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Inhibition of the replication of hepatitis B virus *in vitro* by 2′,3′-dideoxy-3′-thiacytidine and related analogues

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ABSTRACT **Several 2′,3′-dideoxy-3′-thiapyrimidine nucleosides were studied for their ability to inhibit hepatitis B virus (HBV) DNA replication in a HBV-transfected cell line (2.2.15). 2′,3′-Dideoxy-3′-thiacytidine (SddC) and 5-fluoro-2′,3′-dideoxy-3′-thiacytidine(5-FSddC) were found to be the most potent anti-HBV compounds of those examined. Both compounds resulted in nearly complete cessation of viral DNA replication at 0.5 μM, as monitored by the absence of both intracellular episomal and secreted viral DNAs. The HBV-specific RNAs were not reduced at concentrations that completely blocked HBV DNA replication, suggesting that the inhibitory target is HBV DNA synthesis. The antiviral action of SddC and 5-FSddC was reversible. The concentration of SddC and 5-FSddC required to inhibit 50% of 4-day cell growth in culture was 37 μM and more than 200 μM, respectively. Unlike 2′,3′-dideoxycytidine, these two compounds do not affect mitochondrial DNA synthesis in cells at concentrations lower than that required to inhibit cell growth. In view of the potent and selective antiviral activity, both SddC and 5-FSddC should be further evaluated for the treatment of human HBV infection.**

Hepatitis B virus (HBV) causes acute and chronic hepatitis, which affects nearly 300 million people worldwide (1). Chronic infection with HBV has been associated with a high risk for the development of primary hepatocellular carcinoma (2, 3). Effective antiviral therapy against HBV infection has not been fully developed. Studies have been hampered by the extremely narrow host range and limited access to experimental culture systems. Recently, techniques have been developed to propagate hepadnaviruses in tissue culture (4–8), making it possible to study the various aspects of the viral life cycle and to examine the effectiveness of potential antiviral drugs.

Hepadnaviruses replicate through an RNA template that requires reverse transcriptase activity (9). By using computer-assisted DNA and protein sequence analyses, HBV DNA polymerase was shown to share homologies with the reverse transcriptase from retroviruses (10). Though HBV DNA polymerase has not yet been purified, a number of studies (11, 12) have indicated that inhibitors for reverse transcriptase of oncogenic RNA viruses may suppress HBV DNA replication. 2′,3′-Dideoxycytidine (ddC) has been shown to be a potent inhibitor of human immunodeficiency virus replication in cell culture (13). ddC was also shown to have potent antiviral activity against human HBV *in vitro* (14, 15) and duck HBV *in vitro* (12, 16) and *in vivo* (17). Unfortunately, long-term ddC usage causes delayed toxicity such as peripheral neuropathy in patients. Studies in this laboratory suggested that depletion of mitochondrial DNA (mtDNA) in cells treated with ddC could eventually result in cell toxicity; this may be responsible for the peripheral neuropathy associated with ddC observed in clinics (18). Thus it is important to search for effective agents against retroviruses and/or hepadnaviruses with an improved therapeutic index. In this report, 2′,3′-dideoxy-3′-thiacytidine (SddC), known as a potent inhibitor of human immunodeficiency virus replication (¶, 19), is demonstrated to effectively block the production of HBV in 2.2.15 cells *in vitro*. Mitochondrial effects and cytotoxicity were also investigated to evaluate the potential use of those compounds in treatment of HBV infection.

MATERIALS AND METHODS

Compounds. ddC was purchased from Pharmacia. 2′,3′-Didehydro-2′,3′-dideoxycytidine (D4C) was provided by T.-S. Lin (Yale University, New Haven, CT). 3′-Fluoro-2′,3′-dideoxycytidine (3′-FddC) was obtained from T. Kalman (State University of New York, Buffalo). Other nucleosides were synthesized by D. Liotta (Emory University, Atlanta, GA) and were determined to be greater than 99% pure by reversed-phase high-pressure chromatography. All compounds were dissolved in phosphate-buffered saline (0.8% NaCl/0.02% KCl/10 mM Na_2HPO_4/1.76 mM KH_2PO_4, pH 7.4).

***In Vitro* Assay for Antiviral Activity.** The 2.2.15 cells (clonal cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) that secrete hepatitis B virions (7, 20) were kindly provided by G. Acs (Mount Sinai Medical Center, New York, NY). The 2.2.15 cells were maintained in minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum. Cells were incubated at 37°C in a moist atmosphere containing 5% CO_2/95% air. The 2.2.15 cells were inoculated at a density of 3×10^5 cells per 5 ml in 25-cm^2 flask. The compounds studied were added to the medium 3 days after the inoculation. Cells were grown in the presence of drugs for 12 days with changes of medium every 3 days. After incubation, the medium was centrifuged (10 min, 2000 × *g*) and polyethylene glycol (M_r, 8000) was added to the supernatant to a final concentration of 10% (wt/vol). The virus was pelleted (10 min, 10,000 × *g*). The pellet was resuspended at 1% the original volume in TNE (10 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA). The suspension was adjusted to 1% SDS and proteinase K at 0.5 mg/ml and incubated for 2 hr at 55°C. The digest was

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Abbreviations: HBV, hepatitis B virus; SddC, 2′,3′-dideoxy-3′-thiacytidine; 5-FSddC, 5-fluoro-2′,3′-dideoxy-3′-thiacytidine; ddC, dideoxycytidine; D4C, 2′,3′-didehydro-2′,3′-dideoxycytidine; 3′-FddC, 3′-fluoro-2′,3′-dideoxycytidine; $HBID_{50}$, drug concentration inhibiting HBV viral DNA yield in medium by 50%; mtDNA, mitochondrial DNA; $MtID_{50}$, compound concentration causing a 50% reduction in mtDNA content.

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extracted with phenol/chloroform, 1:1 (vol/vol), and the DNA was precipitated with ethanol. The DNA pellet was dissolved in TE_{80} (10 mM Tris·HCl, pH 8.0/1 mM EDTA) and then electrophoresed in a 0.8% agarose gel followed by blotting onto Hybond-N membrane (Amersham). The blot was hybridized with a ^{32}P-labeled HBV DNA [*Bam*HI insert from plasmid Pam6 (American Type Culture Collection)] probe, washed with 2× standard saline citrate (SSC)/0.2% SDS at room temperature for 1 hr and 0.1× SSC/0.2% SDS at 55°C for 30 min, and then autoradiographed. The intensity of the autoradiographic bands was quantitated by a scanning densitometer. The amount of HBV-specific DNAs was similar in separate experiments performed in duplicate. $HBID_{50}$ was defined as the drug concentration that inhibited HBV viral DNA yield in the medium by 50%. The values were obtained by plotting percentage inhibition compared with control versus the drug concentration.

Isolation and Characterization of DNA. Drug-treated cells were lysed in 10 mM Tris·HCL, pH 7.5/5 mM EDTA/150 mM NaCl/1% SDS. The cell lysate was digested with proteinase K (100 μg/ml) at 55°C for 2 hr and deproteinized by phenol extraction. Nucleic acids were precipitated with 2 vol of ethanol. The pellet of nucleic acid was redissolved in TE_{80} followed by RNase A treatment (100 μg/ml) at 37°C for 1 hr. Concentrated ammonium acetate was added to the aqueous phase to yield a final 0.4 M ammonium acetate solution. The nucleic acids were precipitated with ethanol.

Isolation of RNA. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (21). The RNA (20 μg per lane) was electrophoresed through a 1% agarose gel containing 1.1 M formaldehyde and then transferred to a Hybond-N membrane. The immobilized RNA was hybridized with a ^{32}P-labeled HBV DNA probe and the membrane was autoradiographed as described above.

Cytotoxicity. CEM (T-lymphoblastoid cells) cells were seeded in 5 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum at a concentration of 2×10^4 cells per ml. The generation time was approximately 20 hr. The cells were incubated with various concentrations of compounds for 4 days. On day 4, the cell number was determined by using either a Coulter counter or a hemacytometer. It should be noted that the inhibition of mtDNA synthesis does not affect CEM cell growth in 4 days. ED_{50} was defined as the drug concentration that caused a 50% reduction in cell number. The values were determined by plotting the cell number versus the drug concentration.

Determination of mtDNA Content by a Quick-Blot Procedure. The CEM cells (5×10^4 cells) were collected by centrifugation and washed twice with phosphate-buffered saline. The cell pellets were resuspended in 100 μl of 10 mM Tris·HCl (pH 7.5) and subjected to three freeze–thaw cycles. The cell lysate was incubated with RNase A (10 μg/ml) at 37°C for 30 min. The sample was treated with proteinase K (100 μg/ml) at 55°C for 1 hr. A 0.8 vol of saturated NaI (2.5 g of NaI in 1 ml of water at 100°C) was added to the sample and heated at 90°C for 10 min. The DNA was immobilized on nitrocellulose by using a slot-blot apparatus (Schleicher & Schuell). The mtDNA on the blot was detected with a mtDNA-specific probe as described (18). The intensity of the autoradiographic bands was quantitated by a scanning densitometer.

RESULTS

Comparative Potency of Deoxycytidine Analogues as Inhibitors of HBV *in Vitro*. The 2.2.15 cell line was used to evaluate the antiviral activities of ddC analogues—D4C, 3'-FddC, SddC (R = H), and 5-fluoro-2',3'-dideoxy-3'-thiacytidine (5-FSddC; R = F) (Fig. 1). The antiviral effects were measured by analysis of extracellular HBV DNA (Fig. 2). The

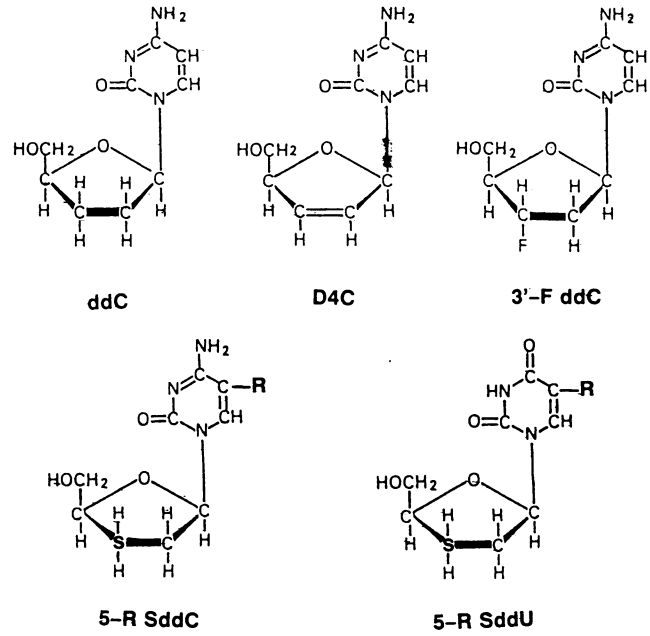

FIG. 1. Structures of ddC and analogues used in this study. ddC, 2',3'-dideoxycytidine; D4C, 2',3'-didehydro-2',3'-dideoxycytidine; 3'-FddC: 3'-α-fluoro-2',3'-dideoxycytidine; 5-RSddC, 5-R-2',3'-dideoxy-3'-thiacytidine; 5-RSddU, 5-R-2',3'-dideoxy-3'-thiauridine, where R = H, F, Cl, Br, I, or CH_3.

experiment revealed that the amount of extracellular HBV DNA decreased in a dose-dependent manner for each drug. The $HBID_{50}$ values of these compounds are presented in Table 1. At 2 μM, both SddC and 5-FSddC completely inhibited the replication of HBV, and approximately 70% inhibition by 3'-FddC and ddC and 40% inhibition by D4C were observed. Episomal HBV DNAs in 2.2.15 cells treated with various concentrations of SddC and 5-FSddC were also examined. Cellular DNAs were digested with *Hin*dIII, which does not cleave the HBV genome, and subjected to Southern blot analysis using a ^{32}P-labeled HBV DNA as probe. The chromosomally integrated HBV DNA genome and the episomal DNA can be separated in the gel and can be differentially quantified. Whereas episomal DNA decreased in a dose-dependent manner as did extracellular HBV DNAs, both the amount and the restriction enzyme pattern of the chromosomally integrated HBV genome were unaltered (Fig. 3).

Comparative Cytotoxicity and Effect on mtDNA Content of 2'-Deoxycytidine Analogues. The ED_{50} of the compounds are shown in Table 1. mtDNA content was measured by slot-blot hybridization analysis. The concentration of compounds which caused a 50% reduction in mtDNA content ($MtID_{50}$) is also shown in Table 1. Both SddC and 5-FSddC inhibited HBV replication at concentrations several orders of magnitude lower than concentrations that inhibit CEM cell growth after 4 days in culture. Moreover, at concentrations that caused cessation of HBV replication, no effect on mtDNA synthesis was observed. In contrast, ddC, a potent DNA polymerase γ inhibitor, had an $HBID_{50}$ of 2.8 μM but a low $MtID_{50}$ at 0.022 μM. D4C and 3'-FddC also had lower values of $MtID_{50}$ than of $HBID_{50}$. Thus, D4C, 3'-FddC, and ddC would be expected to affect mtDNA synthesis before an anti-HBV effect was achieved.

Reversibility of the SddC and 5-FSddC Effects. To determine whether the antiviral effect was reversible, 2.2.15 cells that were treated with 2.0 μM SddC or 2 μM 5-FSddC for 12 days were incubated for an additional 6 or 12 days in the absence of the compound. After 6 days of drug-free incubation, HBV DNA could again be identified extracellularly,

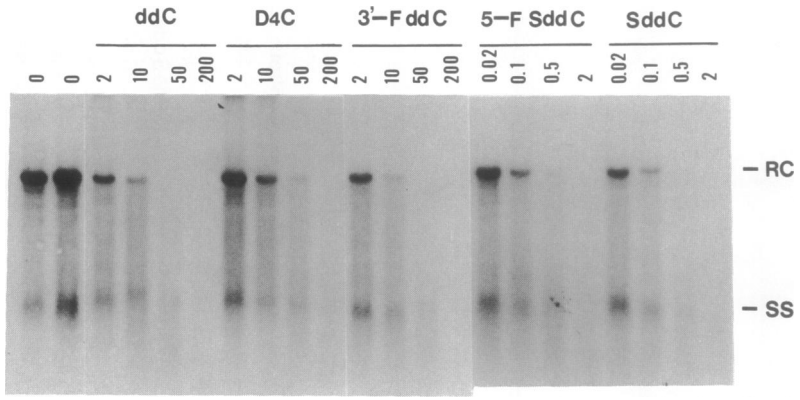

FIG. 2. Comparative potency of 2'-deoxycytidine analogues as inhibitors of HBV. The 2.2.15 cells were incubated with the various concentrations of drugs for 12 days. Medium was harvested. Virions were precipitated with PEG. Nucleic acids were extracted from PEG precipitates and analyzed on Southern blots. RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA.

though not to the same extent as those in control untreated cells (Fig. 4). After 12 days of drug-free incubation, both extracellular (Fig. 4) and intracellular episomal HBV DNAs (Fig. 3) were at a level equal to untreated control.

Effect of Drug on HBV-Specific RNAs in Cells. Northern blot analyses were performed on HBV RNA transcripts. Three major transcripts of approximately 3.5, 2.5, and 2.1 kilobases were detected in the total cellular RNA extract. The HBV-specific transcripts were not affected by 2.0 μM SddC or 2.0 μM 5-FSddC treatment (Fig. 5).

Comparative Potency of 2',3'-Dideoxy-3'-thiapyrimidine Analogues as Inhibitors of HBV *in Vitro*. Various analogues (Fig. 1) of 2',3'-dideoxy-3'-thiapyrimidine nucleosides were tested in 2.2.15 cells for anti-HBV activity. At 1.0 μM, none of the 2',3'-dideoxy-3'-thiauridine analogues was active against HBV replication (Fig. 6). Among the SddC analogues tested, SddC (R = H) and 5-FSddC (R = F) were found to be the most potent inhibitors of HBV replication. At 1.0 μM, the analogues with Br, Cl, or methyl groups at position R were not active, whereas the analogue with I at position R reduced the HBV production by approximately 50% (see Fig. 6).

DISCUSSION

One rational approach to the development of drugs for the treatment of HBV infection in patients is to identify those compounds that specifically inhibit HBV DNA replication. The HBV DNA polymerase has not been well-characterized due to difficulties in purification. Since deoxynucleoside analogues were found to be effective against a variety of viruses, several were tested in culture and *in vivo* against hepadnaviruses (11, 12, 14–17, 22, 23). The selectivity of those compounds against HBV in general is assessed by their relative potency against HBV versus cellular toxicity. The cytotoxicity studies are usually conducted by growth retardation assays that measure cell growth in the presence of compounds tested for three or four generations. This growth retardation assay could not, however, detect delayed cytotoxicity of deoxynucleoside analogues such as ddC that have an effect on cellular mtDNA synthesis (18). Since mitochondria play an important role in organ function, it was hypothesized that the delayed toxicity as shown by peripheral neuropathy (e.g., observed in patients treated with ddC analogs) could be due to decreases in mtDNA. In this study, in addition to assessing their anti-HBV activities, compounds were examined for their effects on 4-day cell growth and on mtDNA.

Among all the ddC analogues analyzed, SddC and 5-FSddC were the most potent inhibitors of HBV replication in 2.2.15 cells. No cell growth retardation or effects on mtDNA were observed after the administration of either of these com-

Table 1. Comparative potencies of ddC analogs as monitored by anti-HBV, cytotoxicity, and mtDNA effects

Compound	$HBID_{50}$, μM	$MtID_{50}$, μM	ED_{50}, μM	SI	$ED_{50}/MtID_{50}$
ddC	2.8	0.022	10	3.57	454
D4C	3.0	2	22	7.3	11
3'-FddC	2.75	0.11	45	16.36	409
SddC	0.05	47	37	740	0.79
5-FSddC	0.1	>200	>200	>2000	NA

SI, selective index ($ED_{50}/HBID_{50}$); NA, nonapplicable.

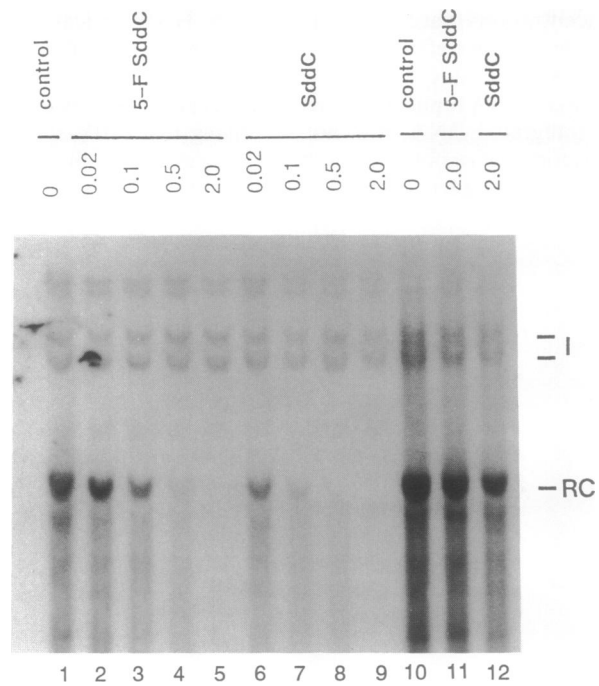

FIG. 3. Action of 5-FSddC and SddC on intracellular HBV DNAs. The 2.2.15 cells were untreated (lanes 1 and 10) or treated with 5-FSddC (lanes 2, 3, 4, 5, and 11) or SddC (lanes 6, 7, 8, 9, and 12) for 12 days. Total cellular DNAs were extracted. DNAs were digested with *Hin*dIII, electrophoresed in 0.8% agarose gel, transferred to Hybond-N membrane, and hybridized with a ^{32}P-labeled HBV probe. Each lane represents 20 μg of total cellular DNA. DNAs from cells untreated (lane 10) or treated with 2 μM 5-FSddC (lane 11) or 2 μM SddC (lane 12) for 12 days and further incubation in the absence of drugs for 12 more days are included. RC, relaxed circular episomal HBV DNA; I, integrated HBV DNAs.

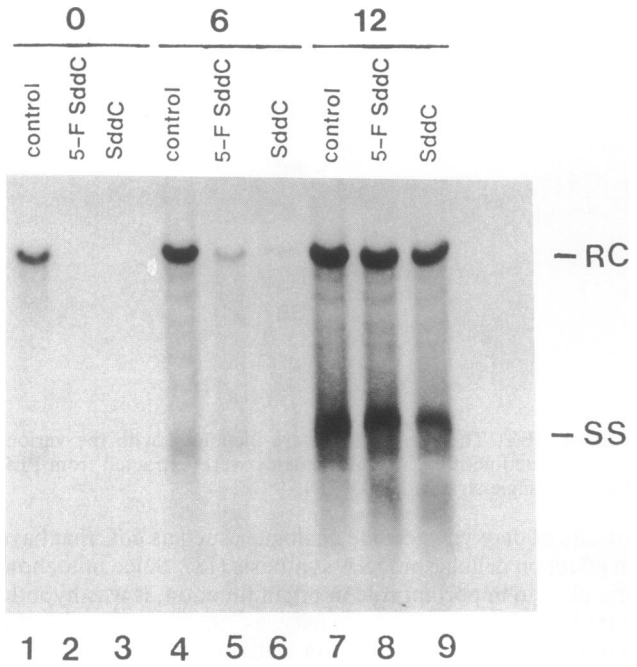

FIG. 4. Reversibility of SddC and 5-FSddC on HBV replication. The 2.2.15 cells untreated (lanes 1, 4, and 7) or treated with 2 μM SddC (lanes 3, 6, and 9) or 2 μM 5-FSddC (lanes 2, 5, and 8) for 12 days were incubated with drug-free medium for 0 (lanes 1, 2, and 3), 6 (lanes 4, 5, and 6), or 12 (lanes 7, 8, and 9) more days. HBV-specific DNAs in the medium were analyzed as described in Fig. 2. RC, relaxed circular HBV DNAs; SS, single-stranded HBV DNAs.

pounds at concentrations at least 100 times higher than concentrations that completely block the HBV replication. In contrast to their effectiveness in inhibiting HBV replication, SddC and 5-FSddC did not affect the integrated HBV DNAs. Since the RNA replicative intermediates are transcribed from the integrated DNA, it is not surprising that HBV-specific transcripts were not affected by drug treatment. Thus, interruption of drug treatment resulted in a return of HBV virus

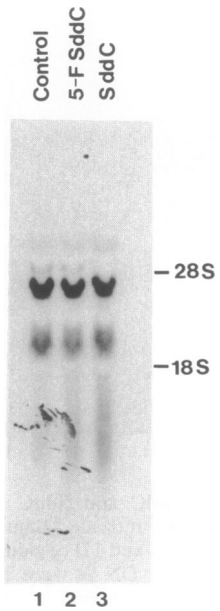

FIG. 5. Effect of 5-FSddC and SddC on HBV RNAs. Total RNAs were extracted from 2.2.15 cells untreated (lane 1) or treated with 2.0 μM 5-FSddC (lane 2) or SddC (lane 3) for 12 days. Each lane represents 20 μg of total RNAs. Positions of 18S and 28S rRNAs are shown.

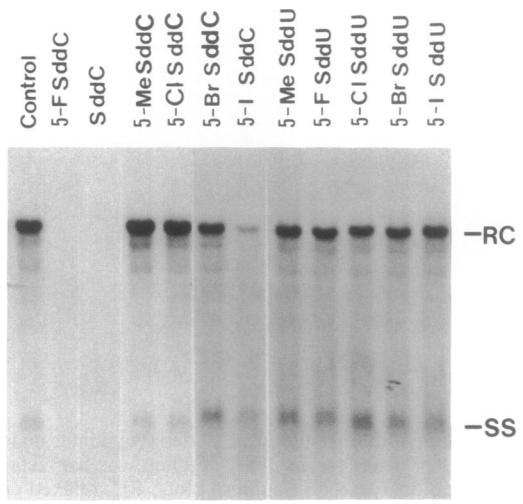

FIG. 6. Comparative potency of various analogues of 2',3'-dideoxy-3'-thiapyrimidine as inhibitors HBV replication. The 2.2.15 cells were treated with various analogues at 1.0 μM for 12 days. Medium was analyzed for the presence of HBV DNAs as described in Fig. 2.

to intra- and extracellular populations, as observed in Figs. 3 and 4. It will be of great interest to know whether the inhibition of HBV replication by those compounds could become irreversible in cells that have only episomal HBV DNA. Nevertheless, long-term usage of these drugs for the treatment of HBV infection is indicated particularly in patients with integrated HBV in their hepatocytes. Then, the delayed toxicity associated with the continuous usage of the drug could become an issue in view of the fact that the impact of drug on cellular mtDNA content is cumulative with increases in drug exposure time. Since SddC and 5-FSddC do not inhibit mtDNA synthesis at concentrations that inhibit virus production, the delayed toxicity, such as peripheral neuropathy, associated with the treatment of ddC may not occur. In addition, both SddC and 5-FSddC are not toxic to proliferating cells at concentrations that completely blocked the synthesis of HBV virion, suggesting that acute bone marrow toxicity may not be a concern either.

Since the HBV-specific transcripts were not affected by SddC and 5-FSddC at concentrations that completely blocked HBV DNA replication, the mechanism of action of SddC and 5-FSddC is likely inhibition of viral DNA polymerase, chain-termination resulting from incorporation into elongated DNA strand, or both. The mechanism has yet to be explored.

2',3'-Dideoxy-3'-thiauridine derivatives were inactive against HBV replication when tested up to 10 μM. This raised the question of whether those cytidine analogues could be deaminated intracellularly to the inactive uracil analogues. However, the addition of tetrahydrouridine or deoxytetrahydrouridine (inhibitors of cytidine/deoxycytidine deaminase) did not increase anti-HBV potency of SddC and 5-FSddC (data not shown), suggesting that catabolic inactivation of these two compounds by deaminase is not important.

Deoxynucleosides such as SddC and 5-FSddC can exist as (+)- or (−)-enatiomers. The compounds evaluated are racemic mixtures and, therefore, it is not presently known if one of these isomers will be superior to another in its activity. This should be further investigated. In summary, SddC and 5-FSddC have potent and selective anti-HBV activity in addition to their anti-human immunodeficiency virus activity, making this class of compounds particularly important in view of the fact that HBV infection is common in patients who develop acquired immunodeficiency syndrome (AIDS) (24). The usage of SddC or 5-FSddC for the treatment of both

groups of viruses in AIDS patients is possible. Clinical studies should be initiated to address the potential utility of these antiviral agents for the treatment of these severe and life-threatening viral infections in humans.

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