Deoxycytidine Deaminase-resistant Stereoisomer Is the Active Form of (±)-2’,3’-Dideoxy-3’-thiacytidine in the Inhibition of Hepatitis B Virus Replication*

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2’,3’-Dideoxy-3’-thiacytidine (±)-SddC was found to have potent activity against human hepatitis B virus as well as human immunodeficiency viruses in culture. The (+)-form (±)-SddC which is resistant to deoxycytidine deaminase was found to be the more active antiviral stereoisomer than the (-)-form (±)-SddC. The (+)-SddC is susceptible to deamination by deoxycytidine deaminase and is 25- and 12-fold more toxic than (±)-SddC in CEM cells in terms of anti-cell growth and anti-mitochondrial DNA synthesis, respectively. Similar results were obtained using a mixture of their 5-fluoro analogs (±)-FSddC. Unlike 2’,3’-dideoxycytidine, which is a potent inhibitor of mitochondrial DNA synthesis and results in such delayed toxicity as peripheral neuropathy with long term usage, (±)-SddC does not affect mitochondrial DNA synthesis. The (−)-form is phosphorylated to (−)-SddCMP and is subsequently converted to (−)-SddCDP and (−)-SddCTP. One additional major metabolite which has been tentatively assigned the name “(−)-SddCMP sialate” was also identified. No significant difference in terms of the profiles of the metabolites was found between 4 and 24 h. There is an appreciable amount of (−)-SddCTP detectable 24 h after removal of the drug. (−)-SddCTP was also found to be approximately 3-fold more potent than (+)-SddCTP in inhibiting human hepatitis B virus DNA polymerase. This is the first nucleoside analog with the unnatural sugar configuration demonstrated to have antiviral activity.

Hepatitis B virus (HBV) infection is a major health problem. It causes infectious disease and is closely associated with primary hepatocellular carcinoma (1–3). Currently, there are no effective drugs for the treatment of HBV infections without substantial toxicity. Several nucleoside analogs have been found to have activity against HBV (4–10). Among them, 2’,3’-dideoxy-3’-thiacytidine, which was discovered to have potent anti-HIV activity (11–14), and its 5-fluoro analog were found to be two of the most potent and selective compounds against HBV replication in culture (14). The drugs examined, however, were the racemic mixture of the cis-isomers (Fig. 1). There are four possible stereoisomers each for SddC or 5-FSddC: a pair of cis-isomers and a pair of trans-isomers (Fig. 1). With the exception of cis-d-(+)-SddC, the isomers have unnatural sugar configurations. It was not clear whether one, or all of these stereoisomers were responsible for the inhibition of HBV replication.

In this report, a novel methodology to prepare (−)-SddC and (−)-FSddC from (±)-SddC and (±)-FSddC, respectively, is described. This allowed us to address the issue of whether one or both of the two forms of the stereoisomers were responsible for anti-HBV activity and cytotoxicity. This was investigated further using chemically synthesized stereoisomers. The metabolism of the active isomer as well as the inhibitory effect of its triphosphate metabolite against HBV-associated DNA polymerase is also reported.

MATERIALS AND METHODS

Compounds and Deaminase—The stereoisomers of SddC were synthesized by J. W. Beach, L. S. Jeong, and C. K. Chu, Department of Chemistry, University of Georgia. 5-FSddC was synthesized by D. Liotta and sent by R. F. Schinazi (Emory University, Atlanta, GA). 5′-SddC (BCH-189-5, 6′-H, mixture of two cis-isomers, (+)SddC and (−)-SddC, 20 Ci/mmol, was purchased from Moravek Biochemica, Inc., Brea CA. Human deoxycytidine deaminase was partially purified from human liver through a DEAE-cellulose column using a previously published procedure (15) with the specific activity of 2 units/mg. The unit is defined as the amount of enzyme which converts 1 μmol of dCyd in 1 min at 37°C.

Assay for Antiviral Activity—The procedure was essentially the same as that published previously (14). The 2215 (HBV-transfected cell line) cells were incubated with various concentrations of drug and grown for 12 days with medium changes every 3 days. At designated times, the cells were removed by low speed centrifugation, and polyethylene glycol (PEG) was added to the supernatant media to precipitate the virions. Nucleic acids were extracted from PEG precipitates and analyzed on Southern blots.

Endogenous HBV Polymerase Assay—For the assay of viral polymerase activity, the 2215 cells were incubated under conditions described previously (14). Cells were grown to confluence with changes of medium every 3 days. The medium was centrifuged (2,000 X g, 10 min), and an equal volume of 20% polyethylene glycol solution containing 1 M NaCl was added to the supernatant. After 1 h of incubation at 4°C, virions were pelleted by centrifugation (10,000 X g, 10 min). The pellet was resuspended in 50 mM Tris-Cl, pH 7.5. Endogenous DNA polymerase activity was measured as described (16, 17) with some modifications. The assay mixture contained, 42 mM Tris-Cl, pH 7.5, 34 mM MgCl2, 340 mM KCl, 22 mM mercaptoethanol, 0.4% Nonidet P-40, 70 μM each of dATP, dGTP, and dCTP.
acid-soluble material was prepared by centrifuging the extract at 4°C. The supernatant was neutralized with 100 μl of acetonitrile to stop the reaction, and the proteins were removed by centrifugation. The supernatant was lyophilized to dryness and reconstituted with HPLC mobile phase buffer (pH 6.5, with a flow rate of 1 ml/min). The HPLC was connected to a fraction collector. Fractions were collected at 1-min intervals, and used directly for scintillation counting.

Preparation of SddCMP and SddCTP—The procedure was a modification of the procedure described by Ruth and Cheng (18). Approximately 5 mg of SddC (22 μmol) was dissolved in 50 μl of triethyl phosphate (Aldrich, 13,219–S) 10 μg/ml, and stirred on methanol-ice for approximately 10 min. Then one equivalent of phosphorus oxychloride (Fisher P106) was added. The progress of the monophosphosphate formation was monitored using HPLC analysis with a Whatman SAX column using 0.03 M potassium phosphate buffer, pH 6.5, as solvent at a flow rate of 1 ml/min. When the reaction was maximized, three to four volumes of dimethyl formamide containing 5–8 equivalents of the triphosphoryl ammonium pyrophosphate was added. The formation of triphosphate nucleotide was monitored by HPLC with a Whatman SAX column and 0.16 M potassium phosphate buffer, pH 6.5, as the solvent with a flow rate of 2 ml/min. When the amount of triphosphate nucleotide appeared to be at a maximum the reaction was stopped by the addition of ice-cold water and neutralized to approximately pH 7.0 by the addition of triethylamine.

Incorporation into Nucleic Acid—DNA and RNA were purified from 2 x 10^7 cells treated for 24 h with 0.5 μM [3H]-SddC (1000 mCi/mmol). They were centrifuged in CsSO4 gradients as previously described (19), except that the rate of centrifugation was at 40,000 rpm for 44 h. Gradients were fractionated from top to bottom, and the positions of the DNA and RNA peaks were determined by ethidium bromide fluorescence as described (20).

RESULTS

Susceptibilities of cis- and trans-Dde Stereoisomers to Human Deoxycytidine Deaminase—Previous studies (14) using the mixture of cis-isomers of SddC and FSddC showed potent activity against HBV replication. By incubating the (+)-cis-isomers with human dCyd deaminase for 16 h, approximately 50% of the (+)-SddC can be deaminated based on HPLC analysis (Fig. 2A). Longer incubation does not increase the conversion. The deaminated product, SddU, eluted off the HPLC column at approximately 8.5 min while SddC eluted off the column at 7.0 min. The identity of SddU was ensured

Fig. 1. Structures of ddC analogs used in this study.

0.175 μM dCTP including 10 μCi of [α-32P]dCTP (3,000 Ci/mmol, Amersham Corp.), inhibitor and virus suspension in a final volume of 50 μl. After incubation at 37°C for 2 h, the reaction was stopped by the addition of sodium dodecyl sulfate to a final concentration of 1%, then together with 10 μg of yeast tRNA, and 20 μg of proteinase K with a final total volume of 100 μl. It was incubated at 50°C for 30 min. The 32P-labeled viral DNA was then isolated by phenol-chloroform extraction and ethanol precipitation. The reaction product was analyzed by electrophoresis on a 0.7% agarose gel dried onto 3MM paper, and subjected to autoradiography. The radioactive areas were then cut from the gel and quantitated in a liquid scintillation counter.

Cytotoxicity—The procedure was essentially the same as previously published (14). CEM or MT2 (T-lymphoblastoid cells) cells were grown in 5 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum at an initial cell number of 2 x 10^4 cells/ml. The doubling time was approximately 20 h. The cells were incubated with various concentrations of the compounds for 4 days. On day 4, the cell number was determined by using either a Coulter counter or a hemacytometer.

Susceptibility to Deoxycytidine Deaminase—The reaction mixture contained 25 mM Tris-HCl, pH 8.0, 0.2 mM of nucleoside, and approximately 0.04 units of human dCyd deaminase in a final volume of 50 μl and was incubated at 37°C for the period indicated. At the end of incubation, 100 μl of acetonitrile was added to stop the reaction, and the proteins were removed by centrifugation. The supernatant was lyophilized to dryness and reconstituted with HPLC mobile phase buffer. Nucleosides were separated by HPLC using an Alltech RP-18 column with the detector monitor at wave length 260 and 270 nm. The solvent at a flow rate of 1 ml/min. A step gradient system was employed using 0.03 M buffer from 0–12 min followed by 0.15 M buffer from 12–52 min. At 52 min, the buffer concentration and flow rate were increased to 0.3 M and 2 ml/min, respectively. The HPLC was connected to a fraction collector, fractions were collected at 1-min intervals, and used directly for scintillation counting.

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RESULTS

Susceptibilities of cis-(k)-SddC Stereoisomers to Human Deoxycytidine Deaminase—Previous studies (14) using the mixture of cis-isomers of SddC and FSddC showed potent activity against HBV replication. By incubating the (+)-cis-isomers with human dCyd deaminase for 16 h, approximately 50% of the (+)-SddC can be deaminated based on HPLC analysis (Fig. 2A). Longer incubation does not increase the conversion. The deaminated product, SddU, eluted off the HPLC column at approximately 8.5 min while SddC eluted off the column at 7.0 min. The identity of SddU was ensured

Fig. 2. HPLC profiles of the deamination of cis-SddC stereoisomers by human dCyd deaminase. The reaction mixture contained 25 mM Tris-HCl, pH 8.0, 0.2 mM of (±)-SddC, (+)-SddC and (−)-SddC and approximately 0.04 units of partially purified human CDd deaminase in a final volume of 50 μl. The reaction time was 16 h, and incubation temperature was 37°C. The reaction products were analyzed by HPLC as described under "Materials and Methods." Under these conditions cis(+)- and (−)-SddC eluted off the HPLC column at approximately 8.5 min with UV λmax = 270 nm; however, the deaminated product SddU has a retention time of 8.5 min with UV λmax of 260 nm.

Fig. 1. Structures of ddC analogs used in this study.
Table I

The rate of deamination for SddC stereo isomers relative to deoxycytidine using partially purified human deaminase

| Substrate | Rate of deamination | $K_m$ | Product |
|-----------|---------------------|-------|---------|
| dCyd      | 1.8 ± 0.3           | 38    | dUrd    |
| cis-(±)-SddC | 0.17 ± 0.02   | cis-(+)SddU |
| cis-(+)SddC | 0.27 ± 0.03   | cis-(+)SddU |
| cis-(−)-SddC | <0.001$^a$  |        | ddU     |
| ddC       | <0.001$^a$         |       | ddU     |
| trans-(+)SddC | <0.001$^a$ |        |         |
| trans-(−)-SddC | <0.001$^a$ |        |         |

$^a$ The rates of deamination were determined using HPLC analysis.

$^b$ Product of the reaction was not detected.

Table II

Comparative potencies of dC analogs as monitored by anti-HBV, cytotoxicity, and anti-mtDNA effects

The compounds tested here are all cis-isomers.

| Compound | Anti-HBV | Cytotoxicity | Anti-mtDNA |
|----------|----------|--------------|------------|
|          | $IC_{50}$ | $IC_{50}$ | $IC_{50}$ |
| (+)-SddC | 50       | >20          | >50        |
| (-)-SddC | 1         | 13           | 4          |
| (-)-SddC | >50      | >200         | >200       |
| (+)-SddC | 2.8      | 10           | 0.022      |

$^a$ The compound was purified from HPLC after deamination from their racemic mixtures.

Identification of the Active Stereoisomers of SddC and FSddC as Anti-HBV Compounds—The 2215 cell line was used to evaluate the antiviral activities of stereoisomers of SddC and FSddC. The antiviral effects were measured by analysis of extracellular HBV DNA (Fig. 3). The experiments revealed that the amount of extracellular HBV DNA decreased in a dose-dependent manner for each compound. Each dose was done in duplicate including the solvent control. The cis-(−)-SddC and cis-(−)-FSddC were prepared from cis-(-)-SddC and cis-(−)-FSddC, respectively, after dCyd deaminase treatment and purification by HPLC. At 0.1 μM cis-(−)-SddC almost completely inhibited the replication of HBV, however, less than 10% of the inhibition was observed by chemically synthesized cis-(−)-SddC under the same conditions. Although cis-(+)SddC was not identified, the identification of cis-(−)-FSddC as the active stereoisomer in the mixture of cis-(−)-FSddC can be deduced from the result shown in Fig. 3B. At 0.05 μM cis-(−)-FSddC was much more potent than cis-(−)-FSddC. If cis-(−)-FSddC was the same potency as cis-(−)-FSddC, one would expect to see a similar intensity for the two lanes treated with 0.1 μM of (±)-FSddC and 0.05 μM of (−)-FSddC. The concentrations of the stereoisomers of SddC that inhibit 50% ($IC_{50}$, μM) of the secreted HBV DNA, the growth of CEM cells or MT2 cells, and the content of mitochondrial DNA from CEM cells are presented in Table II. cis-(−)-SddC had an HBV $IC_{50}$ of 0.01 μM, 50-fold more potent than cis-(+)SddC (HBV $IC_{50}$ = 0.5 μM) (see Table II). Whereas, cis-(−)-SddC was approximately 25-fold less toxic than cis-(+)SddC with regard to the inhibition of CEM cell growth after 4 days in culture. cis-(+)SddC could also decrease mitochondrial DNA content in cells at a concentration lower than cis-(−)-SddC. The trans-isomers (see Fig. 1) are relatively inactive as anti-HBV or anti-cell growth compounds (data not shown).

Metabolism of cis-(−)-SddC in 2215 Cells—The HPLC profiles of the acid-soluble fraction of 2215 cells treated with 0.5 μM 3H-cis-(−)-SddC for 4 or 24 h are shown in Fig. 4, A and B, respectively. Peak A was confirmed to be Sdc. The identity of the other $^3$H-labeled peaks was assessed by subjecting them to alkaline phosphatase and snake venom phosphodiesterase digestion. Peaks E, D, and C were digested by alkaline phosphatase and were confirmed to be SddC, SddCDP, and SddCTP, respectively, after dCyd deaminase digestion. Peaks E, D, and C can all be digested to regenerate peaks A, C, and D. A similar experiment was done using peak D, and it was found to also regenerate peak A and C. The elution positions of these peaks were also compared with chemically synthesized SddCMP, SddCDP, and SddCTP nucleotides, and it was concluded that peak C was (−)-SddCMP, peak D was (−)-SddCDP, and peak E was (−)-SddCTP. Peak B was a poor substrate for alkaline phosphatase and based on the elution position, it was tentatively identified as (−)-FSddC.
assigned as SddCMP-sialate. This will require further confirmation. Under these conditions the cellular ATP eluted off the HPLC column at approximately 24 h (A). Medium was removed after 24 h, replaced with fresh medium without drug, and incubated for an additional 24 h (C). At the time and condition indicated, cells were harvested, extracted, and analyzed by HPLC according to the procedures described under "Materials and Methods."

fig. 4. The metabolism studies of (−)-SddC in 2215 cells. Cells were incubated in the presence of 0.5 μM [3H]−SddC (2.3 mCi/mmol) for 4 h (A) and 24 h (B). Medium was removed after 24 h, replaced with fresh medium without drug, and incubated for an additional 24 h (C). At the time and condition indicated, cells were harvested, extracted, and analyzed by HPLC according to the procedures described under "Materials and Methods."

Behavior of (−)-SddCTP and (+)-SddCTP Toward HBV-Associated DNA Polymerase—Chemically synthesized (−)-SddCTP and (+)-SddCTP were examined as inhibitors of the HBV endogenous DNA polymerase activity. At 0.175 μM dCTP (−)-SddCTP could inhibit HBV DNA synthesis in a dose-dependent manner (Fig. 5). Less inhibitory action of (+)-SddCTP was indicated. (−)-SddCTP exhibited approximately a 3-fold higher potency than (+)-SddCTP in inhibiting HBV DNA replication with 50% inhibitory dosage less than 0.05 μM.

**DISCUSSION**

Nucleoside analogs are a major chemical entity in the field of viral chemotherapy because they utilize the subtle differences between the viral DNA synthesis apparatus and the host cellular apparatus. It has always been assumed that the active stereoisomer of these analogs would be the one which most closely mimicked the natural nucleoside. Since most enzymes are stereospecific with respect to their substrates, many stereoisomers were found to have excellent selectivities against certain types of enzymes. In this report, it has been demonstrated that although cis-(−)-SddC, the natural form of the stereoisomer, could be deaminated by dCyd deaminase and was more cytotoxic, it was the cis-(−)-SddC which was the active stereoisomer against HRV. The metabolism studies of (−)-SddC indicate the formation of (−)-SddCMP, (−)-SddCDP, (−)-SddCTP, and an unidentified metabolite tentatively assigned as SddCMP-sialate in (−)-SddC-treated cells. The intracellular half-life of (−)-SddCTP is complicated due to the interconversion of SddC nucleotides and may require further studies. Basically, the kinetics of the drug metabolism seemed to be different between before and after removing the drug. The enzymes responsible for the formation of these metabolites have yet to be identified. Preliminary studies indicate the cytoplasmic dCyd kinase could phosphorylate (−)-SddC. Whether this is the only enzyme responsible for the phosphorylation of (−)-SddC to (−)-SddCMP is not clear. The active metabolite of (−)-SddC is likely to be (−)-SddCTP since this metabolite was shown to have potent inhibitory activity against HBV-associated DNA polymerase. The mode of the inhibition of (−)-SddCTP is competitive.
with dCTP. A detailed study will be published later. (+)-SddC is less active than (-)-SddCTP, but this may not be sufficient to account for the 50-fold difference of inhibition of HBV replication between (-) and (+)-SddC. Differences in the metabolism between these two stereoisomers could also account for their inhibition. It was noted that the difference in antiviral activity between (-) and (+)-SddC or (-) and (+)-FSddC is more than 5-fold. This suggests the possibility of an interaction of the (-) and (+)-forms of SddC or FSddC in terms of metabolism. This will be further investigated. Currently, one stereoisomer of SddC is under phase I clinical trial for HIV chemotherapy, however, it is not evident which isomer is being used. The unpublished results indicate the (-)-isomer is 5-fold more active than the (+)-isomer in terms of anti-HIV effect, therefore, the potential use of (-)-isomer for both anti-HIV and anti-HBV therapy should be entertained.

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