when NaCl was replaced with KCl in the transport buffer. A K_m of 77 μM and a V_{max} of 23 pmol/min/10^6 cells were determined. Transport was inhibited 91% by 10 μM nitrobenzylthioinosine (NBMPR).

Intracellular metabolism of (−)-FTC. HepG2 derivative 2.2.15 (subclone P5A) cells were incubated for 24 h with [^3H](−)-FTC at an initial concentration of 1.0 μM. Figure 3A shows an ion-exchange high-performance liquid radiochromatogram of an extract from these cells. (−)-FTC and its respective 5′-mono-, 5′-di-, and 5′-triphosphates were initially identified by comparison of their retention times with those of authentic standards. Their identities were confirmed by enzymatic digestion with alkaline phosphatase, which resulted in peak shifting on HPLC on a SAX column (Fig. 3C). The

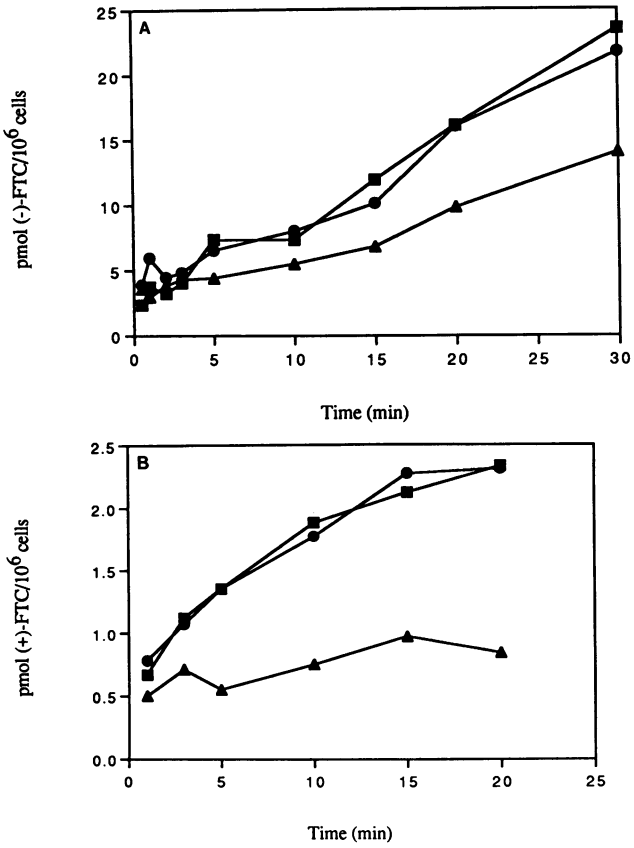

FIG. 2. (A) Time dependence of (−)-FTC influx into HepG2 cells. Monolayers were incubated with 10 μM [^{3}H](−)-FTC in Na^+ transport buffer (●), transport buffer with 120 mM KCl instead of NaCl (■), or Na^+ transport buffer containing 10 μM NBMPR (▲), as described in the text. (B) Time dependence of (+)-FTC influx into HepG2 cells. Monolayers were incubated with 1 μM [^{3}H](+)-FTC in Na^+ transport buffer (●), transport buffer with 120 mM KCl instead of NaCl (■), or Na^+ transport buffer containing 10 μM NBMPR (▲), as described in the text.

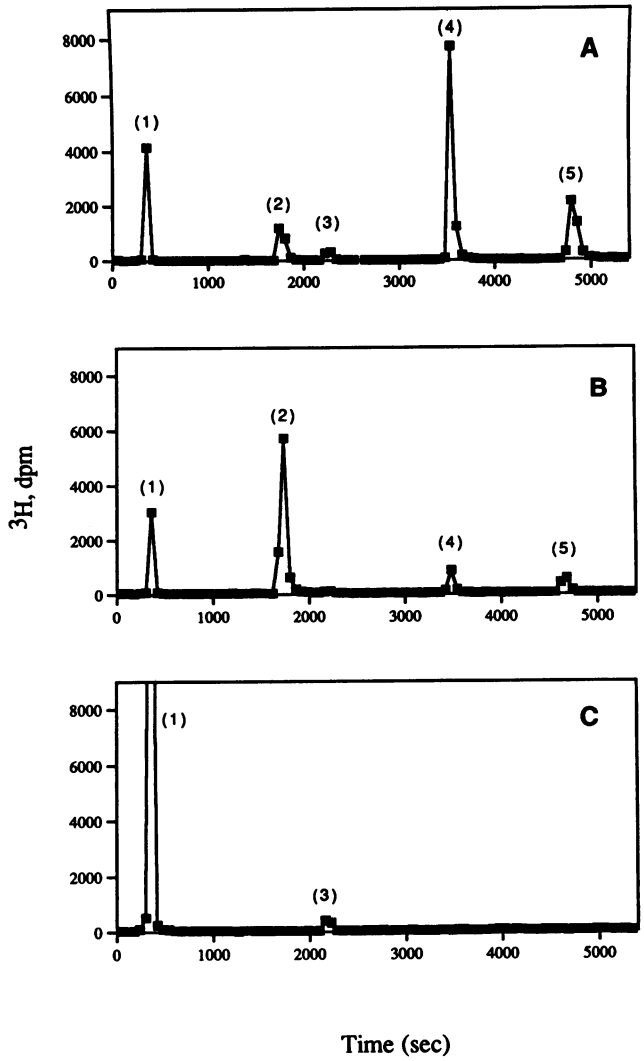

FIG. 3. Intracellular metabolites of (−)-FTC in HepG2 derivative 2.2.15 (subclone P5A) cells. Cells were incubated for 24 h with 1.0 μM (−)-FTC prior to their extraction for analysis. Aliquots of the cell extract received no treatment (A) or were digested with snake venom phosphodiesterase I (B) or alkaline phosphatase (C) and were chromatographed by HPLC on a SAX column. Individual peaks are identified as follows: 1, (−)-FTC; 2, (−)-FTC 5′-monophosphate; 3, metabolite M2280; 4, (−)-FTC 5′-diphosphate; and 5, (−)-FTC 5′-triphosphate.

intracellular concentrations of the individual metabolites are given in Table 2. The deaminated form of (−)-FTC, 2′,3′-dideoxy-5-fluoro-3′-thiauridine (FTU), was not observed as an intracellular metabolite. An FTU standard was generated via deamination of FTC (19). (−)-FTC was the sole radiolabeled analyte found in the extracellular medium.

An unknown metabolite (M2280) eluted at 2,280 s, between the 5′-mono- and 5′-diphosphates of (−)-FTC. Comparison of this metabolite profile with that of ddC suggests that M2280 may be the diphosphocholine derivative of (−)-FTC (12, 34). In order to characterize M2280 further, aliquots of the cell extract were digested with either snake venom phosphodiesterase I or alkaline phosphatase. Phosphodiesterase I cleaves only phosphodiester bonds, whereas alkaline phosphatase is a monoesterase. If M2280 were a diphosphocholine derivative of (−)-FTC, it would be susceptible to phosphodiesterase I cleavage but insusceptible to alkaline phosphatase digestion. The 5′-di- and 5′-triphosphates of (−)-FTC, but not the 5′-monophosphate, should be susceptible to phosphodiesterase I, whereas all three phosphates should be susceptible to alkaline phosphatase digestion.

As shown in Fig. 3B, treatment of the cellular extract with phosphodiesterase I resulted in the digestion of M2280 and the 5′-di- and 5′-triphosphates of (−)-FTC. However, as shown in

TABLE 1. Inhibition of 10 μM (−)-FTC influx into HepG2 cells by inhibitors of nucleoside transport, nucleosides, and adenine at 20°C

Agent	Concn (μM)	% Inhibition	No. of expts
NBMPR	10	45	6
Dipyridamole	10	48	6
Dilazep	10	35	6
Cytidine	500	37	3
Uridine	500	69	2
Inosine	500	60	2
Adenine	500	14	3

TABLE 2. Concentration of intracellular anabolites in HepG2 derivative 2.2.15 (subclone P5A) cells incubated with [6-^{3}H](−)-FTC or [6-^{3}H](+)-FTC at the specified concentrations for 24 h

Sample	Concn (μM)	Concn of intracellular anabolites (pmol/10^6 cells)[a]						
		Nucleoside	M1380[b]	M1560[b]	Mono-PO_4	M2280[b]	Di-PO_4	Tri-PO_4
(−)-FTC	0.01	0.04 ± 0.01			0.05 ± 0.02	0.01 ± 0.00	0.17 ± 0.08	0.11 ± 0.04
(−)-FTC	0.1	0.20 ± 0.00			0.20 ± 0.01	0.09 ± 0.01	0.78 ± 0.25	0.51 ± 0.12
(−)-FTC	1.0	3.10 ± 0.15			1.84 ± 0.31	0.58 ± 0.16	8.16 ± 3.20	3.58 ± 0.63
(−)-FTC	10.0	33.5 ± 0.6			7.31 ± 0.92	2.63 ± 0.56	34.2 ± 3.4	20.2 ± 8.9
(+)-FTC	1.0	3.46 ± 0.37	0.32 ± 0.01	0.21 ± 0.04	0.02 ± 0.00	0.11 ± 0.02	0.07 ± 0.01	0.18 ± 0.00

[a] Each value represents the mean ± standard deviation for the analysis of triplicate cell extracts.
[b] Metabolites of (−)- and (+)-FTC which eluted from a SAX column at 1,380, 1,560, or 2,280 s are identified as follows: M1380, FTC diphosphoethanolamine; M1560, FTU 5′-monophosphate; M2280, FTC diphosphocholine.

Fig. 3C, M2280 was uniquely resistant to digestion with alkaline phosphatase. Further analysis of each individual metabolite by digestion with a combination of alkaline phosphatase and phosphodiesterase I and then reversed-phase HPLC showed that all metabolites were derivatives of (−)-FTC (data not shown). Comparison of the relative retention times of the diphosphocholine, diphosphoethanolamine, and the 5′-mono-, 5′-di-, and 5′-triphosphate derivatives of cytidine in this analysis system shows an elution order of CDP-ethanolamine, CMP, CDP-choline, CDP, and CTP. The relative elution order of the metabolites of (−)-FTC on ion-exchange HPLC was similar to that seen with the metabolites of cytidine and ddC (35). This comparison, together with the enzymatic digestion data, led to the tentative identification of M2280 as the diphosphocholine derivative of (−)-FTC.

Concentration dependence of intracellular metabolites. HepG2 derivative 2.2.15 (subclone P5A) cells were incubated for 24 h with [^{3}H](−)-FTC at an initial concentration of 0.01, 0.1, 1.0, or 10 μM. As shown in Table 2, the levels of the mono-, di-, and triphosphates of (−)-FTC as well as that of the putative diphosphocholine derivative (M2280) increased with increasing extracellular (−)-FTC concentrations, suggesting that anabolism was not saturated at the concentrations tested. The intracellular water volume of HepG2 cells was estimated to be 2.6 μl/10^6 cells. Therefore, the intracellular concentration of (−)-FTC is approximately equivalent to the concentration (−)-FTC in the extracellular medium at all concentrations tested. The level of (−)-FTC 5′-diphosphate was observed to be slightly higher than that of the other anabolites, particularly in the incubations with 1.0 and 10.0 μM [^{3}H](−)-FTC.

Determination of intracellular (−)-FTC 5′-triphosphate half-life. HepG2 derivative 2.2.15 (subclone P5A) cells were incubated for 48 h with 20 μM (−)-FTC, washed free of exogenous (−)-FTC, and then incubated in drug-free medium. Table 3 shows the concentration of (−)-FTC 5′-triphosphate during the accumulation and washout phases of the experiment. The intracellular levels of (−)-FTC 5′-triphosphate increased with time for 48 h postdose, reaching a value of 37.7 pmol/10^6 cells, or approximately 15 μM. The intracellular half-life of (−)-FTC 5′-triphosphate was determined to be 2.4 h by fitting an exponential curve containing a constant to the data. However, (−)-FTC 5′-triphosphate concentrations reached a constant low level 24 h after the change to drug-free medium. Although the cells were initially washed twice with drug-free medium to remove the extracellular (−)-FTC, a slow release of (−)-FTC into the medium occurred over the first few hours after drug washout. The level of (−)-FTC in the medium reached 0.35 μM after 6 to 12 h and remained at that level through 48 h. Reanabolism of (−)-FTC by the cells may result in the new steady-state level of (−)-FTC 5′-triphosphate observed by 24 h post washout. As shown in Table 2, incubation of cells with 0.1 μM (−)-FTC resulted in a (−)-FTC 5′-triphosphate level of 0.51 pmol/10^6 cells.

Intracellular metabolism of (+)-FTC. Figure 4A shows an ion-exchange high-performance liquid chromatogram of an extract from cells that had been incubated for 24 h with 1 μM [6-^{3}H](+)-FTC. (+)-FTC and its 5′-mono-, 5′-di-, and 5′-triphosphates were initially identified by comparison of their respective retention times with authentic standards. Analogous to (−)-FTC, the identities of these anabolites of (+)-FTC were confirmed by alkaline phosphatase enzymatic peak shifting by HPLC on a SAX column. The intracellular concentrations of the individual anabolites are given in Table 2. Although the levels of intracellular (+)-FTC and (−)-FTC were similar, the levels of the phosphorylated metabolites of (+)-FTC appeared to be much lower than those of the phosphorylated metabolites of (−)-FTC. Analysis of peak 1 (Fig. 4A) by reversed-phase HPLC showed that approximately 4% of (+)-FTC was deaminated to (+)-FTU.

Digestion of the metabolite M1560 with alkaline phosphatase and reanalysis by reversed-phase HPLC resulted in the recovery of (+)-FTU. This result suggested that metabolite M1560 is (+)-FTU 5′-monophosphate. (+)-FTU 5′-monophosphate standard was generated by deaminating (+)-FTC 5′-monophosphate (19). Two other metabolites, M1380 and M2280, were eluted from the column by HPLC on a SAX column immediately prior to (+)-FTU 5′-monophosphate and between (+)-FTC 5′-mono- and 5′-diphosphates, respectively. Each metabolite was found to be susceptible to phosphodiesterase I cleavage (Fig. 4B), but insusceptible to alkaline

TABLE 3. Concentration of (−)-FTC 5′-triphosphate in HepG2 derivative 2.2.15 (subclone P5A) cells incubated with 20 μM (−)-FTC for 48 h and then incubated in drug-free medium

Incubation time (h)	Washout period (h)	pmol of (−)-FTC 5′-triphosphate/10^6 cells[a]
12		21.7 ± 1.7
24		29.9 ± 7.8
48		37.7 ± 2.5
	2	26.3 ± 1.9
	4	14.9 ± 1.0
	6	7.7 ± 1.7
	12	2.4 ± 0.4
	24	1.0 ± 0.3
	36	0.8 ± 0.3
	48	1.4 ± 0.4

[a] Each value represents the mean ± standard deviation for the analysis of triplicate cell extracts.

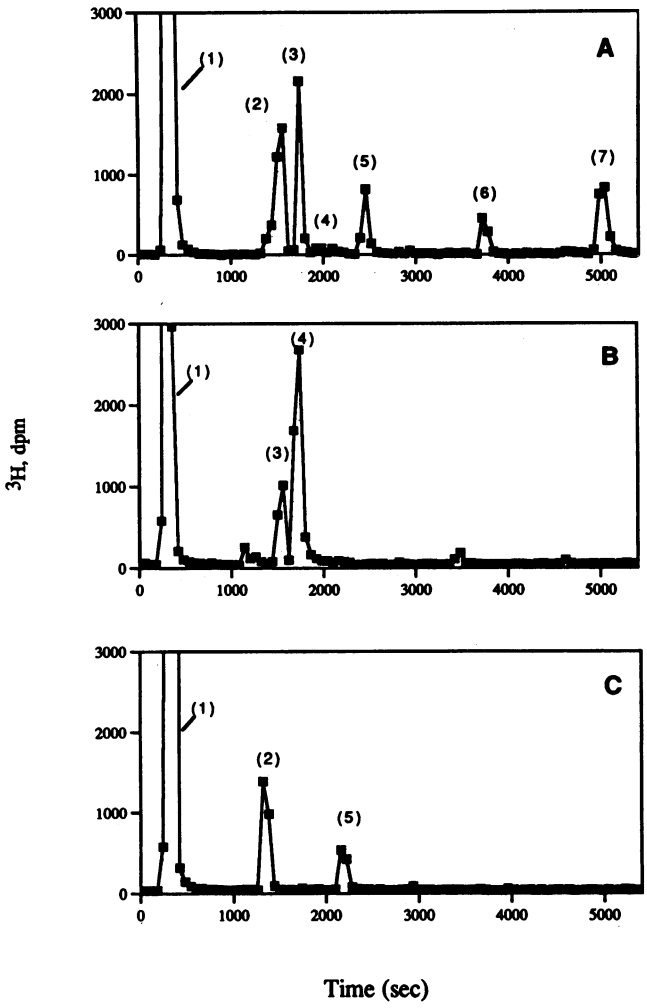

FIG. 4. Intracellular metabolites of (+)-FTC (1 μM) incubated for 24 h with HepG2 derivative 2.2.15 (subclone P5A) cells. Aliquots of the cell extract received no treatment (A) or were digested with snake venom phosphodiesterase I (B) or alkaline phosphatase (C) and were chromatographed by HPLC on a SAX column. Individual peaks are identified as follows: 1, (+)-FTC and (+)-FTU; 2, metabolite M1380; 3, metabolite M1560; 4, (+)-FTC 5′-monophosphate; 5, metabolite M2280; 6, (+)-FTC 5′-diphosphate; and 7, (+)-FTC 5′-triphosphate.

phosphatase digestion (Fig. 4C). As was the case for M2280 from (−)-FTC, this selective enzymatic digestion, in combination with the known metabolic profiles of cytidine and ddC, led to the tentative identification of M1380 and M2280 as (+)-FTC diphosphoethanolamine and (+)-FTC diphosphocholine, respectively.

Intracellular biosynthesis of FTC diphosphoethanolamine (M1380) and FTC diphosphocholine (M2280). In Fig. 5A and C, it can be seen that both the enantiomers of FTC combined with choline intracellularly to make a ^{3}H and ^{14}C double-labeled FTC diphosphocholine product (peak 4). The (−)-FTC diphosphocholine standard (see Materials and Methods) coeluted with the ^{3}H and ^{14}C double-labeled FTC diphosphocholine product (peak 4). These data, in combination with the results of the peak-shifting experiments shown in Fig. 3 and 4, indicate that M2280 is FTC diphosphocholine.

As shown in Fig. 5B and D, an endogenous peak resulting from the metabolism of [^{14}C]ethanolamine alone obscured the elution region (20 to 22 min) of the putative (+)-FTC diphosphoethanolamine metabolite (M1380). To characterize this region further, peaks 1 in Fig. 5B and D were isolated and rechromatographed by reversed-phase HPLC (data not shown). Only peak 1 from Fig. 5D resulted in a phosphodiesterase-susceptible, alkaline phosphatase-resistant [^{3}H and ^{14}C] double-labeled peak which eluted at 360 s. Following the phosphodiesterase treatment, the expected products from the enzymatic degradation of [^{3}H](+)-FTC diphospho[^{14}C]ethanolamine, [^{3}H](+)-FTC 5′-monophosphate and [^{14}C]ethanolamine 5′-monophosphate, were generated and eluted at 240 and 480 s respectively. An FTC diphosphoethanolamine HPLC standard was not available; however, the organic synthesis of FTC diphosphoethanolamine is under investigation in our laboratory. These data, in combination with the results of the peak-shifting experiment shown in Fig. 4, indicate that M1380 is (+)-FTC diphosphoethanolamine. Although Fig. 5B appears to indicate the presence of (−)-FTC diphosphoethanolamine, no such double-labeled peak could be identified by reversed-phase HPLC. If a (−)-FTC diphosphoethanolamine is synthesized under these conditions, the level is below the limit of detection of the assay.

DISCUSSION

(+)-FTC appears to enter HepG2 cells by means of the NBMPR-susceptible equilibrative nucleoside transporter. Influx is saturable, nonconcentrative, and largely inhibited by NBMPR, a transport profile which is characteristic of physiological nucleosides and several of their analogs (26, 27). In contrast, the influx of (−)-FTC was incompletely inhibited by inhibitors of nucleoside transport, indicating that this compound may have multiple transport mechanisms. Stereochemically, (−)-FTC corresponds to a nucleoside with an L conformation and (+)-FTC corresponds to a nucleoside with a D conformation. Enantiomers of adenosine, thymidine, and uridine with L conformations were found to be poor permeants of the nucleoside transporter of S49 mouse lymphoma cells, while the corresponding enantiomers with D conformations were good substrates (27). Incomplete inhibition of L-adenosine transport by NBMPR and D-adenosine was found in mouse erythrocytes and L1210 cells, suggesting that the transport of L-adenosine occurred partially by the nucleoside transporter as well as by nonfacilitated diffusion (16). Little or no inhibition of (−)-FTC transport by the protein modification agents *N*-methylmaleimide, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid, and 4-acetamido-4′-isothiocynatostilbene-2,2′-disulfonic acid (data not shown) suggests that a component of (−)-FTC entry into HepG2 cells is not transporter mediated.

Analogous to the difference in in vitro activity against human HBV (15, 21), intracellular metabolism was found to differ significantly between the (−) and (+) enantiomers of FTC. Furman et al. (15) have shown that (−)-FTC has a 10-fold greater antiviral potency against HBV in HepG2 derivative 2.2.15 (subclone P5A) cells. The intracellular levels of (−)- and (+)-FTC were comparable to the extracellular levels, consistent with the results of the membrane transport studies discussed above. Both (−)- and (+)-FTC were phosphorylated inside the cell to their respective 5′-mono-, 5′-di-, and 5′-triphosphates. Deoxycytidine kinase has been shown to phosphorylate FTC to its 5′-monophosphate (15, 31). Since the levels of (+)-FTC 5′-di- and 5′-triphosphates are higher than the concentration of (+)-FTC 5′-monophosphate, the initial phosphorylation of (+)-FTC by deoxycytidine kinase appears to be rate limiting. Calf thymus and human deoxycytidine kinases have been shown to be stereoselective in the phosphor-

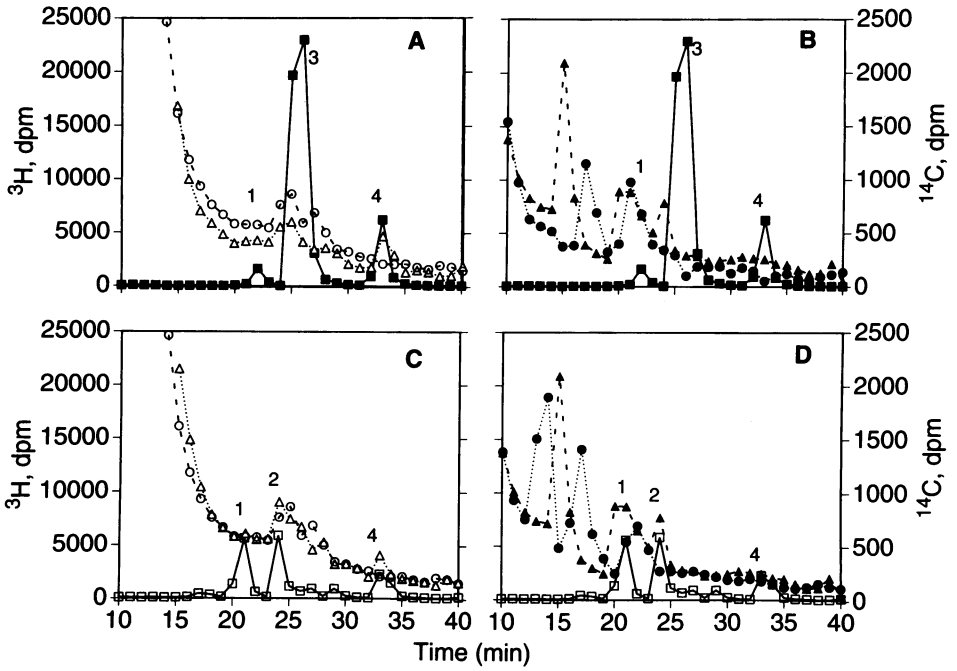

FIG. 5. Intracellular metabolites of [^{3}H](−)-FTC and [^{3}H](+)-FTC (10 μM) incubated in the presence of either [^{14}C]choline (40 μM) or [^{14}C]ethanolamine (40 μM) for 24 h with HepG2 derivative 2.2.15 (subclone P5A) cells. Cells were also incubated with either [^{14}C]choline alone or [^{14}C]ethanolamine alone as controls. Aliquots of cell extracts were chromatographed by HPLC on a SAX column, and the relevant portions of those chromatograms are shown. (A and C) [^{14}C]choline alone (○); (B and D) [^{14}C]ethanolamine alone (●); (A) (−)-[^{3}H]FTC (■) plus [^{14}C]choline (△); (B) (−)-[^{3}H]FTC (■) plus [^{14}C]ethanolamine (▲); (C) (+)-[^{3}H]FTC (□) plus [^{14}C]choline (△); (D) (+)-[^{3}H]FTC (□) plus [^{14}C]ethanolamine (▲). Individual peaks are identified as follows: 1, FTC diphosphoethanolamine; 2, (+)-FTU 5′-monophosphate; 3, FTC 5′-monophosphate; and 4, FTC diphosphocholine.

ylation of FTC, preferring the (−) form over the (+) form (15, 31). dCMP kinase isolated from calf thymus has shown similar selectivity with these enantiomers (15). It therefore follows that cellular phosphorylation of FTC to both the 5′-mono- and 5′-diphosphates may be stereoselective. This selectivity may contribute to the fact that the concentration of the 5′-triphosphate derivative of FTC is higher in cells incubated with 1 μM (−)-FTC (3.6 pmol/10^6 cells) versus 1 μM (+)-FTC (0.2 pmol/10^6 cells). The levels of (−)-FTC 5′-triphosphate were comparable to the amount of ddCTP reported earlier for CEM cells incubated for 6 h with 1 μM ddC (3.5 pmol/10^6 cells) (35), but much higher than the level of ddATP seen when Molt-4 cells were incubated for 6 h with 5 μM dideoxylnosine (0.04 pmol/10^6 cells) (2) or of 2′,3′-dideoxy-3′-thiacytidine (3TC) 5′-triphosphate seen when HepG2 (derivative 2.2.15) cells were incubated with 0.5 μM (−)-3TC for 24 h (~0.1 pmol/10^6 cells) (9).

The intracellular level of (−)-FTC 5′-triphosphate increased in a dose-dependent manner when HepG2 derivative 2.2.15 (subclone P5A) cells were incubated with 0.01 to 10 μM (−)-FTC. The 50% inhibitory concentration of (−)-FTC for the inhibition of HBV in the same cell line was reported to be 10 to 20 nM (9, 15, 21). Following a 24-h incubation of HepG2 derivative 2.2.15 (subclone P5A) cells with a 50% inhibitory concentration of (−)-FTC (10 nM), the intracellular concentration of (−)-FTC 5′-triphosphate was 0.10 pmol/10^6 cells, or approximately 40 nM. This intracellular concentration is sufficient to interrupt the replication of the HBV genome by a single chain termination event (13a).

The intracellular half-life of the (−)-FTC 5′-triphosphate in HepG2 derivative 2.2.15 (subclone P5A) cells was observed to be 2.4 h. (−)-FTC 5′-triphosphate appeared to reach a new, lower steady-state concentration after 12 h in drug-free medium. This may be due to the efficient rephosphorylation of the (−)-FTC formed during the catabolism of intracellular (−)-FTC nucleotides. FTC was shown to be efficiently phosphorylated at very low concentrations, which could account for the persistent levels of the 5′-triphosphate found beyond 12 h. The initial rate of decay is similar to that seen with other dideoxynucleotides which have been studied in log-phase lymphoid cell systems. The 5′-triphosphates of both ddC and zidovudine have been reported to have half-lives of approximately 3 h each in Molt-4 and CEM cells (20, 33). In contrast, ddATP has been reported to have an extraordinarily long half-life of approximately 24 h in human T cells (1). The half-life of BCH-189 5′-triphosphate [(±)-3TC 5′-triphosphate] in human peripheral blood lymphocytes was reported to be biphasic, with an initial half-life of 5 h and then a longer half-life of 24 h after 10 h of incubation in drug-free medium (8). Recently, however, the half-life of (−)-3TC 5′-triphosphate in stimulated human peripheral blood lymphocytes was reported to be about 14 h (7). Evidence has also been reported that (−)-3TC 5′-triphosphate exhibits a long half-life in HepG2 derivative 2.2.15 (subclone P5A) cells (9).

Several intracellular metabolites, in addition to the 5′-mono-, 5′-di-, and 5′-triphosphates, were formed when HepG2 derivative 2.2.15 (subclone P5A) cells were incubated with (−)- and (+)-FTC. An unknown metabolite (M2280) of (−)-FTC was found in (−)-FTC-treated cells at lower levels than the corresponding 5′-mono-, 5′-di-, and 5′-triphosphates of (−)-FTC. A metabolite with similar chromatographic properties was reported when ATH8 and CEM cells were incubated with ddC (13, 35). This latter metabolite,

corresponding to M2280, elutes chromatographically between ddCMP and ddCDP and was conclusively shown to be ddC diphosphocholine (18). The similarities in the anabolic pathways for ddC and (−)-FTC (13, 35) led us to propose that the unknown metabolite described here (M2280), which eluted chromatographically between (−)-FTC 5′-monophosphate and (−)-FTC 5′-diphosphate, was (−)-FTC diphosphocholine. The ^{3}H and ^{14}C double-label experiments with [^{3}H](−)-FTC and [^{14}C]choline indicated that M2280 is [^{3}H](−)-FTC diphospho[^{14}C]choline. In addition, the authentic (−)-FTC diphosphocholine synthesized in our laboratory had an elution profile on HPLC on a SAX column identical to that of [^{3}H](−)-FTC diphospho[^{14}C]choline. A metabolite with similar chromatographic properties was seen following the incubation of human peripheral blood lymphocytes with (−)-3TC (7), but it was not identified. In addition, an analogous metabolite was formed following the incubation of (−)-3TC with HepG2 (derivative 2.2.15) cells (9). This latter metabolite was tentatively identified as (−)-3TC 5′-monophosphosialate. Since M2280 appeared to contain an internal phosphodiester bond, similarity to the monophosphosialate derivative of (−)-3TC was ruled out.

Three anionic metabolites, in addition to the 5′-mono-, 5′-di-, and 5′-triphosphates, were found when HepG2 derivative 2.2.15 (subclone P5A) cells were incubated with (+)-FTC. Comparison of the metabolic profile and relative retention times of these metabolites with those of ddC suggested that M1380 and M2280 are the diphosphoethanolamine and diphosphocholine derivatives of (+)-FTC, respectively. The ^{3}H and ^{14}C double-label experiments with [^{3}H](+)-FTC, [^{14}C] ethanolamine, and [^{14}C]choline indicated that M1380 and M2280 are [^{3}H](+)-FTC diphospho[^{14}C]ethanolamine and [^{3}H](+)-FTC diphospho[^{14}C]choline, respectively. FTC diphosphocholine was detected in extracts from cells incubated with both enantiomers. The absolute level of FTC diphosphocholine, however, was higher in the (−)-FTC cell extract. FTC diphosphoethanolamine was only found in the (+)-FTC cell extract. This derivative appears to be a stereoselective metabolite of (+)-FTC, which is analogous to a ribonucleoside with a D conformation. Metabolite M1560 was identified by isolation of the individual peak and subsequent digestion with phosphodiesterase I. Rechromatography of the digestion products identified the original peak as (+)-FTU 5′-monophosphate. It is not known at this time whether (+)-FTU 5′-monophosphate is derived via deamination of the (+)-FTC with subsequent phosphorylation or by direct deamination of the (+)-FTC 5′-monophosphate. No similar metabolites were observed following the incubation of human peripheral blood lymphocytes with (+)-3TC (7). However, the chromatographic conditions used in the analysis of intracellular metabolites of (+)-3TC were not optimized for the separation of 3TC from the 5′-monophosphate. It is possible that, because of coelution with the 3TC 5′-monophosphate peak, these additional minor metabolites of 3TC were masked.

In summary, the intracellular metabolism of (−)-FTC was studied in cultured human hepatoma cells which chronically produce HBV. (−)-FTC is readily phosphorylated in these cells to the active 5′-triphosphate form with minimal formation of additional metabolites. We have also shown in our laboratory that primary human hepatocytes have identical metabolic profiles following a 24-h incubation with (−)-FTC (4). The low level of cytotoxicity (9, 14, 15) and the lack of an effect on mitochondrial DNA (9, 14) give FTC distinct advantages over ddC as an antiviral therapy. In addition, the low 50% inhibitory concentration of (−)-FTC for the inhibition of HBV, combined with the high level and appreciable half-life of the intracellular (−)-FTC 5′-triphosphate, makes (−)-FTC a strong candidate for clinical investigation as an anti-HBV agent.

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