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Potent Inhibition of Hepatitis B Virus Production In Vitro by Modified Pyrimidine Nucleosides

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2',3'-Dideoxy-3'-fluorothymidine (FddThd), 2',3'-didehydro-2',3'-dideoxythymidine (ddeThd), and 3'-fluoro-5-methyl-deoxycytidine (FddMeCyt) are, in their triphosphate forms, selective inhibitors of human immunodeficiency virus type 1 reverse transcriptase. We report that 0.3 μM FddThd, FddMeCyt, or ddeThd as well as 3'-chloro-5-methyl-deoxycytidine (CldMeCyt) or 3'-amino-5-methyl-deoxycytidine (AddMeCyt) almost completely blocked production of hepatitis B virus (HBV) particles by HBV DNA-transfected cell line 2.2.15 in vitro. Only at an at least 10-fold-higher concentration was a cytotoxic effect observed. These results indicate that FddThd, FddMeCyt, CldMeCyt, AddMeCyt, and ddeThd are potent anti-HBV agents in vitro.

Hepatitis B is a disease with continuously increasing occurrence and is caused by the hepatitis B virus (HBV) (11). Vaccination against hepatitis B is one way of effectively preventing HBV infection (2). Two lines of treatment of hepatitis B infection have been followed: (i) treatment with cytokines (8) to substitute the impaired production of peripheral blood mononuclear cells (7, 32) and (ii) treatment with antiviral agents, e.g., adenine arabinoside (18, 21, 29). The rationale for a chemotherapeutic treatment for hepatitis B is the inhibition of the viral DNA polymerase (4).

Recently, we found that the triphosphate of 2',3'-dideoxy-3'-fluorothymidine (FddThd) is a potent inhibitor of reverse transcriptase of human immunodeficiency virus, a weak inhibitor of cellular DNA polymerase β, and not effective at all against DNA polymerase α (12). In addition, we established that FddThd is well phosphorylated to the 5'-triphosphate in relevant cell lines (13). Moreover, it was found that the triphosphate of FddThd and the triphosphates of 2',3'-didehydro-2',3'-dideoxythymidine (ddeThd) and 3'-fluoro-5-methyl-deoxycytidine (FddMeCyt) are potent inhibitors of endogenous HBV DNA polymerase (14; E. Matthes, M. von Janta-Lipinski, K. Reimer, H. Meisel, J. Schildt, and C. Lehmann, World Patent Application 61K/3310512, 1989). Hence, it appeared to be promising to determine whether FddThd and related modified nucleosides are also effective as agents against HBV infection in an intact cell system. Results of the experiments reported here indicate that the modified pyrimidine nucleosides FddThd, FddMeCyt, 3'-chloro-5-methyl-deoxycytidine (CldMeCyt), 3'-amino-5-methyl-deoxycytidine (AddMeCyt), and ddeThd effectively block production of HBV in HepG2 cells transfected with HBV DNA in vitro.

MATERIALS AND METHODS

Compounds. [^{3}H]deoxyadenosine ([^{3}H]dAdo; generally labeled with a specific radioactivity of 14 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, United Kingdom.

FddThd, FddMeCyt, CldMeCyt, AddMeCyt, and ddeThd were synthesized as described previously (6, 9, 30; E. Matthes, C. Lehmann, D. Scholz, M. von Janta-Lipinski, K. Gaertner, P. Langen, and H. A. Rosenthal, European Patent 0254268A2, 1987).

In vitro assay for antiviral activity. The human hepatoblastoma cell line HepG2 was transfected with pDolTHBV-1, a vector which contains HBV (27). The clonal line of cells was designated 2.2.15 and was found to secrete both hepatitis B surface antigen (HBsAg) and HBV DNA (27). The 2.2.15 cells were kept in RPMI 1640 medium supplemented with 2 mM glutamine and 10% (vol/vol) fetal bovine serum. The cultures were incubated at 37°C in 5% CO_2 in air.

Cells were planted at a density of 5×10^5/ml in plastic flasks. One hour later the compounds were added and incubation was continued for 4 days; the cultures reached confluence on day 5. On day 2 fresh serum was added to the culture medium and the respective compound concentration was renewed.

Cell growth was determined by two methods. First, after the 4-day incubation period, the cultures were incubated for an additional 24 h with 3 μCi of [^{3}H]dAdo per ml. In order to avoid direct interference of the precursor with the intracellular pool sizes of the pyrimidine nucleosides, [^{3}H]dAdo instead of, for example, [^{3}H]thymidine was used as a radioactive precursor. The cells were separated from the culture medium by centrifugation (2,000 × *g*, 10 min) and analyzed for acid-insoluble radioactivity (19). In a second approach the growth of the cells was determined by the direct cell count method by using the Cytocomp Electronical Counter (model Michaelis), and the 50% effective dose (ED_{50}; concentration of compounds which caused a 50% reduction of cell density) was determined (16). On day 4 the cell concentration in the control cultures (those not treated with a compound) was $(19.4 \pm 2.1) \times 10^5$/ml.

The medium gathered after day 4 was used to determine the concentration of HBsAg by application of a solid-phase radioimmunoassay (Travenol-Genentech Diagnostics, Cambridge, Mass.); the measurements were performed according to the instructions of the manufacturer. In control studies it was found that the addition of 5 μM nucleosides to the

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immunoassay did not change the sensitivity of the test system.

Recombinant DNA. HBV DNA (3.2-kilobase [kb] full-length clone) (22) was inserted into pAM12. The probe was labeled with ^{32}P to a specific radioactivity of 8×10^7 cpm/μg of DNA (24).

Isolation of DNA. Extracellular DNA was prepared from the culture medium of 2.2.15 cells. The medium samples, which were taken on day 4, were centrifuged (4,000 × *g*, 10 min), and the supernatant was incubated for 1 h at 4°C in the presence of 10% (wt/wt) polyethylene glycol (M_r, 8,000) and centrifuged again (10,000 × *g*, 10 min). The pellet was suspended in 10 mM Tris hydrochloride (pH 7.5; 10 mM EDTA). The DNA was purified by treatment with 400 μg of proteinase K per ml (2 h at 37°C) and deproteinized by two extractions with equal volumes of phenol-chloroform (10).

Episomal DNA was isolated by lysing the 2.2.15 cells in a 20 mM Tris hydrochloride buffer [pH 7.5; 10 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid, 1% sodium dodecyl sulfate] as described previously (5). The samples were suspended in 10 mM Tris hydrochloride (pH 7.5) and incubated with 30 μg of RNase A per ml for 2 h at 37°C. This material was deproteinized by one extraction with equal volumes of phenol-chloroform (10).

Analysis of DNA. Purified DNA samples (20 μg per lane) were electrophoresed through 1% agarose gels and transferred to Zeta Probe nylon (Bio-Rad Laboratories, Richmond, Calif.) and subsequently hybridized with ^{32}P-labeled HBV DNA as described previously (23).

The presence of HBV DNA in the culture medium of 2.2.15 cells was assessed by dot blot analysis as described previously (28). Briefly, the samples (5 μl corresponding to 20 ml of culture medium) were spotted onto nitrocellulose, denatured, neutralized, and baked (30 min, 80°C). Hybridization was performed with nick-translated HBV DNA. The intensities were quantitated by integration of the densitometry tracing obtained from the autoradiograms by using a Shimadzu (CS-910/C-R1A) integrating densitometer.

Statistical evaluation. Student's *t* test was used to determine significance (25).

RESULTS

Antiviral activities of FddThd, FddMeCyt, CldMeCyt, AddMeCyt, and ddeThd in vitro. The 2.2.15 cell line was used to evaluate the antiviral and cytostatic activities of the modified pyrimidine nucleosides FddThd, FddMeCyt, CldMeCyt, AddMeCyt, and ddeThd. Up to a concentration of 0.3 μM, no inhibition of [^{3}H]dAdo incorporation into cellular DNA was found (Table 1); only at concentrations of 1 to 3 μM did reduced incorporation occur. In addition, the cytotoxicity (or the cytostatic activity) of the compounds was determined on the basis of cell growth. The ED_{50}s of the compounds varied between 0.54 (FddMeCyt) and 1.49 (CldMeCyt) μM (Table 2). These data confirm the inhibitory concentrations as deduced from the incorporation studies (Table 1).

The antiviral effects of the compounds were measured by two methods: (i) determination of HBsAg production and (ii) analysis of extracellular HBV DNA. The experiments revealed that concentrations of all compounds tested to as low as 0.01 μM significantly ($P < 0.001$) reduced HBsAg production. The antiviral effects of FddThd and AddMeCyt were found to be more pronounced than those of the other compounds; significant ($P < 0.001$) reductions of HBsAg production were measured at a concentration of 0.003 μM (Table 1).

TABLE 1. Influence of FddThd, FddMeCyt, ddeThd, CldMeCyt, and AddMeCyt on 2.2.15 cell proliferation and HBsAg secretion[a]

Compound	Concn (μM)	Incorporation (% of control)	HBsAg concn (% of control)
None		100.0	100.0
FddThd	0.003	104.1	78.2[b]
	0.01	111.3	23.4[b]
	0.03	117.0	3.7[b]
	0.10	106.2	6.9[b]
	0.30	102.4	4.1[b]
	1.00	86.2[c]	2.0[b]
	3.00	24.2[b]	<1.0[b]
FddMeCyt	0.003	92.7	83.9[c]
	0.01	105.2	35.5[b]
	0.03	108.9	12.2[b]
	0.10	100.3	2.4[b]
	0.30	92.1	<1.0[b]
	1.00	58.0[b]	<1.0[b]
	3.00	17.3[b]	<1.0[b]
CldMeCyt	0.003	99.1	96.3
	0.01	116.2	49.7[b]
	0.03	92.1	37.3[b]
	0.10	113.9	18.0[b]
	0.30	101.2	17.2[b]
	1.00	89.1	1.8[b]
	3.00	56.5[b]	<1.0[b]
AddMeCyt	0.003	86.4	71.8[b]
	0.01	106.3	43.6[b]
	0.03	92.4	35.9[b]
	0.10	102.5	25.1[b]
	0.30	96.8	17.4[b]
	1.00	55.6[b]	2.2[b]
	3.00	17.6[b]	<1.0[b]
ddeThd	0.003	103.6	89.9
	0.01	91.8	67.3[b]
	0.03	106.8	18.2[b]
	0.10	108.2	2.8[b]
	0.30	95.4	<1.0[b]
	1.00	32.5[b]	<1.0[b]
	3.00	19.1[b]	<1.0[b]

[a] The incubation period was 4 days. The absolute incorporation of [^{3}H]dAdo into control cultures was 14,310 ± 1,785 dpm per culture. The absolute concentration of HBsAg produced by untreated cells was 19.3 ± 2.9 ng/ml. The means of five independent experiments each were determined; the standard deviations were less than 15%. The statistical significance was determined.

[b] $P < 0.001$ versus control.

[c] $P < 0.01$ versus control.

The antiviral effects of the compounds on extracellular virion production were also established by the identification and quantification of HBV DNA by using the slot blot hybridization procedure. The experiments revealed that the amount of extracellular HBV DNA decreased in a dose-dependent manner in the presence of FddThd (Fig. 1). At a concentration of 0.3 μM, no HBV DNA could be detected in the medium. To determine whether the antiviral effect was reversible, cells that were treated with 0.03 or 0.3 μM FddThd were incubated for an additional 4 days in the absence of the compound. After that period of incubation, HBV DNA could again be identified extracellularly, even though it was to a lesser extent compared with the controls

TABLE 2. Determination of ED_{50} during an incubation period of 4 days[a]

Compound	ED_{50} (μM)
FddThd	1.22 ± 0.19
FddMeCyt	0.54 ± 0.06
CldMeCyt	1.49 ± 0.22
AddMeCyt	1.07 ± 0.17
ddeThd	0.61 ± 0.08

[a] The means ± standard deviations from five independent experiments were calculated.

(Fig. 1). By setting the amount of extracellular HBV DNA released in the controls (0 μM compound) at 1.0, the quantity of HBV DNA of the cultures treated with 0.003 μM (or 0.03 μM) FddThd was determined to be 0.3 (or 0.1) (Fig. 1). The other modified nucleosides were also determined to inhibit the release of HBV DNA. As summarized in Table 3, 0.3 μM FddThd, FddMeCyt, CldMeCyt, or AddMeCyt and 0.1 μM ddeThd caused a reduction of HBV DNA release from the cells to the point that release was not detectable by this assay. At the low concentration (0.003 μM), a more than 50% reduction of the amount of viral DNA released from the cells was measured (Table 3).

In another set of experiments, episomal HBV DNA in 2.2.15 cells treated with 0, 0.03, or 0.1 μM FddThd was traced. When Southern blot analysis was performed in untreated cells, HBV DNA could be detected in the relaxed-circular, linear, and single-stranded forms (Fig. 2, lane a). In contrast, we failed to detect the relaxed-circular virion DNA if the cells were incubated with compound at 0.03 μM (Fig. 2, lane b). At the higher concentration of FddThd (0.1 μM), no episomal HBV DNA could be detected (Fig. 2, lane c).

DISCUSSION

The HBV RNA pregenome is assumed to function as a template for HBV DNA polymerase (31). It has been suggested that HBV DNA polymerase, which also serves as reverse transcriptase, has a common evolutionary origin with the reverse transcriptases from retroviruses (15). Therefore, it was not very surprising that both noncompetitive inhibitors, e.g., suramin (3, 20) or phosphonoformate (1, 26), and competitive inhibitors, e.g., arabinonucleoside analogs (17, 18, 21) and purine 2′,3′-dideoxynucleosides (29), inhibit reverse transcriptase from oncogenic RNA viruses and the polymerase from HBV with comparably high selectivities.

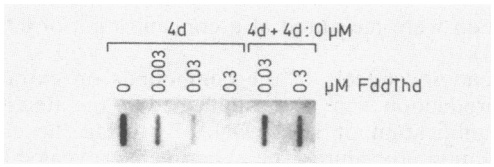

FIG. 1. Slot blot hybridization of DNA from FddThd-treated 2.2.15 cell cultures. The cells were treated with the compound at 0, 0.003, 0.03, or 0.3 μM for 4 days (4d). Then, the medium was collected and subjected to slot blot hybridization by using a ^{32}P-labeled HBV DNA probe (exposure time, 5 days). In an additional set of experiments, the medium from cultures incubated for 4 days with 0.03 or 0.3 μM FddThd was replaced with medium containing the compound at 0 μM, and incubation was continued for an additional 4 days. Then, extracellular DNA was analyzed for HBV DNA.

TABLE 3. Effects of modified nucleosides on HBV DNA release from 2.2.15 cells[a]

Compound	Concn (μM)	Relative amt of HBV DNA released
None		1.00
FddThd	0.003	0.30
	0.03	0.10
	0.10	0.10
	0.30	<0.05
	1.00	<0.05
FddMeCyt	0.003	0.35
	0.03	0.15
	0.10	0.07
	0.30	<0.05
	1.00	<0.05
CldMeCyt	0.003	0.45
	0.03	0.15
	0.10	0.10
	0.30	<0.05
	1.00	<0.05
AddMeCyt	0.003	0.50
	0.03	0.20
	0.10	0.15
	0.30	<0.05
	1.00	<0.05
ddeThd	0.003	0.25
	0.03	0.15
	0.10	<0.05
	0.30	<0.05
	1.00	<0.05

[a] The cells were incubated in the standard assay for 4 days in the absence (none) or the presence of the three different nucleosides. Then, the medium was analyzed for the presence of HBV DNA by using the ^{32}P-labeled HBV DNA probe and the slot blot hybridization procedure described in the text. The intensities of the hybridization signals of the autoradiograms were quantitated by integration of the densitometry tracings; the intensity of the untreated control was set equal to 1.0. In two additional parallel experiments, comparable changes in the intensities of the hybridization signals of HBV DNA were measured; rough estimates showed the standard deviation for each value to be less than 25%.

We have reported here that FddThd, which undergoes intracellular phosphorylation to the triphosphate form (13), and four additional nucleoside analogs (FddMeCyt, CldMeCyt, AddMeCyt, and ddeThd) are potent inhibitors of HBV production in the cellular system (2.2.15 cells) in vitro. We chose this HepG2/2.2.15 cell line as the in vitro system because these cells secrete spherical and filamentous forms of HBsAg, core particles, and virions into the culture medium (27); a reduction of the release of these viral components during treatment with a given compound is a measure of the antiviral activity displayed by the compound. We determined the antiviral effect by measuring both the inhibition of release of HBsAg and the inhibition of HBV DNA release. The first method was applied in a quantitative way, while the data obtained from the second technique can be considered only semiquantitative. The important point to be made is that the antiviral effect in vitro (significant inhibition of HBsAg production) was observed at a concentration of 0.003 μM (FddThd and AddMeCyt [inhibition by 20 to 30%]) or 0.01 μM (for the other three nucleoside analogs [inhibition by 30 to 60%]), which was more than 10-fold lower than the concentration at which the cytostatic effect was observed. In contrast to the inhibition observed in

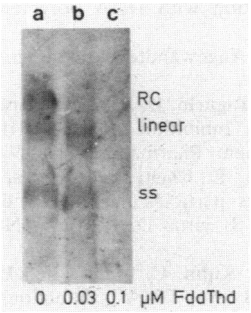

FIG. 2. Southern blot analysis of episomal DNA from cultures of 2.2.15 cells. Episomal DNA from cells treated for 4 days with 0 μM (lane a), 0.03 μM (lane b) or 0.1 μM (lane c) FddThd was prepared from 10^7 cells and subjected to agarose gel electrophoresis. Then, the DNA was transferred to nitrocellulose, hybridized to ^{32}P-labeled HBV DNA, and autoradiographed (exposure time, 2 days). RC, Relaxed-circular DNA; linear, linear DNA; ss, single-stranded DNA.

the assays with HBsAg, a 50% or higher inhibition of HBV DNA release for all compounds was found even at the lowest concentration tested (0.003 μM), indicating that the production of progeny particles was inhibited at the level of HBV DNA synthesis rather than at the level of translation of viral mRNA. More specifically, the finding that after incubation of the cells with FddThd the fraction of relaxed-circular HBV DNA decreased in the first place (a process which requires the HBV DNA-dependent DNA polymerase), while only later a reduction of the fraction of single-stranded HBV DNA was observed (the product of the HBV reverse transcriptase), was taken as a hint that the function of DNA-dependent DNA synthesis of HBV polymerase might be more sensitively affected by the triphosphate of FddThd than by its function as an RNA-dependent DNA polymerase. Because the cytostatic or cytotoxic effects of the compounds were measured by two independent methods, incorporation inhibition studies and direct cell counting, it appears to be unlikely that the antiviral effect that we observed was due to an inhibition of cellular protein synthesis. The high antiviral selectivity could be explained by the high affinity of the triphosphate of FddThd as a competitive inhibitor toward different DNA polymerases (12). While a 50% inhibition of DNA polymerase β was measured at a concentration of 2.2 μM triphosphate of FddThd, the inhibitory concentration of the triphosphate in the DNA polymerase α assay was >200 μM. In contrast, reverse transcriptase from human immunodeficiency virus is inhibited at a concentration of as low as 0.05 μM (12) and the polymerase from HBV is inhibited at 0.15 μM (14). This means that the cellular DNA polymerase β, which is primarily involved in DNA repair (1), is 45-fold less sensitive to inhibition by the compound than the human immunodeficiency virus reverse transcriptase is and 15-fold less sensitive than the HBV DNA polymerase is. The DNA polymerase α, the de novo DNA-synthesizing enzyme (1), is almost insensitive toward the triphosphate of FddThd. Recently, it was found that the triphosphates of the other nucleoside analogs studied here, FddMeCyt and ddeThd, were equally effective and selective inhibitors of HBV DNA polymerase (14).

In a previous study it was reported that pyrimidine 2′,3′-dideoxynucleosides are not inhibitors of hepadnavirus replication, while the purine dideoxynucleosides are very effective inhibitors of duck HBV replication in vitro as well as in vivo (29). A concentration of 4 μM was sufficient to suppress viral DNA synthesis in the in vitro duck hepatocyte culture system, but no cell toxicity data were given in that report (29). Results of the present study, in which we showed that FddThd almost totally suppresses virus production at the lower concentration of 0.1 μM, led us to initiate animal studies with FddThd in the duck HBV-infected duck system. The results of this study will help to determine whether a clinical trial of FddThd in HBV-infected patients is justified.

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