Biochemical Pharmacology, Vol. 53, pp. 75–87, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00653-3

Effect of Stereoisomerism on the Cellular Pharmacology of β-Enantiomers of Cytidine Analogs in Hep-G2 Cells

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ABSTRACT. The β-L enantiomers of 2′,3′-dideoxycytidine (β-L-ddC) and its 5-fluoro derivative, 2′,3′-dideoxy-5-fluorocytidine (β-L-FddC), were demonstrated to be active against human immunodeficiency virus (HIV) and hepatitis B virus (HBV) replication *in vitro*. In the present study, we investigated the cellular pharmacology of β-L-ddC and β-L-FddC and compared it with that of β-D-2′,3′-dideoxy-5-fluorocytidine (β-D-FddC). β-L-FddC (10 μM) was found to be phosphorylated rapidly in Hep-G2 cells to its 5′-mono-, di-, and triphosphate derivatives with intracellular triphosphate levels achieving 26.6 ± 10.9 pmol/10^6 cells after 72 hr. In contrast, the active 5′-phosphorylated derivative of β-D-FddC achieved lower levels with triphosphate levels of only 2.3 ± 0.5 pmol/10^6 cells under the same conditions. β-L-ddC was also phosphorylated rapidly. A 5′-diphosphocholine (18.7 ± 5.8 pmol/10^6 cells) and a 5′-diphosphoethanolamine (13.6 ± 0.9 pmol/10^6 cells) derivative were detected in β-D-FddC-treated cells after 72 hr, whereas in β-L-FddC- and β-L-ddC-treated cells, only the 5′-diphosphocholine derivative (10.9 ± 2.8 and 60.4 ± 5.7 pmol/10^6 cells, respectively) was detected. β-L-FddC-5′-triphosphate (β-L-FddCTP), β-D-FddC-5′-triphosphate (β-D-FddCTP), and β-L-ddC-5′-triphosphate (β-L-ddCTP) followed a single phase elimination process with an intracellular half-life ($T_{1/2}$) of 10.5, 5.7, and 12.3 hr, respectively. Furthermore, β-L-FddCTP, β-D-FddCTP, and β-L-ddCTP levels of 6.7 ± 2.3, 0.3 ± 0.1, and 12.0 pmol/10^6 cells, respectively, were still detectable 24 hr following drug removal. The higher intracellular 5′-triphosphate levels of β-L-FddC and the extended $T_{1/2}$ of its 5′-triphosphate are consistent with the more potent *in vitro* antiviral activity of β-L-FddC in Hep-G2 cells when compared with its β-D enantiomer, β-D-FddC. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;1:75–87, 1997.

KEY WORDS. nucleoside analog; stereoisomer; enantioselective; metabolism; antiviral

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In the search for new antiviral therapies against HIV¶ and HBV infection, nucleoside analogs with the unnatural β-L configuration have been demonstrated to provide, in general, an increased selectivity as compared with their corresponding β-D enantiomers. The recent approval by the United States Food and Drug Administration of 3TC# (Lamivudine) in combination with AZT (Zidovudine) for the treatment of HIV infection [1], the promising results from the preliminary trial of 3TC therapy for chronic HBV infection [2], and the encouraging antiviral features of its 5-fluoro derivative, β-L-FTC [3–5] have prompted investigations with other cytidine analogs characterized by the

¶ *Abbreviations:* AP, alkaline phosphatase; AZT, 3′-deoxy-3′-azidothymidine; BFU-E, human erythroid progenitor cells; dCytK, 2′-deoxycytidine kinase; β-D-ddC, β-D-2′,3′-dideoxycytidine; β-D-FddC, β-D-2′,3′-dideoxy-5-fluorocytidine; β-D-FddCDP-choline, β-D-2′,3′-dideoxy-5-fluorocytidine-5′-diphosphocholine; β-D-FddCDP-ethanolamine, β-D-2′,3′-dideoxy-5-fluorocytidine-5′-diphosphoethanolamine; β-D-FddCMP, β-D-FddC-5′-monophosphate; β-D-FddCTP, β-D-FddC-5′-triphosphate; β-D-FTC, β-D-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; FBS, fetal bovine serum; FddU, 2′,3′-dideoxy-5-fluorouridine; HBV, hepatitis B virus; HBV-RT, HBV-reverse transcriptase; HIV, human immunodeficiency virus; HIV-1, HIV type 1; HIV-2, HIV type 2; HIV-RT, HIV-reverse transcriptase; β-LddC, β-L-2′,3′-dideoxycytidine; β-L-ddCDP, β-L-ddC-5′-diphosphate; β-L-ddCDP-choline, β-L-2′,3′-dideoxycytidine-5′-diphosphocholine; β-L-ddCMP, β-L-ddC-5′-monophosphate; β-L-ddCTP, β-L-ddC-5′-triphosphate; β-L-FddC, β-L-2′,3′-dideoxy-5-fluorocytidine; β-L-FddCDP-choline, β-L-2′,3′-dideoxy-5-fluorocytidine-5′-diphosphocholine; β-L-FddCTP, β-L-FddC-5′-triphosphate; β-L-FTC, β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; MEM, minimum essential medium; PDE I, snake venom phosphodiesterase; SAX, strong anion exchange; $T_{1/2}$, half-life; 3TC, β-L-2′,3′-dideoxy-3′-thiacytidine; 3TC-TP, 3TC-5′-triphosphate.

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Received 22 March 1996; accepted 5 July 1996.

same unnatural β-L configuration. Preclinal studies of the β-L isomers of 3TC and FTC revealed significant biological diversity with their corresponding β-D enantiomers [6–15]. The favorable antiviral selectivity of these β-L isomers of cytidine analogs when compared with their corresponding β-D enantiomers may possibly be attributed to an efficient cellular uptake, higher and sustained intracellular concentrations of the active β-L triphosphates, significant decreased interaction of the β-L isomers with host DNA polymerases as compared with the viral polymerases, and an inability to serve as substrates for degradative enzymes such as deoxycytidine deaminase (EC 3.5.4.14) [4,6,8,10,14, 16–18].

We have demonstrated previously that β-L-ddC and in particular its 5-fluoro derivative, β-L-FddC, are potent anti-HIV compounds in a variety of HIV-infected cell lines [19, 20]. Other groups have also reported that these unnatural β-L nucleoside analogs are active against HIV, with the order of potency being β-L-FddC > β-D-ddC > β-L-ddC [21–23]. Interestingly, β-L-FddC exhibits an extremely higher selective index (SI = 9000) as compared with that of β-L-ddC (SI = 34), β-D-FddC (SI = 16), β-D-ddC (SI = 79), and AZT (SI = 100) when considering the cytotoxicity to BFU-E [19]. β-L-FddC and β-L-ddC were also shown by our group to be cross-resistant with 3TC, and this resistance was associated with the methionine to valine mutation at position 184 (M184V) in the YMDD region of HIV-RT (EC 2.7.7.49) [18]. This M184V mutation in HIV-RT associated with resistance to 3TC has been implicated as a potential mechanism for the sustained suppression of serum HIV-1 RNA concentrations observed with the combination therapy of 3TC and AZT [24].

In addition to their anti-HIV activity, β-L-FddC and β-L-ddC were shown to be potent anti-HBV compounds in the HBV-DNA transfected Hep-G2 cell line 2.2.15 *in vitro* [21–23, 25]. The efficacy of these novel β-L cytidine analogs is absolutely dependent on the cellular tropism of the virus and the ability of hepatic cells to activate these unnatural β-L analogs via host cellular kinases. Furthermore, when inside the cell, the selectivity of these analogs is determined by the affinity of the nucleoside's respective triphosphate to HBV-RT and the affinity to host cellular polymerases. The anti-HBV activity and the cytotoxicity of β-L-ddC and its 5-fluoro derivative reflected an extremely favorable selective index for both β-L-ddC (SI = 136) and β-L-FddC (SI = 156) as compared with β-D-ddC (SI = 27) [25]. To better understand the favorable antiviral features of β-L-FddC, we investigated its cellular pharmacology and compared it with its defluorinated analog, β-L-ddC, and with its corresponding β-D enantiomer, β-D-FddC, in Hep-G2 cells.

MATERIALS AND METHODS

Materials

The stereoselective synthesis of β-L-FddC and β-L-ddC from the starting material L-xylose has been reported elsewhere [20]. β-D-FddC was supplied by Dr. Victor Marquez (National Institutes of Health, Bethesda, MD). Each of their respective 5′-triphosphate derivatives, β-L-FddCTP, β-L-ddCTP, and β-D-FddCTP, were synthesized by a standard phosphorylation method from their corresponding nucleosides [18]. All compounds were fully characterized by nuclear magnetic resonance (^1H, ^{31}P), fast atom bombardment mass spectroscopy, HPLC, and UV spectroscopy. [6-^{3}H]-β-L-FddC (2.5 Ci/mmol), [2′,3′-^{3}H]-β-D-FddC (50 Ci/mmol), and [5,6-^{3}H]-β-L-ddC (1.8 Ci/mmol) were purchased from Moravek Biochemical (Brea, CA), and their purity was determined to be in excess of 97% as assessed by the chiral HPLC method described below. [*methyl*-^{14}C]-Choline chloride (55 mCi/mmol) and [2-^{14}C]-ethanolamine hydrochloride (53 mCi/mmol) were obtained from Amersham Life Science (Elkgrove, IL). All other chemicals and reagents were of the highest analytical grade available.

Isomeric Purity

Chiral HPLC was used to determine the isomeric purity of [^{3}H]-β-L-FddC, [^{3}H]-β-D-FddC, and [^{3}H]-β-L-ddC using a modified methodology of a previously published technique [12]. A Chiralpak AS column (J. T. Baker Inc., Phillipsburg, NJ), which has an amylose tris [(S)-α-methylbenzyl carbamate] coated silica gel was used as the stationary phase. Column temperature was maintained at 10°, and an isocratic elution was performed at 1 mL/min with a 30%: 70% isopropyl alcohol:hexane (v/v) mixture with 0.3% diethylamine (v/v) modifier for the separation of β-L- and β-D-FddC. Their retention times were 8.5 and 15.4 min, respectively. The separation of β-L- and β-D-ddC required a 40%:60% ratio of isopropyl alcohol:hexane (v/v) with 0.3% diethylamine (v/v) modifier. Their retention times were 11.2 and 16.0 min, respectively. Fractions were collected every 0.5 min and combined with an Econo Safe II scintillation fluid (Research Products International Corp., Mount Prospect, IL). Radioactivity was quantitated on a Beckman LS5000 TA counter. The purity of [^{3}H]-β-L-FddC and [^{3}H]-β-D-FddC, as determined by radioactivity, was greater than 99%, and the purity of [^{3}H]-β-L-ddC was 97%.

Cell Culture, Exposure, and Harvest

The well characterized human hepatoblastoma cell line Hep-G2 (American Type Culture Collection, Rockville, MD) was cultured in MEM supplemented with 10% FBS (v/v), 1 mM sodium pyruvate, and 1 mM penicillin G/streptomycin sulfate and maintained at 37° with a humidified atmosphere of 5% CO_2. Confluent cells were harvested with an initial 15- to 20-min exposure to trypsin + EDTA for the detachment of the adherent monolayer. Cells were then washed three times with medium and spun down at 350 *g*. Cell medium was changed every 72 hr. Approximately 20×10^6 cells/mL were exposed to 10 μM (sp. act., 100 dpm/pmol) and 1 μM (500 dpm/pmol) [^{3}H]-β-L-FddC,

[^{3}H]-β-D-FddC, and [^{3}H]-β-L-ddC for 6, 12, 24, 48, and 72 hr. At the termination of exposure, cells were washed three times with 10 mL of cold PBS. Cells were then counted on a hemacytometer, and viability, as assessed by trypan blue uptake, was greater than 95%. Intracellular nucleotide pools were extracted twice with a total volume of 1.5 mL of 60% methanol:distilled water (v/v). Extracts were then dried under a gentle stream of nitrogen and reconstituted with 160 μL of distilled water. Reconstituted extracts were frozen at −20° until analysis by the anion exchange HPLC method as described below.

Primary Cultured Human Hepatocytes

Human primary hepatocytes were obtained from fresh human liver samples and cryopreserved in L15 medium using a Nicool ST20 apparatus. Cells were thawed and seeded as previously described [26, 27]. Human hepatocytes were incubated with 10 μM [^{3}H]-β-L-FddC (100 dpm/pmol) for 32 hr. Following that incubation time period, the extracellular medium was sampled, and cells were scraped in a 50% acetonitrile:distilled water (v/v) mixture. Aliquots of cell extracts were then dried under nitrogen and reconstituted in 200 μL of distilled water. Intracellular and extracellular aliquots were analyzed by anion exchange and reverse-phase HPLC.

HPLC Analysis of Intracellular Metabolites

The intracellular metabolism of β-L-FddC, β-D-FddC, and β-L-ddC in Hep-G2 cells was assessed by ion exchange HPLC using a Partisil SAX 10 μm column (Jones Chromatography, Lakewood, CO). A gradient elution was carried out at 1 mL/min with 15 mM and 1 M potassium phosphate buffer as the mobile phase. The gradient started at 10 min, proceeded linearly to 100% 1 M potassium phosphate until 55 min, and was maintained for 15 min to allow elution of the 5′-triphosphate derivative. Fractions were collected every minute and combined with a mixture of Econo Safe II scintillation fluid and the high salt capacity scintillation fluid Ultima Flo AP (Packard Instrument Co., Meriden, CT). Under these conditions, the retention times of the 5′-mono-, di-, and triphosphate derivatives of β-L-FddC and β-D-FddC were 18, 35, and 56 min, respectively. The retention times of β-L-ddCMP, β-L-ddCDP, and β-L-ddCTP were 7, 29, and 45 min, respectively. In addition to the 5′-phosphorylated derivatives, another radiochromatogram peak eluted between the 5′-monophosphate and the 5′-diphosphate at 26 min for both β-L and β-D isomers of FddC and at 13 min for β-L-ddC. This peak (peak 3) was denoted as β-L-FddC-X, β-D-FddC-X, and β-L-ddC-X, respectively. Finally, another radiochromatogram peak, in addition to the 5′-phosphorylated derivatives of β-D-FddC and β-D-FddC-X metabolite, eluted at 15 min just prior to β-D-FddCMP. This peak (peak 6) was denoted as β-D-FddC-Y. This metabolite was not detected in extracts obtained from cells that had been exposed to either β-L-FddC or β-L-ddC.

Deamination of β-L-FddC and β-D-FddC

The susceptibility of β-L- and β-D-FddC to deoxycytidine deaminase was assessed in Hep-G2 cells following a 72-hr exposure to either β-L-FddC or β-D-FddC. The isolated parent nucleoside peak from the above SAX HPLC method was analyzed using a C-18 reverse-phase HPLC analysis with a Hypersil ODS 5 μm column (Jones Chromatography). An elution gradient was carried out at a flow rate of 1 mL/min with 50 mM phosphoric acid, pH 3.0, and acetonitrile. The gradient started at time zero and proceeded linearly to 3.6% acetonitrile at 30 min. Under these conditions, the retention times of FddC and FddU were 21.7 and 24.4 min, respectively.

Identification of Intracellular Metabolites

Intracellular metabolites were identified by a combination of authentic cold standards, enzyme digestion of whole cell extracts, or ^{14}C/^{3}H double-labeled incorporation experiments. Approximately 20,000–30,000 dpm of whole cell extracts were digested with approximately 90 U of calf intestine AP (EC 3.1.3.1) and 6 U of PDE I (EC 3.1.4.1), which were obtained from the Worthington Biochemical Corp. (Freehold, NJ). The reaction mixture, which was titrated to the pH optimum of the particular enzyme (pH 9.75 for AP and pH 9.00 for PDE I), consisted of 0.11 M Tris free base, 0.11 M NaCl, and 15 mM $MgCl_2$. In different experiments, cells were also exposed simultaneously to a 10 μM concentration of the ^{3}H-nucleoside (β-L-FddC, β-D-FddC, or β-L-ddC) at a specific activity of 100 dpm/pmol and 15 μM [^{14}C]-choline chloride (122 dpm/pmol) or [^{14}C]-ethanolamine hydrochloride (117 dpm/pmol). In control experiments, cells were incubated with either [^{14}C]-choline chloride or [^{14}C]-ethanolamine hydrochloride alone.

Intracellular Metabolite Decay Experiments

Approximately 1.4×10^8 Hep-G2 cells were incubated for 24 hr in an upright 225 cm^2 cell culture flask with either 10 μM β-L-FddC, 10 μM β-D-FddC, or 10 μM β-L-ddC in a final volume of 100 mL. At the termination of exposure, cells were washed three times with fresh medium, and aliquots of approximately 20×10^6 cells/10 mL were resuspended in upright 75 cm^2 flasks in drug-free medium for the following indicated time periods. Cells exposed to both isomers of FddC and to β-L-ddC were incubated in drug-free medium for 0, 1, 2, 4, 6, 12, and 24 hr. Extraction procedures and HPLC analysis were identical to those as described above. Intracellular metabolite concentrations versus time curves were generated using the SIPHAR/Base software package (SIMED, Creteil, France). An initial concentration versus time curve was generated by using a peel-

ing of algorithm [28] and assuming a zero order input with a first order elimination process. A refined curve, from which the intracellular $T_{1/2}$ values were calculated, was obtained by using a weighted least squares algorithm with a weighing factor equal to $1/y(calc)^2$.

RESULTS

HPLC Analysis of [^{3}H]-β-L-FddC, [^{3}H]-β-D-FddC, and [^{3}H]-β-L-ddC Intracellular Metabolites in Hep-G2 Cells

Figure 1 shows the HPLC radiochromatograms of Hep-G2 cell extracts exposed to [^{3}H]-β-L-FddC (A), [^{3}H]-β-D-FddC (B), and [^{3}H]-β-L-ddC (C). The retention times of the parent nucleosides, monophosphates, and triphosphates were identical to that of authentic unlabeled standards synthesized in our laboratories. The diphosphates were identified by their susceptibility of AP and PDE I as illustrated in Fig. 2. Furthermore, an additional radiochromatogram peak (peak 3) eluted for all three cytidine analogs between the mono- and diphosphates. These unknown metabolites were referred to as β-L-FddC-X, β-D-FddC-X, and β-L-ddC-X. A second unknown radiochromatogram peak (peak 6) eluted just prior to β-D-FddCMP and was referred to as β-D-FddC-Y.

Characterization of β-L-FddC-X, β-D-FddC-X, β-D-FddC-Y, and β-L-ddC-X

β-L-FddC-X and β-L-ddC-X were resistant to catabolism by AP as shown in Fig. 2D and 2F, which indicated that a terminal phosphate group was not present. Furthermore, their susceptibility to PDE I digestion as shown in Fig. 2A and 2C indicated that a phosphodiester bond was present. β-D-FddC-X and β-D-FddC-Y were susceptible to AP catabolism as shown in Fig. 2E and PDE I digestion as shown in Fig. 2B. The susceptibility of β-D-FddC-X and β-D-FddC-Y to AP catabolism would indicate the presence of a terminal phosphate group, but there was evidence of PDE I contamination in the AP as indicated by the hydrolysis of 5′-thymidine monophosphate-*p*-nitrophenyl ester. Based on previous work with other cytidine analogs [3, 6, 29], we hypothesized that the X moiety was possibly choline and the Y moiety was possibly ethanolamine linked to the nucleoside analogs by a phosphodiester bond. Consequently, double-labeled incorporation experiments were performed with [^{3}H]-nucleosides and [^{14}C]-choline or [^{14}C]-ethanolamine. The radiochromatograms which illustrate the coelution of the [^{3}H]-nucleoside and either [^{14}C]-choline or [^{14}C]-ethanolamine are presented in Fig. 3 and led to the tentative identification of the X metabolite as the 5′-diphosphocholine derivative of β-L- and D-FddC and β-L-ddC and the Y metabolite as the 5′-diphosphoethanolamine of β-D-FddC.

Time Course Accumulation of β-L-FddC, β-D-FddC, and β-L-ddC Metabolites in Hep-G2 Cells

The metabolite accumulation profiles obtained after a 0- to 72-hr exposure of a 10 μM concentration of each of the cytidine analogs to cells are illustrated in Fig. 4. β-L-FddC, β-D-FddC, and β-L-ddC were phosphorylated rapidly to their respective 5′-mono-, -di-, and -triphosphate derivatives, and steady-state levels were reached within 24 hr. All three nucleosides led to the formation of approximately equal intracellular concentrations of their corresponding 5′-monophosphates. In contrast, concentrations of the β-L nucleoside 5′-diphosphate and 5′-triphosphate derivatives formed were much greater than their counterparts of the β-D nucleoside. β-L-FddCTP and β-L-ddCTP achieved intracellular levels of 26.6 ± 10.9 and 19.3 ± 4.4 pmol/10^6 cells, respectively, which were approximately ten times greater than the intracellular concentration of β-D-FddCTP with a value of 2.3 ± 0.5 pmol/10^6 cells. A major difference in the metabolism of β-L-ddC as compared with its 5′-fluoro derivative was the enhanced formation of the diphosphocholine derivative. β-L-ddCDP-choline was by far the major intracellular metabolite, accumulating to a steady-state level of 60.4 ± 5.7 pmol/10^6 cells within 72 hr, whereas β-L-FddCDP-choline only achieved a 6-fold lower concentration in the same time period and β-L-FddCTP was the predominant intracellular metabolite. The two liponucleotide metabolites of β-D-FddC, β-D-FddCDP-choline and β-D-FddCDP-ethanolamine, were predominant intracellularly with concentrations of 18.7 ± 5.8 and 13.6 ± 0.9 pmol/10^6 cells, respectively. In contrast, the pharmacologically active β-D-FddCTP was the least formed metabolite under similar conditions. A metabolic pathway that summarizes the enantioselective intracellular metabolism of β-L-FddC and β-D-FddC is illustrated in Fig. 5.

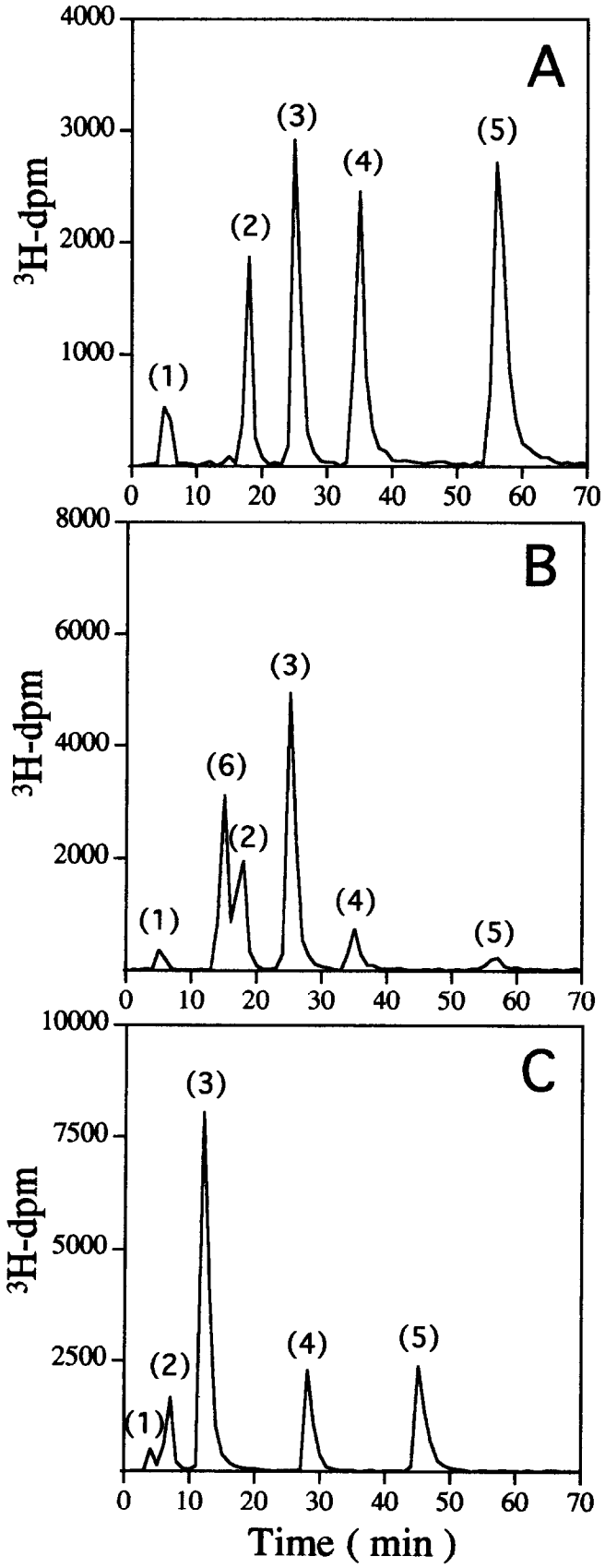
The effect of concentration was studied by exposing cells to either a 1 or a 10 μM concentration of each nucleoside for 72 hr (Table 1). The formation of the intracellular metabolites of β-L-ddC and β-D-FddC increased linearly as a function of the extracellular nucleoside concentration, while the concentration of 5′-phosphorylated β-L-FddC was increased by only 3-fold when incubations of 1 and 10 μM concentrations of the nucleoside were compared.

β-L-FddC and β-D-FddC Deamination in Hep-G2 Cells

Following a 72-hr exposure to either [^{3}H]-β-L-FddC or [^{3}H]-β-D-FddC in Hep-G2 cells, the extent of deamination was determined by analysis of intracellular FddC and FddU levels. β-L-FddC-exposed cells had no detectable β-L-FddU. In contrast, β-D-FddC-exposed cells had detectable levels of the deaminated product, β-D-FddU, and it accounted for approximately 5% of the recovered intracellular [^{3}H]-β-D-FddC. Furthermore, no data suggested the presence of any of the 5′-phosphorylated deaminated derivatives for either β-L-FddC- or β-D-FddC-treated cells.

HPLC Analysis of β-L-FddC Metabolites in Primary Cultured Human Hepatocytes

Reverse-phase HPLC analysis of extracellular samples detected no metabolite in the extracellular medium, whereas



three metabolites were detected intracellularly. Anion exchange HPLC analysis of the intracellular extracts revealed these metabolites to be the 5′-mono-, di-, and triphosphate derivatives of β-L-FddC. After 32 hr of cell exposure to 10 μM [^{3}H]-β-L-FddC, intracellular levels of β-L-FddC-5′-monophosphate, -diphosphate, and -triphosphate were 0.8, 6.0, and 4.0 pmol/10^6 cells, respectively. In contrast to Hep-G2 cells, neither the 5′-diphosphocholine nor the 5′-diphosphoethanolamine derivative was detected in human primary hepatocytes.

Rates of Decay of 5′-phosphorylated Metabolites in Hep-G2 Cells

To determine the intracellular $T_{1/2}$ of the 5′-phosphorylated derivatives of the three cytidine analogs, Hep-G2 cells were incubated with either 10 μM [^{3}H]-β-L-FddC, [^{3}H]-β-D-FddC, or [^{3}H]-β-L-ddC for 24 hr, after which cells were washed and incubated in drug-free medium for specified time periods. Intracellular metabolite levels were then plotted against time on a semi-log graph. A monophasic curve was initially fitted to the data using a peeling algorithm and then was refined as described in Materials and Methods. A $T_{1/2}$ could not be calculated for either β-L-FddCMP or β-L-FddCDP because their intracellular levels either did not digress below half the original concentration or a decay trend was not observed. Therefore, their $T_{1/2}$ was assumed to be greater than 24 hr with 2.9 ± 2.7 and 5.0 ± 1.3 pmol/10^6 cells, respectively, remaining intracellularly at 24 hr after drug removal. β-L-ddCMP and β-L-ddCDP each displayed a decay trend and had similarly extended $T_{1/2}$ values of 15.4 and greater than 24 hr, respectively. In contrast, the 5′-monophosphate and 5′-diphosphate of β-D-FddC exhibited a significantly more abbreviated $T_{1/2}$ of 3.7 and 3.4 hr with only 1.1 ± 0.5 and 0.9 ± 0.1 pmol/10^6 cells remaining within the cell, respectively. For the above decay curves, the minimum correlation coefficient was 0.945. The correlation coefficients were 0.995, 0.955, and 0.919 for β-L-FddCTP, β-D-FddCTP, and β-L-ddCTP decay curves, respectively. β-L-FddCTP underwent a monophasic decay with an intracellular $T_{1/2}$ approximating 14.8 hr and accounted for as much as 6.7 ± 2.3 pmol/10^6 cells after 24 hr of incubation in drug-free medium. β-L-ddCTP demonstrated a monophasic intracellular decay with an intracellular $T_{1/2}$ of 12.3 hr, and β-D-FddCTP also underwent a monophasic decay but exhibited a significantly shorter intracellular $T_{1/2}$ of 5.7 hr with only 0.3 ± 0.1 pmol/10^6 cells remaining intracellularly 24 hr after drug removal. The 5′-diphosphocholine derivative of both β-L-ddC and its

FIG. 1. Resolution by SAX HPLC of intracellular metabolites detected in Hep-G2 cells exposed to either (A) 10 μM [^{3}H]-β-L-FddC, (B) 10 μM [^{3}H]-β-D-FddC, or (C) 10 μM [^{3}H]-β-L-ddC. The metabolites were identified as: (1) nucleoside, (2) nucleoside monophosphate, (3) nucleoside-5′-diphosphocholine, (4) nucleoside diphosphate, (5) nucleoside triphosphate, and (6) nucleoside-5′-diphosphoethanolamine. In the 10 μM [^{3}H]-β-D-FddC radiochromatogram (B), peak 1 represents β-D-FddC and β-D-FddU.

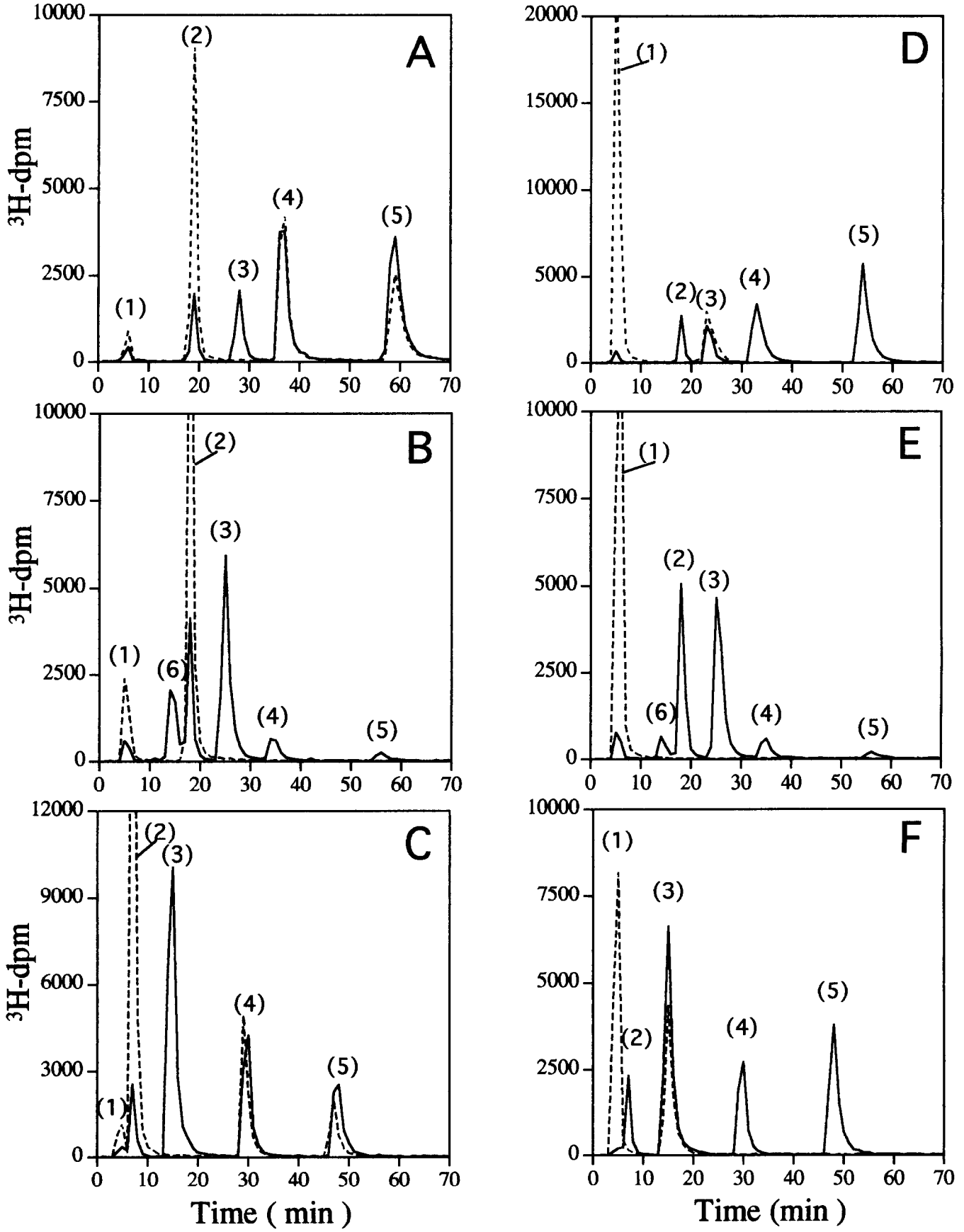


FIG. 2. Radiochromatograms of intracellular metabolites detected in Hep-G2 cell extracts after digestion with either PDE I or AP (-----), and control (—). Key: (A) 10 μM β-L-FddC-exposed cell extracts digested with PDE I, (B) 10 μM β-D-FddC-exposed cell extracts digested with PDE I, (C) 10 μM β-L-ddC-exposed cell extracts digested with PDE I, (D) 10 μM β-L-FddC-exposed cell extracts digested with AP, (E) 10 μM β-D-FddC-exposed cell extracts digested with AP, and (F) 10 μM β-L-ddC-exposed cell extracts digested with AP. Metabolites are numbered as in Fig. 1.

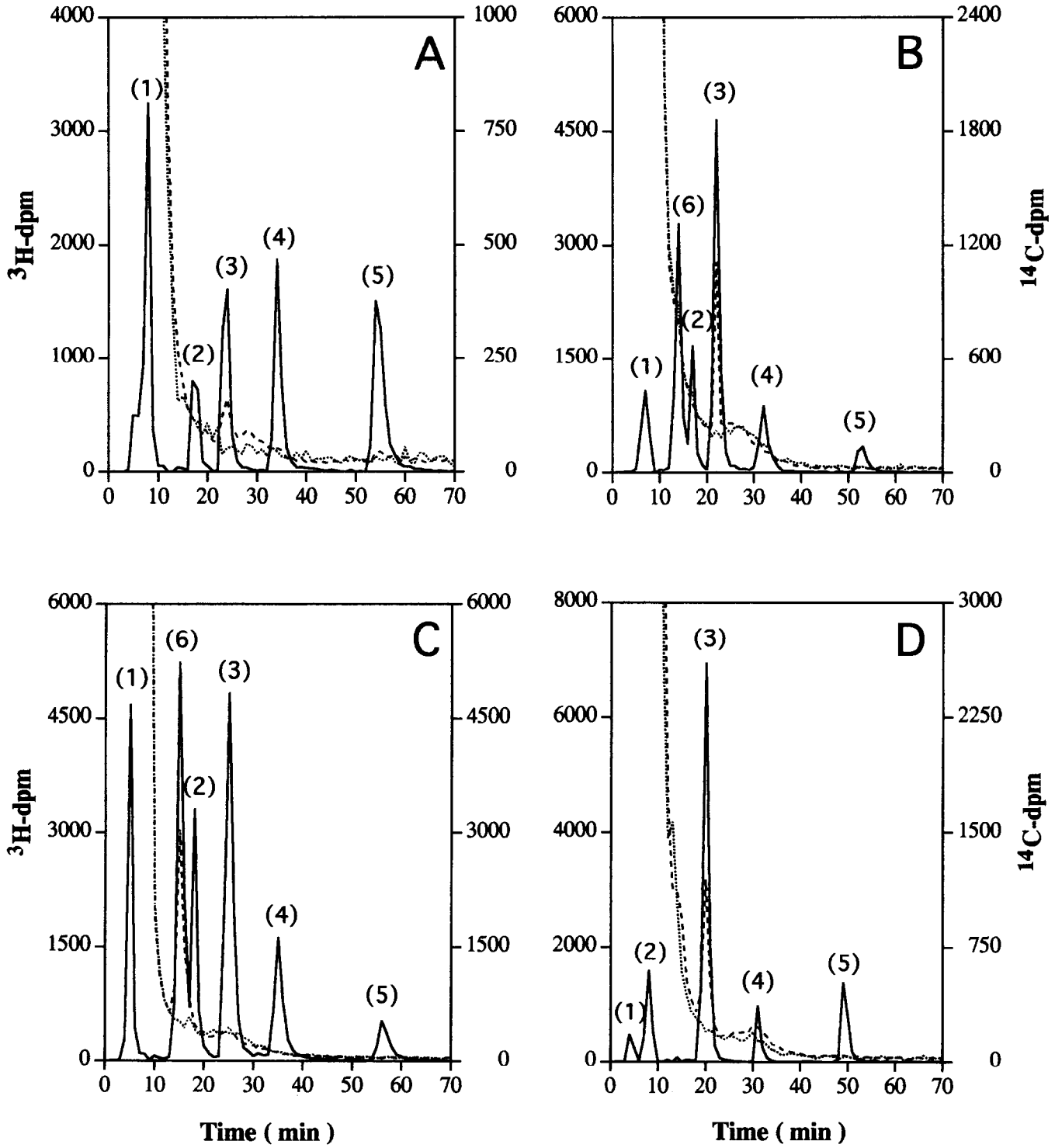


FIG. 3. Radiochromatograms of intracellular metabolites detected in Hep-G2 cells after incubation with 15 μM [^{14}C]-choline in the presence (-----) or absence (.....) of 10 μM [^{3}H]-β-L-FddC (A), 10 μM [^{3}H]-β-D-FddC (B), or 10 μM [^{3}H]-β-L-ddC (D). Furthermore, Hep-G2 cells were incubated with 15 μM [^{14}C]-ethanolamine in the presence (-----) or absence (.....) of 10 μM [^{3}H]-β-D-FddC (C). Tritiated anabolites (—) were identified as: (1) nucleoside, (2) nucleoside monophosphate, (3) nucleoside-5′-diphosphocholine, (4) nucleoside diphosphate, (5) nucleoside triphosphate, and (6) nucleoside-5′-diphosphoethanolamine.

5-fluoro derivative, β-L-FddC exhibited relatively short intracellular $T_{1/2}$ values of 6.6 and 3.8 hr with 6.0 and 2.1 pmol/10^6 cells remaining intracellularly, respectively. In contrast, the 5′-diphosphocholine and the 5′-diphosphoethanolamine derivatives of β-D-FddC had intracellular $T_{1/2}$ values that were greater than 24 hr with appreciable levels of 3.5 ± 1.0 and 5.7 ± 1.3 pmol/10^6 cells, respectively, remaining intracellularly 24 hr after drug removal. The cor-

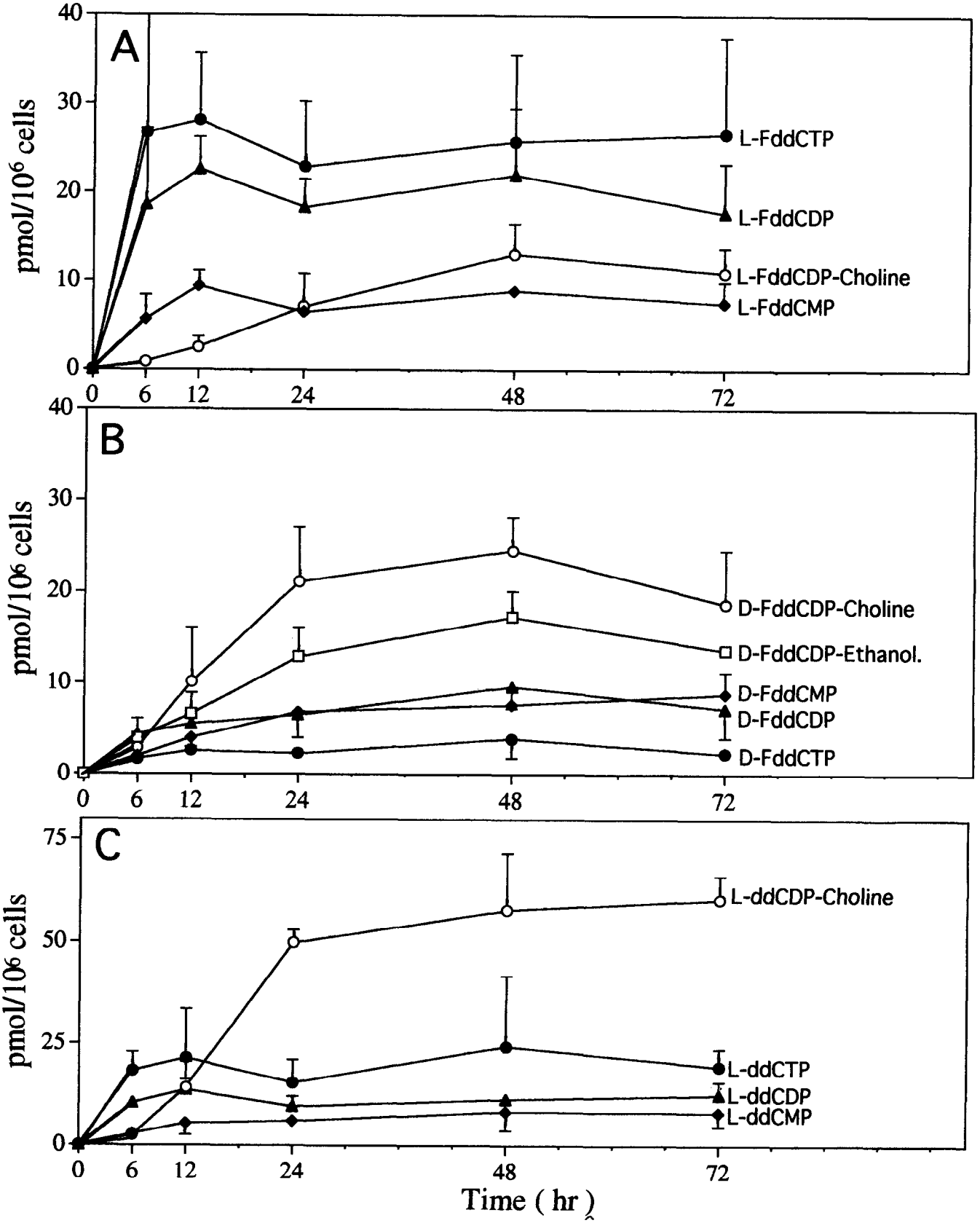


FIG. 4. Time course of intracellular metabolites detected in Hep-G2 cells after incubation with 10 μM β-L-FddC (A), 10 μM β-D-FddC (B), or 10 μM β-L-ddC (C). Each experimental value represents the mean ± SD of three experiments.

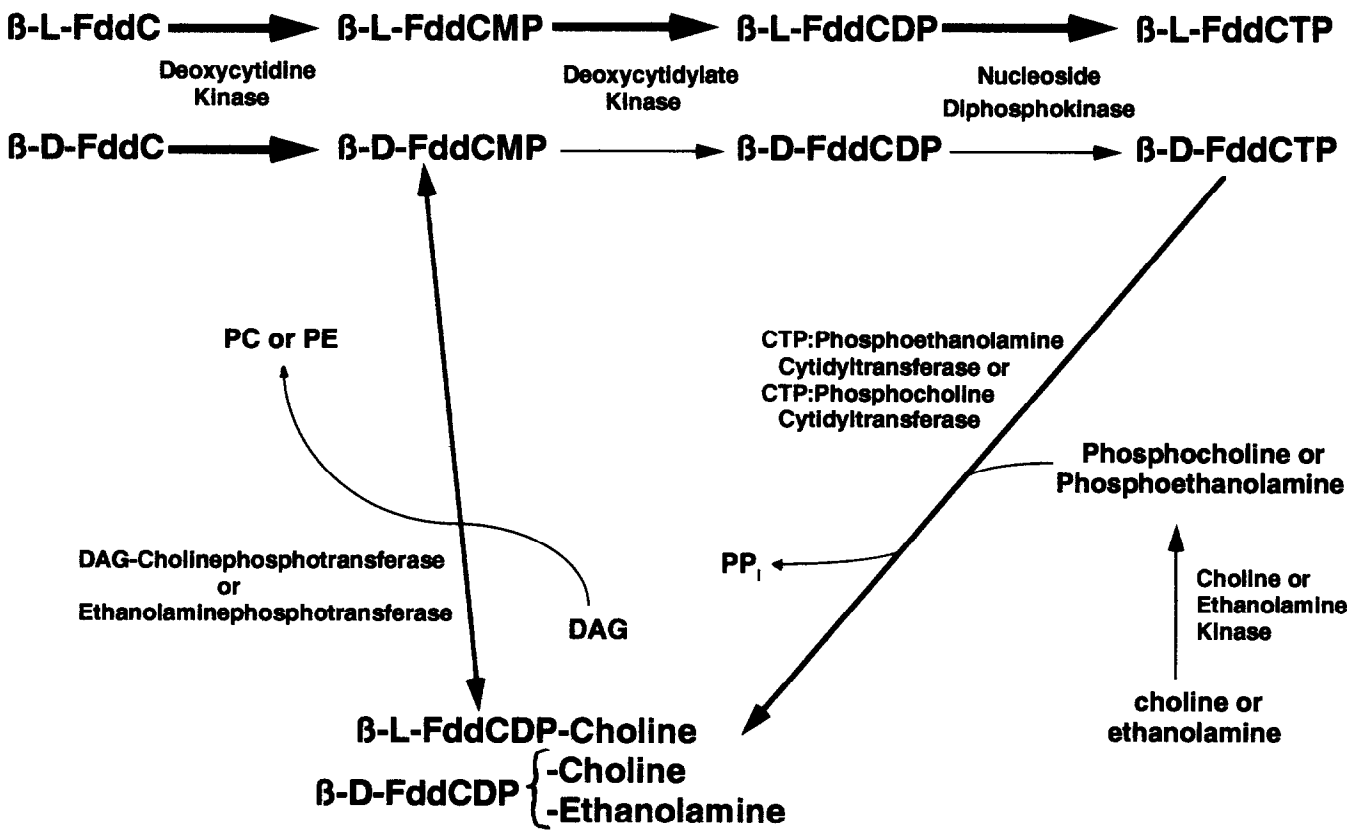


FIG. 5. Enantioselective intracellular metabolism of β-L-FddC and β-D-FddC in Hep-G2 cells. Abbreviations not used previously: DAG, 1,2-diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PP_i, inorganic pyrophosphate.

relation coefficients for the 5′-liponucleotide decay curves ranged from 0.829 to 0.990.

DISCUSSION

The discovery of the antiviral activity of unnatural nucleoside analogs with a β-L configuration has attracted much attention in recent years with a primary reason being their increased selectivity when compared with their corresponding β-D enantiomers [8, 11]. The impressive selective indices of 3TC and its 5-fluoro derivative, β-L-FTC, has prompted the biological evaluation of other β-L-cytidine structural analogs. Recent reports by our group and others have shown that β-L-ddC and its 5-fluoro derivative possess potent antiviral activity against HIV-1, HIV-2, and HBV *in vitro* [19–23, 25, 30], but the effect of stereoisomerism on the antiviral activities has not been fully clarified. Stereoselective antiviral activity of nucleoside analogs can result primarily from a transport mechanism(s), cellular activation by host cell kinases, inhibition of viral polymerase by the active 5′-triphosphate derivative, and catabolism of the latter intracellular metabolite by host cellular enzymes.

The mechanism of anti-HIV activity of β-L-ddC and β-L-FddC is a competitive inhibition of HIV-RT's RNA

TABLE 1. Intracellular concentrations of metabolites detected in Hep-G2 cells after 72 hr of incubation with either [^{3}H]-β-L-ddC, [^{3}H]-β-L-FddC, or [^{3}H]-β-D-FddC, at the indicated concentrations

Compound	Concentration (μM)	Concentration* (pmol/10^6 cells)				
		-DP-Ethanolamine	-MP	-DP-Choline	-DP	-TP
β-L-ddC	1	ND†	0.5 ± 0.4	4.9 ± 2.8	0.9 ± 0.4	2.4 ± 0.1
β-L-ddC	10	ND	8.1 ± 3.4	60.4 ± 5.7	12.4 ± 5.6	19.3 ± 4.4
β-L-FddC	1	ND	2.5 ± 0.5	2.9 ± 1.2	5.9 ± 0.5	8.9 ± 1.4
β-L-FddC	10	ND	7.5 ± 2.4	10.9 ± 2.8	17.7 ± 5.6	26.6 ± 10.9
β-D-FddC	1	1.8 ± 0.4	0.9 ± 0.1	2.9 ± 0.3	0.7 ± 0.1	0.3 ± 0.1
β-D-FddC	10	13.6 ± 0.9	8.8 ± 2.4	18.7 ± 5.8	7.2 ± 3.2	2.3 ± 0.5

* Values are means ± SD of three independent experiments.
† ND: not detected.

directed DNA polymerase activity by their respective 5′-triphosphate [18]. Furthermore, β-L-ddCTP and β-L-FddCTP also have been shown to inhibit the DNA polymerase activity of woodchuck hepatitis virus [25], and β-L-FddCTP has been shown to also inhibit duck hepatitis B virus DNA polymerase [30]. Interestingly, when comparing the woodchuck hepatitis virus DNA polymerase inhibition by β-L and β-D isomers of ddCTP and FddCTP, the β-L triphosphates were much more potent inhibitors [18], and this stereoselective inhibition correlated with the observed anti-HBV activity of the corresponding nucleoside parents in cell-based assays [8, 19, 22, 25]. This competitive inhibition of viral reverse transcriptase is also supplemented by the ability of the respective triphosphates of these dideoxynucleoside analogs to serve as alternate substrates for incorporation into viral DNA. Consequently, viral DNA synthesis is terminated due to the lack of a 3′-hydroxyl group. Due to the lack of a purified HBV-RT system to evaluate the chain termination capacity of various nucleoside analogs, results from studies using the HIV-RT have been a major assessment system used to determine alternate substrate acceptance. Using a poly(rI)n · oligo$(dC)_{10-15}$ as a template in a chain termination assay, Faraj *et al.* [18] demonstrated the ability of both isomers of ddC and FddC to serve as alternate substrates for wild-type HIV-RT with the respective β-D isomers exerting a stronger more premature chain terminating ability. The HIV-RT acceptance of the structurally similar cytidine analog 3TC and its corresponding β-D enantiomer as alternate substrates has also been demonstrated, with both isomers equally causing chain termination [17]. The racemic mixture of FTC, the 5-fluoro derivative of (±)-2′,3′-dideoxythiacytidine, has also been demonstrated by Schinazi *et al.* [5] to lead to chain termination more frequently than that of β-D-ddC at concentrations of 0.01 and 0.1 μM. In addition to the stereoselective inhibition of HBV DNA polymerase and the chain terminating ability of these nucleoside analogs, the triphosphate levels achieved in Hep-G2 cells indicated a significant difference in the host cellular activation of the β-L and β-D enantiomers of FddC (Table 1).

The cellular activation by dCytK (EC 2.7.1.74) of these unnatural β-L-cytidine analogs and their corresponding β-D enantiomers has been demonstrated to be significantly different. β-L-FddC and β-L-ddC had a 6- and 3-fold higher affinity, respectively, for calf thymus dCytK when compared with β-D-ddC [23]. The β-L enantiomer 3TC and its 5-fluoro derivative, FTC, are much more efficient substrates for human dCytK than their corresponding β-D enantiomers with either ATP or UTP as the phosphate donor [14]. The intracellular concentration of the nucleoside analog rapidly establishes an equilibrium with the extracellular concentration of the nucleoside. Upon washing, the intracellular nucleoside concentration will decrease dramatically to levels that do not reflect the true intracellular concentration at the exact time of exposure termination. This trend was observed in the washout experiments in which the intracellular nucleoside concentration decreased by greater than 50% after 1 hr of incubation in drug-free medium (data not shown). Therefore, the intracellular nucleoside levels could not be assessed accurately, but they would be expected to far exceed the levels of their corresponding 5′-monophosphate derivative. On the basis of the intracellular anabolite levels (Table 1) and the likely high intracellular nucleoside levels before washing, the cellular activation of β-D-FddC by dCytK is probably the rate-limiting step, while the activation of β-L-FddC and β-L-ddC to their respective triphosphates may be limited by nucleoside diphosphokinase (EC 2.7.4.6). Similar conclusions have been suggested regarding the rate-limiting activation enzyme of β-L-FTC and β-D-FTC [6]. Therefore, the enantioselective phosphorylation of the β-L nucleosides to their respective triphosphates represents a primary factor responsible for the very potent antiviral activity of some β-L enantiomers. Our data concerning β-L-ddC and its 5-fluoro derivative, β-L-FddC, may not be exclusive to reported cytidine analogs [8], and recent data would suggest that stereoselective cellular activation of nucleosides can also be applied to β-L-thymidine derivatives.**

Studies comparing the intracellular metabolism of 3TC to its respective β-D enantiomer noted a 2-fold higher level of accumulation of the β-L-5′-triphosphate in H-9 cells [9]. A 10-fold higher intracellular triphosphate concentration for β-L-FTC versus β-D-FTC was also reported [6]. Furthermore, 3TC was not deaminated by a partially purified human deoxycytidine deaminase, whereas under the same conditions β-D-2′,3′-dideoxy-3′-thiacytidine (β-D-BCH-189) was deaminated rapidly to β-D-2′,3′-dideoxy-3′-thiauridine [10]. Intracellular metabolism studies of FTC also demonstrated deamination of only the β-D enantiomer of FTC [6, 14]. In our studies, none of the isolated β-L-FddC remaining intracellularly after washing was detectably deaminated as assessed by reverse-phase HPLC, whereas under the same conditions 5% of the isolated β-D-FddC remaining intracellularly after washing was determined to be β-D-FddU. The 5′-monophosphate derivative of β-D-FddU was not detected intracellularly, and this is probably due to the poor affinity of this substrate for cellular kinases. The poor affinity of uridine analogs for cellular kinases has been demonstrated in studies investigating the cellular activation of 2′,3′-dideoxyuridine in ATH8, Molt-4, and CEM cell lines [31]. Therefore, the β-D enantiomers of 3TC, FTC, and FddC nucleoside analogs have the potential to divert available drug from further intracellular 5′ phosphorylation. However, this unattractive feature cannot be attributed to all β-D-cytidine analogs because the β-D enantiomer of ddC was not apparently deaminated in human monocyte-derived macrophages [32].

Another characteristic that contributes to the potent an-

** Data concerning the stereoselective anti-HIV and anti-HBV activity of β-L-AZT and other thymidine analogs will be reported in detail in a paper submitted for publication elsewhere.

tiviral activity of these unnatural configured β-L nucleoside analogs is the extended intracellular $T_{1/2}$ of their active triphosphate. 3TC-TP was shown to have an average intracellular $T_{1/2}$ of 13.8 hr in mock-infected phytohemagglutinin-stimulated peripheral blood lymphocytes, whereas β-D-BCH-189-5′-triphosphate had a substantially shorter $T_{1/2}$ of 3.5 hr [33]. In our study using Hep-G2 cells, β-L-FddCTP and β-L-ddCTP also exhibited extended intracellular $T_{1/2}$ of values 14.8 and 12.3 hr, respectively, with β-D-FddCTP having a shorter intracellular $T_{1/2}$. Furthermore, the 5′-mono- and diphosphate derivatives of β-D-FddC had markedly more abbreviated intracellular $T_{1/2}$ values of 1.0 and 0.9 hr, respectively, whereas both β-L-FddCMP and β-L-FddCDP had extended intracellular $T_{1/2}$ values of greater than 24 hr. These prolonged intracellular $T_{1/2}$ values, which have been shown to be inherent features of other β-L triphosphates such as FTCTP and 3TCTP [6, 8, 33], probably reflect a poor affinity for pyrimidine nucleoside phosphorylases, phosphatases, 5′-nucleotidases, and also possibly for CTP:phosphocholine or phosphoethanolamine cytidyltransferase.

Two unknown metabolites were detected in β-D-FddC-exposed Hep-G2 cells, whereas only one additional metabolite to their 5′-phosphorylated derivatives was detected with β-L-FddC and β-L-ddC. Characterization of these metabolites by double-labeled incorporation and enzymatic digestion experiments revealed their identity as the 5′-liponucleotides β-D-FddCDP-choline, β-D-FddCDP-ethanolamine, β-L-FddCDP-choline, and β-L-ddCDP-choline. Of note, the 5′-ethanolamine derivative was not detected in either β-L-FddC- or β-L-ddC-exposed Hep-G2 cells consistent with the previously proposed enantioselective metabolism of β-L-FTC and β-D-FTC to these 5′-liponucleotides in Hep-G2 (2.2.15) cells [6].

These 5′-diphosphocholine and/or 5′-diphosphoethanolamine derivatives have also been detected in a variety of cell lines that have been exposed to either deoxycytidine [32], β-D-ddC [29], or (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) [34]. These 5′-liponucleotides have been suggested to be involved in two possible roles including indirect anti-HIV activity and toxicity of the clinically approved drug, β-D-ddC [29]. In Molt-4 cells, Hao *et al.* [29] documented an intracellular $T_{1/2}$ for β-D-ddCDP-choline of 6.35 hr, and 14.8 hr for the corresponding 5′-diphosphoethanolamine derivative of β-D-ddC. Indeed, these liponucleotides may act as intracellular precursors or reservoirs for the active nucleoside triphosphate and also be potential inhibitors of lipid biosynthesis which would affect the membrane integrity as their endogenous cytidine counterparts, cytidine diphosphocholine and cytidine diphosphoethanolamine, are vital intermediates in phospholipid biosynthesis [35]. The biochemical pathway (Fig. 5) responsible for lipid biosynthesis permits these activated liponucleotides to reenter the triphosphate activation pathway at the monophosphate level via 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) or ethanolaminephosphotransferase (EC 2.7.8.1).

To determine the potential of these 5′-liponucleotides of β-L- and β-D-FddC and β-L-ddC to serve as prolonged intracellular reservoirs of the active 5′-triphosphate, their intracellular $T_{1/2}$ values were calculated in Hep-G2 cells. The 5′-diphosphoethanolamine derivatives of β-D-FddC had substantially longer intracellular $T_{1/2}$ of greater than 24 hr when compared with 3.8 hr for β-L-FddCDP-choline and 6.6 hr for β-L-ddCDP-choline. Two explanations may explain the different stereoselective intracellular $T_{1/2}$ of the respective 5′-liponucleotides. The 5′-liponucleotide derivatives of β-D-FddC and β-L-FddC are either differentially metabolized to the corresponding 5′-monophosphate and phosphatidylcholine and/or phosphatidylethanolamine, or their levels are readily replenished by the respective CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) or CTP:phosphoethanolamine cytidyltransferase (EC 2.7.7.14). To investigate the contribution of the latter possibility, attempts to inhibit CTP:phosphocholine cytidyltransferase with sphingosine and the effect on intracellular 5′-triphosphate levels were performed. This analog of the predominant neurophospholipid sphinomyelin was identified by Sohal and Cornell [36] as a potent inhibitor of purified rat liver CTP:phosphocholine cytidyltransferase, but this compound was extremely toxic to Hep-G2 cells. Therefore, no definite conclusions about the contribution of the 5′-liponucleotides can be ascertained.

Interestingly, when primary cultured hepatocytes were exposed to 10 μM β-L-FddC, substantial phosphorylation to its 5′-mono-, di-, and triphosphate was detected, but β-L-FddCDP-choline levels were below our limit of detection. In contrast, Condreay *et al.* [3] detected both the 5′-diphosphocholine and the 5′-diphosphoethanolamine derivative in β-L-FTC-exposed primary cultured hepatocytes, although earlier experiments performed in Hep-G2 (2.2.15) cells led only to the detection of the 5′-diphosphocholine derivative [6]. Both 5′-liponucleotides were detected in β-D-FTC-exposed Hep-G2 (2.2.15) cells [6]. The apparent difference in the consistency of enantioselective metabolism of β-L-FddC and β-L-FTC in Hep-G2 cells and in primary cultured hepatocytes may reflect differences in cells and medium conditions. Indeed, addition of exogenous choline and ethanolamine to the culture medium may interact with lipid metabolism [37], and the disease state of the donated liver from which the hepatocytes are obtained may also contribute to the degree of formation of these 5′-liponucleotides. It is not yet clear whether these 5′-liponucleotides may be possible contributing factors in the cytotoxicity of β-L-FddC, β-D-FddC, and β-L-ddC. As depicted in Fig. 5, their phosphocholine and phosphoethanolamine moieties being transferred via 1,2-diacylglycerol cholinephosphotransferase or ethanolaminephosphotransferase to 1,2-diacylglycerol generate the prominent phospholipids phosphatidylcholine and phosphatidylethanolamine, and subsequent inhibition of either phosphatidylcholine or phosphatidylethanolamine formation may have effects on lipoprotein secretion [37].

In this report we have demonstrated that β-L-FddC, β-D-FddC, and β-L-ddC are phosphorylated rapidly in Hep-G2 cells to their respective 5′-mono-, di-, and triphosphate derivatives. The 5′-triphosphate derivatives of the two β-L nucleoside enantiomers, β-L-FddC and β-L-ddC, achieved substantially higher intracellular levels as compared with the 5′-triphosphate of β-D-FddC. Furthermore, both β-L-FddCTP and β-L-ddCTP had a significantly longer intracellular $T_{1/2}$ when compared with β-D-FddCTP. The higher intracellular triphosphate levels and the extended intracellular $T_{1/2}$ of the β-L triphosphates may be contributing factors in the pronounced antiviral activity of β-L- versus β-D-cytidine nucleoside analogs. Important phosphorylation of β-L-FddC in resting primary cultured hepatocytes was also demonstrated, further suggesting that β-L-FddC may be a good candidate for further development as an anti-HBV agent. Lastly, the major difference in the presence and extent of accumulation of 5′-diphosphocholine and 5′-diphosphoethanolamine derivatives of β-L cytidine analogs versus β-D cytidine analogs supports an enantioselectivity of that metabolic pathway, but the relevance of the 5′-liponucleotide enantioselectivity toward the pharmacological effects of cytidine analogs remains to be established.

This work was supported, in part, by Public Health Service Grants AI-33239 (J-P. S.), AI-25899 (R. F. S.) and by the Department of Veterans Affairs. J-P. S. is the recipient of a Faculty Research Award from the American Cancer Society.

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