Biochemical Pharmacology of (+)- and (-)-2',3'-Dideoxy-3'-thiacytidine as Anti-hepatitis B Virus Agents*

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2',3'-Dideoxy-3'-thiacytidine (cis-2)-SddC was found to have potent activity against hepatitis B virus and human immunodeficiency viruses in culture. Recent studies by us identified (-)-SddC as the stereoisomer responsible for the antiviral effect and showed that the cytotoxicity was mainly caused by (+)-SddC. Metabolism studies showed that these drugs were converted to their monophosphates, diphosphates, and triphosphates. The enzyme responsible for the formation of monophosphates was identified to be cytoplasmic deoxycytidine kinase in CEM cells. Uptake studies showed that the intracellular concentration of (-)-SddC and its metabolites was approximately 5-fold higher than that of (+)-SddC metabolites. (-)-SddCTP was more potent than (+)-SddCTP in inhibiting hepatitis B virus replication; (+)- and (-)-SddCTP exhibited minimal inhibition on polymerases α and δ, more inhibition on β, and strong inhibition on γ. In all cases, (+)-SddCTP was found to be more inhibitory than (-)-SddCTP to all four polymerases. (+)-SddCMP competed with dCTP for incorporation into DNA by DNA polymerase γ and β and served as a chain terminator; however, similar incorporation was not detected using other polymerases. The selective inhibition of DNA synthesis in isolated mitochondria by (+)- and (-)-SddCTP suggests a stereospecificity on the mitochondrial uptake of deoxynucleoside triphosphates.

Hepatitis B virus (HBV) infection commonly results in acute and chronic hepatitis and has also been shown to be closely related to the development of hepatocellular carcinoma (1-3). HBV is a double-stranded DNA virus; its DNA polymerase catalyzes both DNA-dependent as well as RNA-dependent DNA synthesis (4, 5). The life cycle of this unique DNA virus involves the enzyme reverse transcriptase in its DNA replication (6). Although vaccination has been used for the prevention of HBV infection, there are no effective drugs available for the treatment of HBV infection.

Several 2',3'-dideoxycytidine analogs were examined for their inhibition of HBV DNA replication using HBV-transfected cell lines (2215) (7, 8). Among them, 2',3'-dideoxy-3'-thiacytidine ((+)-SddC) and 5-fluoro-2',3'-dideoxy-3'-thiacytidine ((±)-SddC) were found to be the most potent inhibitors of HBV DNA replication with ID₅₀ equal to 0.05 and 0.1 µM, respectively, as monitored by the amount of both intracellular episomal and secreted viral DNAs (7). At concentrations that completely inhibited HBV DNA replication, no detectable changes were found on the HBV-specific RNAs (7). Therefore the inhibitory target is most likely the HBV DNA synthesis. Upon drug removal, the antiviral effects of SddC and 5-FSddC were reversed (7), suggesting that continuous usage of the drugs may be necessary in the treatment.

By incubating the racemic mixture of cis-SddC stereoisomers (Fig. 1) with human deoxycytidine deaminase at 37 °C for 16 h, approximately 50% of the mixture could be deaminated (9). No further deamination were observed upon prolonged incubation. A detailed kinetic study showed that only the (+)-SddC could be deaminated; the other stereoisomers were not substrates for the human deoxycytidine deaminase (9). (-)-SddC, which was resistant to deoxycytidine deamination, was found to be a more potent antiviral stereoisomer than (+)-SddC (HBV ID₅₀ = 0.5 µM, 50-fold higher than that of (-)-SddC). The (+)-form, which was susceptible to the deamination, was 25-fold and 12-fold more toxic than the (-)-SddC in CEM cells in terms of anti-cell growth and antimitochondrial DNA synthesis, respectively (9). Compared with ddC, which had an anti-mitochondrial DNA ID₅₀ of approximately 0.022 µM, the (+)- and (-)-SddC were relatively ineffective in inhibiting the mitochondrial DNA metabolism.
(mtDNA) synthesis (9). Peripheral neuropathy has been suggested to be the result of inhibition of mitochondrial DNA synthesis (10). Therefore, delayed toxicity, such as peripheral neuropathy, observed in patients treated with ddC, may not be an issue using (-)-SddC for antiviral drug therapy. Once (-)-SddC enters the cell, it can be phosphorylated to SddCMP and subsequently converted to SddCDP and SddCTP. The identification of each of the (-)-SddC metabolites has been published previously (9).

In this report, identification of cytoplasmic deoxycytidine kinase as the enzyme responsible for the monophosphorylation of (+)- and (-)-SddC and their kinetic studies are described. Detailed comparison of nucleoside uptakes and the efflux of metabolites using (+)- and (-)-SddC as well as the inhibition of human DNA polymerases and mitochondrial DNA synthesis using (+)- and (-)-SddCTP are presented. The antiviral effects and the cytotoxicities of these two SddC stereoisomers are explained based on these studies. The possible stereospecificity of the whole cell and the mitochondria membranes for these stereoisomers at either nucleoside or nucleotide level is also discussed.

MATERIALS AND METHODS

Compounds and Chemicals—The stereoisomers of cis- (+)- and (-)-SddC were kindly provided by Dr. C. K. Chu, Department of Medicinal Chemistry, University of Georgia (12). The (+)-SddCTP and (-)-SddCTP were synthesized from their nucleoside counterparts and purified from HPLC using a previously published method (9). (+)-[3H]SddC (RCH=185-2,4,5OH, mixture of two cis-isomers, (+)-SddC and (-)-SddC, 20 Ci/mmol, was purchased from Moravek Biochemicals, Inc., Brea, CA; (+)- and (-)-[3H]SddC were separated from this mixture using a Cyclobond I acetylated HPLC column (Advanced Separation Technologies Inc., NJ) similar to a published procedure (13).

Enzymes—Human DNA polymerases α, δ, and γ were purified from either K562 (chronic myelogenous leukemia cell line) or CEM (T-lymphoblastoid cell line) cells; polymerase β was purified from AML (acute myelogenous leukemia cell line) cells. In general, 10 ml of cell pellet was resuspended in 5 volumes of a buffer containing 10 mM potassium phosphate, pH 7.5, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM (PMFSF), 1 mM KCl, and 10% glycerol. The cells were frozen and thawed three times, then sonicated three times, for 30 s, at a setting of 4 on a Bronson model 200 cell disruptor, followed by centrifugation at 40,000 g for 30 min. The supernatant fraction was passed through a 50-ml DEAE-cellulose column equilibrated with buffer A (400 mM potassium phosphate, pH 7.5, 2 mM DTT, 1 mM EDTA, 1 mM PMFSF, and 10% glycerol). Unabsorbed fractions were pooled and dialyzed overnight against buffer B (substituted 400 mM potassium phosphate with 50 mM Tris-HCl, pH 7.5, from buffer A). The dialysate was applied to a 25-ml single-stranded DNA (ssDNA) cellulose column equilibrated with buffer B. The column was washed with 50 ml of buffer B, and eluted with a 100-ml linear gradient of 0-1 M KCl in buffer B. DNA polymerase α, δ, γ, and β eluted off the column at approximately 0.2, 0.3, 0.45, and 0.8 M KCl, respectively. Fractons containing polymerases β and γ were pooled and dialyzed against buffer B for 2 h with 2 changes of buffer B and applied to a 5-ml DEAE-cellulose column equilibrated with buffer B. The column was washed with 10 ml of buffer B and eluted with a 50-ml linear gradient of 0-0.6 M KCl in buffer B. DNA polymerase γ and δ eluted off the column at approximately 0.35 and 0.5 M KCl, respectively. The first several fractions that contain polymerase α from ssDNA column were pooled, and a second ssDNA column was repeated to assure that possible contamination of polymerases δ and ε was removed. The specific activities of these partially purified DNA polymerases α, β, γ, and δ were 280, 25, 40, and 32 units/mg, respectively. One unit of enzyme activity is defined as the amount which catalyzes the incorporation of 1 nmol of [3H]dTMP into acid-insoluble DNA within 60 min at 37 °C. The unabsorbed fractions from ssDNA column were pooled and directly applied to a 15-ml hydroxylapatite column preequilibrated with buffer C (10 mM potassium phosphate, 5 mM MgCl2, 5 mM CaCl2, 0.5 mM dithiothreitol, and 20% glycerol), and eluted with a 100 ml linear gradient of 0-200 mM potassium phosphate. Deoxycytidine kinase activity eluted off the column at approximately 75 mM potassium phosphate. The peak activities were pooled and applied to a thymidine kinase affinity column using published procedures (14). The specific activity of the partially purified dCDK kinase was 240 units/mg. The unit definition was the amount of enzyme which produced 1 nmol of product/h at 37 °C under our assay conditions.

Standard Assay for the Activity of dCDK Kinase—The reaction mixture contained 0.1 M Tris-HCl, pH 7.5, 2 mM DTT, 6 mM MgCl2, 6 mM ATP, 0.2 mM tetrahydrodiamine, 1 unit of creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 0.5 mM dCDK, and 0.1 Ci of [3H]dCTP (66 Ci/mmol) in a total volume of 0.1 ml. The assays were performed as described by Cheng et al. (14). The incubation was at 37 °C for 60 min.

Standard Assay for the Analysis of the Acid-soluble Metabolites—The assay condition was virtually the same as the procedure described by Chang et al. (9). In general, the cells were incubated in a specified concentration of [3H]SddC for the time of indicated. Medium was removed, and cells washed for 5 min and loaded onto 15% polyacrylamide DNA sequencing gel. Electrophoresis was carried out at 80 volts/1 w per lane for 18 h followed by autoradiography using Kodak XRP-5 x-ray film.

Standart Assay for the Analysis of DNA Polymerase Activity Using M13 mp19 DNA—The assay was done as was previously described by our laboratory (16). The M13 primer was labeled by incubating 0.005 (A260) of the primer with 50 µCi of [32P]ATP (5000 Ci/mmol) and 9 units of T4 polynucleotide kinase (Boehringer Mannheim) in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, and 5 mM DTT in a total volume of 30 µl for 15 min at 37 °C, followed by boiling for 5 min. The primer was annealed to 75 µl of M13 mp19(+)-ssDNA by heating at 65 °C for 10 min followed by gradual cooling to room temperature, usually overnight. The sequence of the primer-template substrate for DNA elongation assays is shown below.

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\begin{align*}
\text{TACCGGCGACAGAATTG} & \quad 3' \\
\text{TACCGACCGTAGAATTGC} & \quad 5' \\
\text{TACCGACCGAGCAATTGC} & \quad 3' \\
\text{TACCGACCGAGCAATTGC} & \quad 5'
\end{align*}
\]

The DNA elongation reactions were done with 0.005 units of DNA polymerases α, δ, and ε, 0.0005 units of γ, and 0.0003 units of β at 37 °C for 40 min. The reaction mixture contained, in a total volume of 10 µl, 0.005 M Tris-HCl, pH 8.0, 2 mM MgCl2, 2 mM DTT, 5 or 50 µM of each dATP, dGTP, and dTTP, and 100 µCi of [32P]-labeled M15 template/primer, and the indicated concentration of dCTP or analog. The reactions were terminated by the addition of 4.0 µl of 98% formamide, 10 mM EDTA, and 0.25% bromophenol blue; samples were denatured by boiling for 3 min and loaded onto 15% polyacrylamide DNA sequencing gel. Electrophoresis was carried out at 80 volts/1 w per lane for 18 h followed by autoradiography using Kodak XRP-5 x-ray film.
RESULTS

Nucleoside Uptake of (+)- and (-)-SddC in 2215 Cells—The uptakes of (+)- and (-)-SddC nucleosides in 2215 (HBV transfected cell line) cells at different time points were compared, and the results are shown in Fig. 2A. The nucleoside concentrations used was 0.2 μM. The differences of the intracellular concentrations between (+)- and (-)-SddC metabolites increased with time, and at 8 h, a difference of approximately 5-fold was observed. The HPLC profiles of the acid-soluble fraction of 2215 cells treated with 0.5 μM (+)- and (-)-SddC for 24 h are shown in Fig. 2 (B and C, respectively). There is no qualitative difference between the two profiles except that the intracellular concentration of (-)-SddC nucleotides is much higher than that of (+)-SddC nucleotides. It is interesting to note that the retention time of mono- (13 min), di- (23 min), and triphosphates (50 min) metabolized from both (-)-SddC and (+)-SddC were identical under our HPLC conditions.

The Cytoplasmic Deoxycytidine Kinase Is Responsible for the Monophosphorylation of the (+)- and (-)-SddC Nucleosides—Fig. 3 shows the HPLC analysis of (-)-SddC acid-soluble metabolites following incubations of 2215 cells with 0.5 μM (-)[3H]SddC for 1 h at 37 °C. In the presence of 2.5

Fig. 2. Uptake studies of (+)- and (-)-SddC in 2215 cells. 2215 cells (5 × 10⁶) were incubated in the presence of 0.2 μM (A) or 0.5 μM (B and C) of (+)- and (-)[3H]SddC (2.3 mCi/mmol) at 37 °C for 0.5, 1.5, 4, and 8 h (A) or 24 h (B and C). At the end of each time point, medium was removed, the cells were washed, and the acid-soluble metabolites were extracted according to the standard method described under "Materials and Methods." The radioactivities of the acid-soluble metabolites were either measured directly by scintillation counting (A) or analyzed by HPLC (B and C).

Fig. 3. The effect of dCyd on the formation of acid-soluble metabolites of (-)-SddC in 2215 cells and the metabolism studies of (-)-SddC in wild type and Ara-Cyd-resistant CEM cells. 2215 cells (5 × 10⁶) were incubated in the presence of 0.5 μM (-)[3H]SddC (2.3 mCi/mmol) with (A) or without (B) 2.5 μM dCyd at 37 °C for 1 h. Wild type CEM cells (5 × 10⁶) (C) were also incubated in the presence of the same concentration of (-)[3H]SddC for 8 h. Medium was removed, cells were washed twice with phosphate-buffered saline, and the acid-soluble metabolites were extracted and analyzed by HPLC.
μM deoxycytidine (Fig. 3B), the production of acid-soluble metabolites of (-)-SddC was significantly inhibited as compared with the metabolites observed in the absence of deoxycytidine (Fig. 3A). Similar results were also obtained when the incubations were extended to 3 h (results not shown); however, the extent of inhibition was slightly lower. The HPLC profile of the acid-soluble metabolites of CEM cells treated with 0.5 μM (-)-[3H]SddC for 8 h is shown in Fig. 3C. The metabolites obtained from wild type CEM cells were identical to those observed in 2215 (Fig. 2B) or HepG2 (data not shown) cells based on the retention time of each metabolite. However, when Ara-Cyd-resistant CEM cells (dCyd kinase-deficient) were used, no acid-soluble metabolites other than the (-)-SddC nucleoside were observed intracellularly (results not shown). These results indicate that dCyd kinase is responsible for the monophosphorylation of (-)-SddC nucleoside in both 2215 and CEM cell lines. 3H-Labeled dCyd, dideoxycytidine, (+)-SddC, and (-)-SddC were used to determine the kinetic constants of monophosphorylation by partially purified cytoplasmic dCyd kinase. The $K_m$ and $V_{max}$ values for these substrates are summarized in Table I. Whereas the $K_m$ values were virtually the same, differences of 15- and 30-fold in the $V_{max}$ values were obtained for (-)-SddC and (+)-SddC, respectively, relative to the $V_{max}$ value of dCyd.

**Accumulation and Degradation of (-)-SddC Metabolites in 2215 Cells after Drug Removal—** Upon measuring the half-life of (-)-SddCTP after drug removal, an interesting result was observed (Fig. 4, A–D). The experiment was done by incubating 0.5 μM [3H]labeled (-)-SddC at 37 °C for 24 h (Fig. 4A). Fresh medium without drug was replaced at the end of 24 h, and the incubation was continued. The acid-soluble metabolites were analyzed at different time points using HPLC (Fig. 4, B–D). In the first 2 h, most of the intracellular (-)-SddC nucleoside (retention time, 5 min) was converted to (-)-SddCDP (retention time, 24 min) (Fig. 4B). At the 4-h time point (Fig. 4C), most of the diphosphate was converted to (-)-SddCTP (retention time, 50 min). At later time points, the triphosphate and diphosphate were gradually degraded (Fig. 4D) and effluxed out of the cells.

**Inhibition of Human DNA Polymerases and Endogenous HBV DNA Polymerase by (+)- and (-)-SddCTP—** Human DNA polymerases $\alpha$, $\beta$, $\gamma$, and $\delta$ were partially purified, and the sensitivity of (+)- and (-)-SddCTP against each of the four DNA polymerases were examined. As shown in Fig. 5, at 0.175 μM both dCTP and inhibitors, only DNA polymerase $\gamma$ showed potent inhibition. (+)-SddCTP was a more potent inhibitor than (-)-SddCTP of all four polymerases. The $K_m$ values of (+)- and (-)-SddCTP for human DNA polymerases $\alpha$, $\beta$, $\gamma$, and $\delta$ as well as for endogenous HBV DNA polymerase are summarized in Fig. 5 (lower panel). It is interesting to note that for the inhibition of polymerase $\gamma$, (+)-SddCTP is 5-fold more potent than (-)-SddCTP. On the other hand, for the inhibition of endogenous HBV DNA polymerase, (+)-SddCTP is approximately 5-fold less potent than (-)-SddCTP.

**Table I**

Comparison of dCyd, ddCyd, (+)SddC, and (-)SddC phosphorylation by cytosolic dCyd kinase

| Substrate | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) |
|-----------|------------|-------------------------|
| dCyd      | 8 ± 3      | 4 ± 1                   |
| ddCyd     | 160 ± 30   | 1.6 ± 0.3               |
| (+)-SddC  | 7 ± 1      | 0.13 ± 0.02             |
| (-)-SddC  | 7 ± 1      | 0.25 ± 0.03             |

A primer extension assay utilizing 32P-labeled M13 primer annealed to M13 mp19 ssDNA was employed to assess the effect of the dCTP analogs ddCTP, (+)-SddCTP, and (-)-SddCTP on partially purified human DNA polymerases. In the case of polymerase $\alpha$, reducing the concentration of dCTP from 50 μM to 0.175 μM results in an increased accumulation of DNA products terminated with dTTP one base before the incorporation of dCTP (Fig. 6, lanes 1–3). In the absence of dCTP, there is a small amount of misincorporation indicated by DNA products 26 bases long (lane 4). All analogs inhibit DNA elongation past the first “C” site in reactions without dCTP. Under these conditions, ddCTP, (+)-SddCTP, and (-)-SddCTP are not incorporated into the DNA by polymers.
erase α (lanes 5, 7, and 9). The DNA elongation is restored to control levels when equimolar dCTP is included in the assay (compare lane 3 with lanes 6, 8, and 10). Similar observations were made with DNA polymerase δ.

In the case of DNA polymerase γ, the activity was processive on the M13 primer/template under optimal conditions (lane 1). There was a substantial amount of misincorporation by this enzyme when dCTP was left out of the reaction (lane 4). The results in lane 5 show that (+)-SddCTP is incorporated into the DNA and results in the accumulation of products that are terminated at the first “C” site. This effect is partially reversed in the presence of dCTP, indicating that (+)-SddCTP competes with dCTP as a substrate for polymerase γ (lane 6). Similar observations were made with (-)-SddCTP; however, this stereoisomer is less inhibitory as indicated by a lower extent of DNA termination. Furthermore, in the presence of dCTP, there is minimal incorporation of the analog at the first “C” site (lane 8). The incorporation of the ddCTP into the DNA proceeds to the same extent as that of (+)-SddCTP and is also partially inhibited by dCTP (lanes 9 and 10).

In the case of DNA polymerase β, the assay conditions had to be changed in order to determine whether this enzyme can incorporate any of the three analogs. Assays utilizing 0.175 µM dCTP resulted in DNA products which were elongated to 22 bases just prior to the first “C” site (results not shown). Since the $K_m$ for dCTP is 2.6 µM, the concentration of dCTP was increased to 5 µM to attain sufficient reaction velocity. Similar kinetic considerations were taken into account in choosing the concentrations of the dCTP analogs. At 0.8 µM (+)-SddCTP, there is evidence of incorporation at the appropriate “C” site (lane 4). As was noted with polymerase γ, dCTP competes with (+)-SddCTP for incorporation into DNA. (-)-SddCTP and ddCTP are both less efficient chain terminators than (+)-SddCTP at concentrations close to their $K_i$ values (lanes 6 and 8). At 5 µM, ddCTP is a potent chain terminator in the absence of dCTP. The ability of these analogs to serve as substrates for polymerases γ and β could account for their inhibitory effect on these enzymes.

**Inhibition of DNA Synthesis in Isolated Mitochondria by (+)- and (-)−SddCTP**—(+)-SddCTP and (-)-SddCTP were tested for their effect on mitochondrial DNA (mtDNA) synthesis in comparison with ddCTP. (-)-SddCTP, (+)-SddCTP, and ddCTP inhibited mtDNA synthesis in a dose-dependent manner (Fig. 7). Aphidicolin (25 µg/ml), a specific inhibitor of DNA polymerase α, was also used to demonstrate...

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**Fig. 5. Inhibition of DNA polymerases and endogenous HBV DNA polymerase by ddCTP analogs.** The enzyme activity assays and the determinations of kinetic constants were performed using standard methods as described under “Materials and Methods.”

**Fig. 6. Inhibition of DNA polymerases by ddCTP analogs using M13 mp19 DNA.** For polymerases α, δ, and γ: lane a, reaction without enzyme; lane 1, reaction with 50 µM dATP, dGTP, dTTTP (dNTP), and dCTP; lane 2, reaction with 5 µM dNTP and dCTP; lane 3, reaction with 5 µM dNTP and 0.175 µM dCTP; lane 4, 5 µM dNTP, no dCTP (C− reaction); lanes 5, 7, and 9, C− reactions and 0.175 µM (+)-SddCTP, (-)-SddCTP, and ddCTP, respectively; lanes 6, 8, and 10, similar to reactions in lanes 5, 7, and 9, respectively, except 0.175 µM dCTP was included. For β polymerase: lane 1, 50 µM dNTP and dCTP; lane 2, 50 µM dNTP and 5 µM dCTP; lane 3, C− reaction; lanes 4, 6, 8, and 10, C− reactions with 0.8 µM (+)-SddCTP, 1.5 µM (-)-SddCTP, 0.9 µM ddCTP, and 5 µM ddCTP, respectively; lanes 5, 7, 9, and 11, similar to reactions in lanes 4, 6, 8, and 10, respectively, except 5 µM dCTP was included.
that the nuclear DNA synthesis was absent in the assay conditions. The inhibition of mtDNA synthesis by (+)-SddCTP at 0.1 μM was more potent than the inhibition by (-)-SddCTP at 3 μM. The relative potencies among these three nucleoside triphosphates were in the following order: ddCTP > (+)-SddCTP >> (-)-SddCTP.

**DISCUSSION**

Our unpublished studies showed that diprydamole (a potent inhibitor of nucleoside transport system) effectively suppressed the transport of SddC, indicating that the nucleoside transport system is operative. However, a majority of the SddC nucleoside enters the cell through nonfacilitated passive diffusion or other mechanisms after prolonged incubation. The intracellular concentration of (-)SddC acid-soluble metabolites were approximately 5-fold higher than that of (+)-SddC at steady-state levels. Nonetheless, the HPLC profiles of the metabolites from (-)- and (+)-SddC show no intrinsic differences.

Deoxycytidine inhibits the formation of SddC acid-soluble metabolites in 2215 cells, suggesting that SddC and deoxycytidine share a common kinase for their monophosphate phosphorylation. Further investigations using wild type and Ara-Cyd-resistant (deoxycytidine kinase-deficient) CEM cell lines showed that only the wild type CEM cells produced SddC metabolites with a profile similar to the one obtained from 2215 cells. Ara-Cyd-resistant CEM cells have no capability of converting SddC nucleoside to its corresponding nucleotides, indicating that deoxycytidine kinase is responsible for the SddC monophosphorylation. Although the Vmax values of (-)- and (+)-SddC examined from monophosphorylation of cytoplasmic dCyd kinase were approximately 30- and 15-fold lower than that of dCyd, their Km values were virtually the same.

The stability study on the acid-soluble metabolites, when monitored by HPLC profiles after drug removal (Fig. 4), showed that (-)-SddCTP had an extended half-life. However, after removing the drug, instead of effluxing out of the cell, SddC nucleoside was further converted to SddCDP and di-phosphate accumulation occurred. This observation suggests that the rate-limiting step is the conversion of SddCDP to SddCTP. Phosphorylations of SddC to SddCMP and SddCMP to SddCDP were relatively rapid inside the cell. Further conversion from SddCDP to SddCTP continued, and a net accumulation of SddCTP was observed, indicating that the rate of degradation of SddCTP to SddCDP was slower than the rate of conversion of SddCDP to SddCTP. According to Fig. 4, the apparent half-life of SddCTP was approximately 24 h; however, the real half-life was estimated to be approximately 12 h.

Evaluation of the relative potency of chemically synthesized (+)- and (-)-SddCTP as inhibitors of the endogenous HBV DNA polymerase activity showed that (-)-SddCTP (Km = 21 nM) was approximately 5-fold more potent than (+)-SddCTP (Km = 110 nM). The 50-fold difference of anti-HBV effects between (-) and (+) SddCTP is 50-fold more inhibitory than (-)-SddCTP to all four polymerases. (+)-SddCMP and (-)-SddCMP can be incorporated into M13 DNA in competition with dCTP by DNA polymerase γ and β and served as chain terminators; however, similar incorporation was not detected for polymerases α and δ. These results are consistent with the cytotoxicity data published previously.

There is an approximate 12-fold difference in the ID50 values between (+)-(4 μM) and (-)-SddCTP (50 μM) in terms of auto-inhibitory DNA polymerase synthesis (9). Since the only DNA polymerase responsible for the mitochondrial DNA synthesis is DNA polymerase γ, and since the intracellular concentration of (+)-SddCTP was 5-fold less than that of (-)-SddCTP, one would expect that (+)-SddCTP should be approximately 60-fold more inhibitory than (-)-SddCTP to DNA polymerase γ activity. However, inhibition of DNA polymerase γ by (+)-SddCTP was not that dramatic compared with that by (-)-SddCTP, according to the Km values in Fig. 5. The results of experiments to examine DNA synthesis in isolated mitochondria (Fig. 7) provide a logical explanation. The inhibition of mitochondrial DNA synthesis by (+)-SddCTP at 0.1 μM was more potent than the inhibition by (-)-SddCTP at 3 μM. This result accounts for the above apparent discrepancy, and, more importantly, it also suggests a potential stereospecificity of the transport on the membrane of mitochondria. It should be noted that we have shown previously that ddCTP is able to enter the mitochondria without going through the process of dephosphorylation (11).

Several groups reported the investigation of the antiviral effect using pure (+)- and (±)-SddC nucleosides as antihuman immunodeficiency virus (HIV) agents in various cell systems (13, 18, 19). A 2-fold difference in the anti-HIV activity between (+)- and (±)-SddC has been reported by Coates et al. (18), whereas (±)-SddC was found to be 4-100-fold more potent than (+)-SddC by Schinazi et al. (13). We previously reported that (+)-SddC could be deaminated by human deoxycytidine deaminase with a Km value approximately 15-fold higher than that of deoxycytidine, while (-)-SddC is not a substrate for the deaminase (9). Therefore, variable intracellular deoxycytidine deaminase levels may explain the variations obtained by these groups. However, in our studies, we did not observe the SddU type of metabolite with incubation up to 24 h in any of the cells examined. Thus, the variations above may reflect differences in the potential selectivities of various cell membranes and/or differences in cellular metabolisms.
In summary, the information presented here shows that (-)-SddC is the active stereoisomer against HBV DNA replication, whereas (+)-SddC is mainly responsible for the cytotoxicity observed with the racemic mixture. Metabolism studies as well as direct examinations of the inhibition of endogenous HBV DNA replication and that of human DNA polymerases using chemically synthesized (+)- and (-)-SddCTP elucidated the detailed mechanisms of action by these drugs. Both the whole cell and the mitochondrial membranes exhibited stereospecificities for these stereoisomers at nucleoside or nucleotide levels. The potential competitive interactions between these stereoisomers with their membrane transports and metabolisms should be investigated in greater detail.

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