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INHIBITION OF HEPATITIS B VIRUS PRODUCTION BY MODIFIED 2′,3′-DIDEOXY-THYMIDINE AND 2′,3′-DIDEOXY-5-METHYLCYTIDINE DERIVATIVES

IN VITRO AND *IN VIVO* STUDIES

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Abstract—The effect of analogues of both 2′,3′-dideoxy-3′-fluorothymidine (FddThd) [2′,3′-dideoxy-3′-fluorouridine (FddUrd), 2′,3′-dideoxy-3′-fluoro-5-chlorouridine (FddClUrd), 2′,3′-dideoxy-3′-fluoro-5-bromouridine (FddBrUrd) and 2′,3′-dideoxy-3′-fluoro-5-bromovinyluridine (FddBVUrd)] and 2′,3′-dideoxy-3′-fluorocytidine (FddCyt) [2′,3′-dideoxy-3′-fluoro-5-fluorocytidine (FddFCyt), 2′,3′-dideoxy-3′-fluoro-5-chlorocytidine (FddClCyt), 2′,3′-dideoxy-3′-fluoro-5-methylcytidine (FddMeCyt), 2′,3′-dideoxy-3′-fluoro-5-ethylcytidine (FddEtCyt), 2′,3′-dideoxy-3′-chloro-5-methylcytidine (CldMeCyt), 2′,3′-dideoxy-3′-amino-5-methylcytidine (AmddMeCyt), 2′,3′-dideoxy-3′-azido-5-methylcytidine (AzddMeCyt) and arabinosyl-5-methylcytosine (AraMeCyt)] were tested for their potential antiviral activity *in vitro* using the human hepatoblastoma cell line, Hep G2 2.2.15, which was transfected with a vector containing hepatitis B virus (HBV). It was found that FddThd, FddMeCyt, FddEtCyt, CldMeCyt, AmddMeCyt and AraMeCyt display cytostatic activity at concentrations (CD_{50} values) between 0.54 (FddMeCyt) and 3.93 μM (FddEtCyt), while FddUrd, FddClUrd, FddBrUrd, FddBVUrd, FddCyt, FddFCyt, FddClCyt and AzddMeCyt do not affect cell growth at concentrations of up to 25 μM. Among the thymidine analogues tested, FddThd is the most effective antiviral agent: at a concentration of 0.03 μM a more than 90% reduction of HBV DNA synthesis was measured. On the other hand, the antiviral indexes displayed by FddClUrd, FddBrUrd and FddBVUrd are higher than that of FddThd; FddUrd was completely inactive. The most powerful antiviral agents in the group of cytidine analogues tested *in vitro* were FddMeCyt (more than 90% reduction of HBV DNA synthesis at 0.10 μM) and CldMeCyt (0.10 μM); FddClCyt, FddEtCyt, AmddMeCyt and AraMeCyt were of intermediate activity. None or negligible antiviral activity was determined for FddUrd, FddCyt, FddFCyt and AzddMeCyt. FddThd and FddMeCyt displayed *in vivo* an antiviral effect in the duck/duck HBV (DHBV) animal system. Administration of 10 or 20 mg/kg (total daily dose) of FddThd and 5 or 10 mg/kg of FddMeCyt (i.m. daily) to ducks infected with DHBV for 12 days blocked virus production. Termination of treatment with FddThd of infected animals led to reappearance of the virus in the serum though at lower levels. The *in vitro* and the *in vivo* data suggest that FddThd and FddMeCyt might be promising antiviral agents for the treatment of infection caused by HBV in humans.

Several strategies have been pursued in attempting to prevent and control disease caused by hepatitis B virus (HBV)§; (i) application of an HBV vaccine [1], (ii) immunological manipulation by immune adjuvants (such as levamisole) or by the antiviral immunostimulant interferon [2] and (iii) treatment with compounds that interfere with HBV at different stages in its life cycle, e.g. arabinofuranosyladenine, a nucleoside analogue, which inhibits as triphosphate viral DNA polymerase [3, 4]. This latter approach however turned out to be unspecific. Recently, purine 2′,3′-dideoxynucleosides have been developed which inhibit with comparably high selectivity the synthesis of DNA HBV and oncogenic RNA viruses [5].

We screened for triphosphates of pyrimidine analogues as potential inhibitors of HBV DNA polymerase [6]. Among those tested, 3′-fluoro-thymidine triphosphate was determined to be a strong competitive inhibitor of HBV DNA polymerase with an inhibitor constant (K_i) of 0.04 μM and a Michaelis constant (K_m) for dTTP of 0.18 μM. The compounds, FddThd and FddMeCyt turned out to inhibit HBV production in model cellular systems [7]. For the *in vitro* studies, we used Hep G2 cells

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§ Abbreviations: FddThd, 2′,3′-dideoxy-3′-fluoro-thymidine; FddUrd, 2′,3′-dideoxy-3′-fluorouridine; FddClUrd, 2′,3′-dideoxy-3′-fluoro-5-chlorouridine; FddBrUrd, 2′,3′-dideoxy-3′-fluoro-5-bromouridine; FddBVUrd, 2′,3′-dideoxy-3′-fluoro-5-bromovinyluridine; FddCyt, 2′,3′-dideoxy-3′-fluorocytidine; FddFCyt, 2′,3′-dideoxy-3′-fluoro-5-fluorocytidine; FddClCyt, 2′,3′-dideoxy-3′-fluoro-5-chlorocytidine; FddMeCyt, 2′,3′-dideoxy-3′-fluoro-5-methylcytidine; FddEtCyt, 2′,3′-dideoxy-3′-fluoro-5-ethylcytidine; CldMeCyt, 2′,3′-dideoxy-3′-chloro-5-methylcytidine; AmddMeCyt, 2′,3′-dideoxy-3′-amino-5-methylcytidine; AzddMeCyt, 2′,3′-dideoxy-3′-azido-5-methylcytidine; AraMeCyt, arabinosyl-5-methylcytosine; HBV, hepatitis B virus; DHBV, duck HBV.

[8] which had been stably transfected with the HBV genome. These cells produce hepatitis antigens and HBV DNA as well as virus-like particles. To verify that these two compounds are also effective under *in vivo* conditions, they were applied in the duck/DHBV animal model system [8, 9].

In addition to the *in vivo* data on the antiviral effect of FddThd and of FddMeCyt, we summarize the data gathered *in vitro* of the following FddThd analogues which had been derivatized; FddUrd, FddClUrd, FddBrUrd and FddBVUrd as well as the base and sugar-modified FddMeCyt analogues FddCyt, FddFCyt, FddClCyt, FddEtCyt, CldMeCyt, AmddMeCyt, AzddMeCyt and AraMeCyt. The *in vitro* results revealed that FddClUrd, FddBrUrd and FddBVUrd in particular show a high antiviral activity with a concurrent low cytotoxicity.

MATERIALS AND METHODS

Compounds. FddThd, FddUrd, FddClUrd, FddBrUrd, FddBVUrd, FddCyt, FddFCyt, FddClCyt, FddMeCyt, FddEtCyt, CldMeCyt, AmddMeCyt, AzddMeCyt and AraMeCyt were synthesized as described previously [10–14].

In vitro *assay for antiviral activity.* The human hepatoblastoma cell line, Hep G2, was transfected with pDolTHBV-1, a vector which contains HBV [8]. The clonal line of cells was designated 2.2.15 and found to secrete both HBsAg and HBV-DNA [8]. The 2.2.15 cells were kept in RPMI 1640 medium, supplemented with 2 mM glutamine and 10% (v/v) fetal calf serum. The cultures were incubated at 37° in 5% CO_2 in air as described previously [7].

Cells were seeded at a density of 2.5×10^5 cells/25-cm^2 Petri dish. One hour later the compounds were added at the following concentrations (final): 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 μM; incubation was continued for 4 days [7].

Cell growth was monitored by direct cell count and the 50% cytotoxic dose (CD_{50}) [concentration of compounds which causes a 50% reduction in cell density] was determined [15]. At day 4 the cell concentration in the controls (not treated with a compound) was 3×10^6 cells/dish.

The medium collected after day 4 was used to determine the amount of extracellular HBV DNA.

In vivo *inhibition studies.* One-day-old Peking ducklings, found to be DHBV DNA-negative in serum were injected i.v. with 50 μL of DHBV (type 26)-containing serum. At day 1 or 13 post-infection the animals were treated with FddThd (10 or 20 mg/kg; total daily dose) or FddMeCyt (5 or 10 mg/kg; total daily dose) by i.m. injection twice daily. At different time periods after DHBV infection serum was obtained by bleeding the foot vein and assayed for the presence of DHBV DNA by slot-blot hybridization.

Recombinant DNAs. DHBV-DNA [16] was cloned into bacteriophage M13. Probes were labelled with ^{32}P to a specific radioactivity of 7×10^8 cpm/μg DNA [17].

Table 1. Influence of FddThd, FddUrd, FddClUrd, FddBrUrd, FddBVUrd, FddCyt, FddFCyt, FddClCyt, FddMeCyt, FddEtCyt, CldMeCyt, AmddMeCyt, AzddMeCyt and AraMeCyt on proliferation and HBV DNA released in the medium of the human hepatoblastoma cell line, Hep G2 2.2.15, transfected with pDolTHBV-1

Compound	CD_{50} (μM)	Concn of compound required for the reduction of HBV DNA concn (μM) ≥90%	≥95%
FddThd	1.22 ± 0.19*	0.03*	0.30*
FddUrd	>25	>5.00	>5.00
FddClUrd	>25	0.10	3.00
FddBrUrd	>25	0.30	3.00
FddBVUrd	>25	0.30	3.00
FddCyt	>25	>5.00	>5.00
FddFCyt	>25	>5.00	>5.00
FddClCyt	>25	0.30	1.00
FddMeCyt	0.54 ± 0.06*	0.10*	0.30*
FddEtCyt	3.93 ± 0.10	0.30	1.00
CldMeCyt	1.49 ± 0.22*	0.10*	0.30*
AmddMeCyt	1.07 ± 0.17*	0.30*	0.30*
AzddMeCyt	>25	3.00	>3.00
AraMeCyt	2.94 ± 0.38	0.30	1.00

The respective reference compounds are FddThd and FddMeCyt. The drug concentration which causes a 50% reduction in cell proliferation (CD_{50} concentration) during an incubation period of 4 days is given. The mean values from five independent experiments (±SD) were calculated. In addition the reduction of HBV DNA synthesis, as measured by Southern slot-blot hybridization, by these cells (data in percent of control) is given. The means of five independent experiments each were determined; the standard deviations were less than 17%. Such concentrations are given in the table which caused a reduction of HBV DNA in the culture medium by more than 90 or 95%.

* Data from [7].

Isolation of DNA. Extracellular DNA was prepared from culture medium or from sera of ducks. The samples were centrifuged (4000 *g*; 10 min) and the supernatant was incubated for 1 hr at 4° in the presence of 10% (w/w) polyethylene glycol (M_r 8000), and centrifuged again (10,000 *g*; 10 min). The pellet was suspended in 10 mM Tris–HCl (pH 7.5; 10 mM EDTA). The DNA was purified by treatment with 400 μg/mL of proteinase K (2 hr at 37°) and deproteinized by two extractions with equal volumes of phenol/chloroform [18].

Analysis of DNA. The presence of DHBV DNA in serum of ducks or of HBV DNA in the culture medium of 2.2.15 cells was assessed by slot-blot analysis as described previously [19]. Briefly, the samples (5 μL corresponding to 0.2 mL of serum, pooled from two animals of each group) were spotted onto nitrocellulose, denatured, neutralized and baked (30 min; 80°). Hybridization was performed with nick-translated DHBV DNA or HBV DNA. The intensities were quantitated by integration of the densitometry tracing obtained from the autoradiograms using a Shimadzu (CS-910/C-R1A) integrating densitometer.

ELISA assays. For ELISA assays [20], 5 μL of serum from ducks or of DHBV positive serum [16] were coated onto microtiter wells which were then incubated for 1 hr at 20°. The plates were post-coated with 5% (w/v) of Tween 20 and incubated with the anti-pre S (pre S region of the surface antigen) antiserum from a rabbit for 1 hr at 20°. The bound antibodies were assayed with the aid of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Chemical Co., Deisenhofen, Germany). Alkaline phosphatase activity was determined with paranitrophenyl phosphate that was measured in the microtitre wells.

The anti-pre S antiserum was raised in a rabbit as described previously [16].

RESULTS

Antiviral activity of analogues of FddThd and FddMeCyt in vitro

A modification of FddThd at its base moiety resulted in a pronounced reduction in cytotoxicity (cytostatic activity). While FddThd caused a 50% reduction in cell growth (CD_{50}) at 1.2 μM, its analogues FddUrd, FddClUrd, FddBrUrd, FddBVUrd and AzddMeCyt as well as FddFCyt and FddClCyt were not cytotoxic (cytostatic) even at 25 μM (Table 1). In contrast, only slight changes in the cytotoxic potential were measured for the analogues of the reference compound FddMeCyt (CD_{50} 0.54 μM): FddEtCyt, CldMeCyt, AmddMeCyt and AraMeCyt.

The antiviral activity of the compounds was determined on the basis of inhibition of HBV DNA synthesis by Hep G2 2.2.15 cells. Applying this approach it was not possible to determine unequivocally whether all the HBV DNA identified was released by the cells or was obtained in part from cells which had undergone lysis. The experiments revealed that FddThd caused a 90% reduction of HBV DNA synthesis at 0.03 μM, while

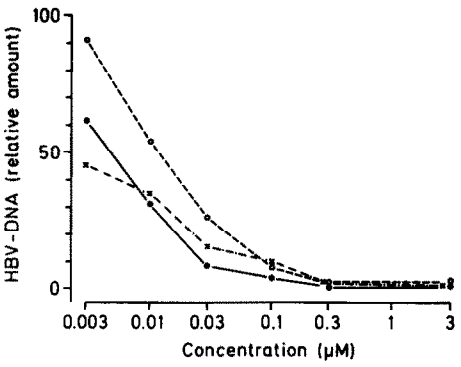

Fig. 1. Dose–response curve for inhibition of extracellular HBV DNA production in the transfected human hepatoblastoma cell line Hep G2 2.2.15 for FddClCyt, FddMeCyt and CldMeCyt. The cells were treated for 4 days with the compounds. FddClCyt (○), FddMeCyt (×) and CldMeCyt (●) were added to the cultures at the indicated concentrations. Then the amount of extracellular HBV-DNA was determined by slot hybridization, as described in Materials and Methods; the amount of HBV DNA in the untreated cultures was set to 100.

approximately 3-fold higher concentrations of FddClUrd and even 10-fold higher concentrations of FddBrUrd and FddBVUrd were necessary to observe the same effect. However, the antiviral index of the latter compounds is higher due to their lack of cytotoxicity. FddUrd was without any noticeable effect (Table 1).

Comparing the antiviral effects of the analogues of FddMeCyt (>90% inhibition of HBV DNA synthesis at 0.1 μM; >95% inhibition at 0.3 μM), FddClCyt, FddEtCyt, AmddMeCyt and AraMeCyt were less potent than CldNeCyt, FddMeCyt, and AzddMeCyt and FddCyt as well as FddFCyt were inactive at the concentrations tested (Table 1 and Fig. 1).

The antiviral effects of FddThd and FddMeCyt in vivo*: level of serum DHBV DNA*

The *in vivo* antiviral effect of FddThd was assessed using Peking ducks infected with DHBV. As shown in Fig. 2 DHBV DNA could be identified in the serum of an infected animal 6–12 days post-infection (Fig. 2, row A); the virus load remained almost unchanged up to an infection period of 36 days (row F). When the animals received a total daily dose of 10 mg/kg from day 1 on, no virus DNA could be detected after 12 days (row B). When the treatment started at day 13 a significant reduction had already occurred 11 days later and viral DNA was almost absent after an additional 12 days treatment (row C). If the dose of the compound was increased to 20 mg/kg the antiviral effect was not augmented significantly (row D); no virus could be detected after 24 days treatment. If the treatment of the animals was stopped after administration of 20 mg/kg of FddThd for 12 days, DHBV DNA could be identified, although at a low concentration, in the serum (row E).

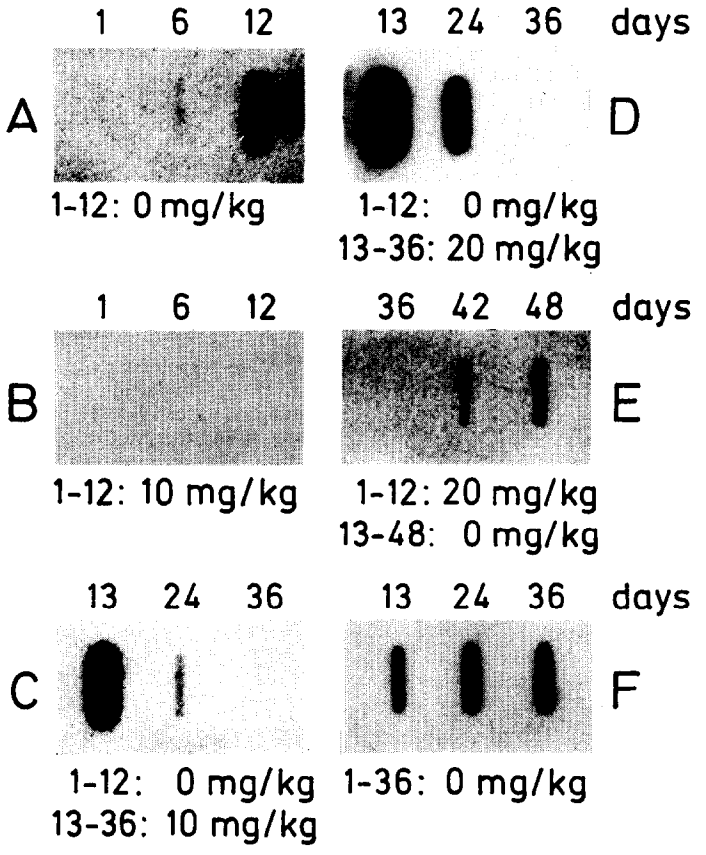

Fig. 2. The antiviral effect of FddThd *in vivo*. One-day-old ducklings were infected with DHBV-positive serum on day 1. One animal each remained untreated (i) for 12 days (row A) or (ii) for 36 days (row F). The infected animals were treated as follows: row B, a daily dose of 10 mg/kg for 12 days, beginning at day 1; row C, a daily dose of 10 mg/kg for 24 days beginning at day 13; row D, a daily dose of 20 mg/kg, beginning at day 13; or row E, the animal was pretreated for 12 days with a daily dose of 20 mg/kg and remained for an additional 36 days without treatment. Pooled sera (from each group) were taken 12 hr after the last injection of the compound (time indicated) and analysed for DHBV DNA by slot-blot hybridization procedure using [^{32}P]DHBV DNA as a probe. Three infected animals were used for each experiment.

The *in vivo* effect of FddMeCyt was found to be more pronounced than that of FddThd. As summarized in Figs 3 and 4 after a compound dose of 5 mg/kg (given daily), administered for the first 12 days after infection, no virus was detectable (Fig. 3, row B). A dose of 10 mg/kg (given daily) and administered for 12 days almost completely cleared the virus from the animals. However, only 36 days after termination of the treatment DHBV DNA could be detected again. The inhibition at day 48 was still 30% (Figs 3, row C and 4). The results of the control group are shown in Figs 3, row A and 4.

In a second series of experiments the ducks were treated with different doses of FddMeCyt starting at day 13 after infection. As summarized in Fig. 5 the DHBV DNA level had decreased by 30% with the low dose (5 mg/kg daily) and by 55% with the high dose (10 mg/kg daily) after 18 days.

The antiviral effect of FddThd and FddMeCyt in vivo*: level of serum DHBV antigen*

In a second approach the amount of DHBV antigen was determined in an ELISA assay system using a polyclonal antiserum directed against the pre S region of the virus surface antigen. In a calibration curve it was determined that a 1:1000 dilution of the antiserum is suitable for a quantification of the antigen in serum of ducks (Fig. 6). In a control using pre-immune serum it was established that the interaction between the antiserum and the viral antigen was specific. Infected ducks had formed a high level of antigen (1.2 A_{405} U) 12 days after infection (Table 2).

The therapeutic studies with FddThd or FddMeCyt revealed that immediate treatment of the infected animals resulted in total elimination of the antigen,

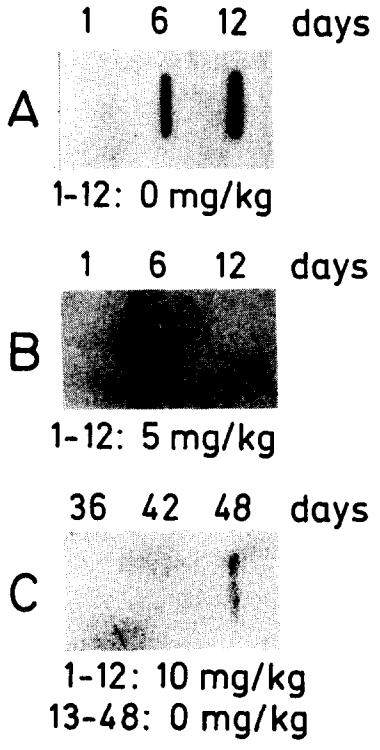

Fig. 3. The antiviral effect of FddMeCyt *in vivo*. Ducklings were infected with DHBV-positive serum on day 1. One series of animals remained untreated for up to 12 days (A). A second series was treated with 5 mg/kg beginning at day 1 for 12 days (B). A third group was treated for 12 days with 10 mg/kg, beginning on day 1, and then remained for an additional 36 days without treatment (C). Further details are given in the legend to Fig. 1.

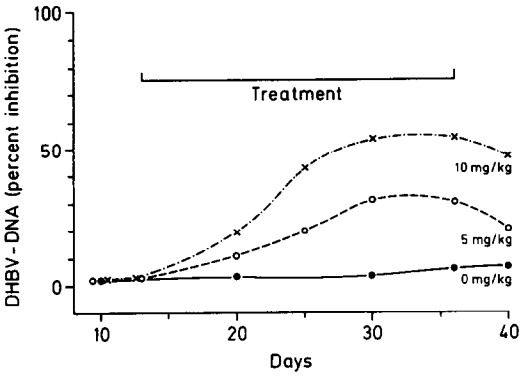

Fig. 5. FddMeCyt, administered to the ducks 13 days after infection for a 24-day course (day 13–36 p.i.) at the following total daily doses: 0 (●), 5 (○) and 10 mg/kg (×).

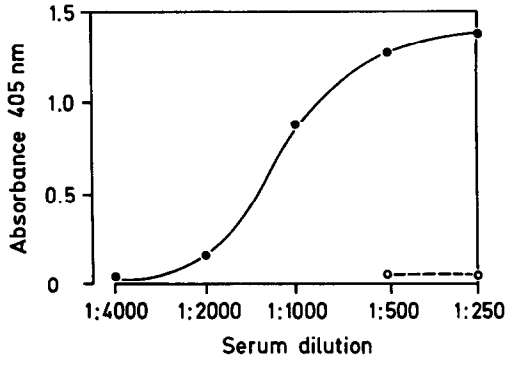

Fig. 6. Binding of anti-pre S antiserum to the antigen, present in a DHBV-positive control serum. Microtiter wells were coated with the control serum and tested with different dilutions of the anti-sera to pre S in the ELISA assay system. Anti-pre S antiserum (●); pre-immune serum (○). Results are means of five parallel determinations; SD was less than 12%.

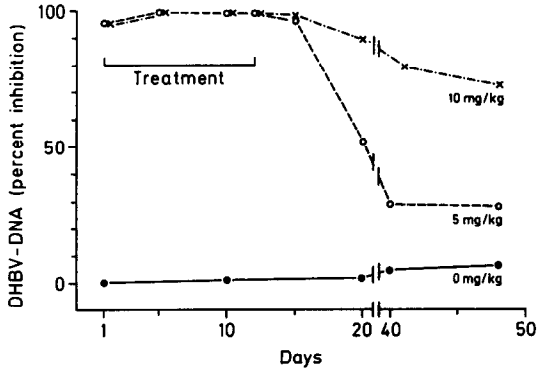

Fig. 4. Changes in mean serum DHBV DNA levels of ducks infected with DHBV receiving a 12-day treatment, begun on the day of infection of the animals, with FddMeCyt at the following total daily doses: 0 (●), 5 (○) and 10 mg/kg (×). Three animals in each group were examined. The DHBV DNA levels were estimated by slot-blot analysis.

while administration of the compound starting at day 12 was less effective (Table 2). A comparison between the two compounds shows that under *in vivo* conditions also FddMeCyt was more potent than FddThd; administration of a total daily dose of 10 mg/kg/day of FddMeCyt given from day 13 to day 36 p.i. resulted in a decrease of the antigen load from 9.3 A_{405} U (untreated and infected control animals) to 1.1 A_{405} U, compared to 3.1 A_{405} U in the experiments with FddThd. Uninfected animals had an antigen level less than 0.05 A_{405} U (not shown). No apparent side effects could be observed during the first 12 days of treatment with either FddThd or FddMeCyt. Likewise, no toxic effects could be seen during the treatment of the animals with 20 mg/kg of FddThd from day 13–36 p.i.; no anorexia followed by a significant loss of body weight could be objectified. No animal died before the end of the treatment.

Table 2. Level of viral antigen in sera of DHBV-infected ducks

Compound	Dose (mg/kg/day)	Schedule (days)	Level of antigen (A_{405})
Control	0	1–12	1.2 ± 0.3
	0	1–36	9.3 ± 2.4
FddThd	10	1–12	<0.05
	10	13–36	3.1 ± 0.6
	20	13–36	1.9 ± 0.5
FddMeCyt	5	1–12	<0.05
	10	1–12	<0.05
	5	13–36	2.9 ± 0.7
	10	13–36	1.1 ± 0.4

The amount is presented in A_{405} U as described in Fig. 6, using an anti-pre S antiserum at a dilution of 1:1000. The ducks were given i.v. inoculation of DHBV on day 1. Treatment was i.m. as indicated. The total dose per day is indicated; the compounds were administered twice daily. Twelve hours after the last injection (day 12 or 36) serum was obtained from the animals. Three animals in each group were examined; the sera at each time point and group were taken and the level of viral antigen was determined. The means of five parallel experiments (±SD) are given.

DISCUSSION

Enzymatic studies have revealed that the modified 2′,3′-dideoxy-thymidine and 2′,3′-dideoxy-5-methyl-cytidine analogues are promising anti-HBV compounds [6]. In this study the triphosphate forms of the modified pyrimidine analogues were found to inhibit potently HBV DNA polymerase. However, a recent publication was less optimistic that these compounds are effective also in intact cell systems or even in animal models; it was reported that 3′-azido-3′-deoxythymidine failed to inhibit replication of DHBV both *in vivo* and *in vitro* [21].

In preceding cellular studies using Hep G2 2.2.15 cells, we established that FddThd (more than 90% reduction of HBV DNA synthesis at 0.03 μM) and FddMeCyt (0.10 μM) display an anti-HBV activity *in vitro*. In this study we extended our structure–activity studies and determined the antiviral effects of the FddThd analogues, FddUrd, FddClUrd, FddBrUrd and FddBVUrd as well as of the FddMeCyt analogues FddCyt, FddFCyt, FddClCyt, FddEtCyt, CldMeCyt, AmddMeCyt, AzddMeCyt and AraMeCyt. The results reveal that a modification of the FddThd at the base moiety improved the antiviral index of the compounds tested. This conclusion can be drawn from the findings showing that the antiviral activity of the latter compounds (FddClUrd, FddBrUrd and FddBVUrd) was approximately 3–10-fold lower than that of FddThd; however, none of these compounds was toxic to the cells. FddUrd was inactive.

Among the FddMeCyt analogues ClddMeCyt was as equally effective a compound as FddMeCyt. With the exception of FddCyt and FddFCyt, which were inactive, FddClCyt, FddEtCyt, AmddMeCyt, AzddMeCyt and AraMeCyt displayed only a moderate antiviral activity. Modifying FddMeCyt both in the base and in the sugar moiety resulted in a lesser cytotoxic effect on the cells.

In the present study we also demonstrated the antiviral activity of FddThd and FddMeCyt *in vivo*, using the duck/DHBV model system. An initial complete protection of the animals could be achieved if the compounds were administered immediately after infection with DHBV. However, the virus reappeared 30 and 36 days, in the studies with FddThd and FddMeCyt, respectively, after an initial 12 days treatment, followed by the indicated period of time without treatment. It remains to be studied whether a change in the schedule of application of the compound improves the therapeutic effect. The antiviral activity of FddMeCyt is, under the conditions used, more pronounced than that of FddThd. From the data obtained it can be concluded that, compared to FddThd, lower doses of FddMeCyt are required to obtain the same therapeutic effect. Under our experimental conditions no toxic effects were observed after treatment of the animals with either FddThd or FddMeCyt; in other animal systems e.g. mice a pronounced toxicity displayed by FddThd has been reported [22].

The data summarized in the present work indicate that an effective protection against hepatitis infection *in vitro* and *in vivo* can be achieved by pyrimidine analogues, carrying at the 3′-position in the sugar moiety a modified group, e.g. halogens or amino moieties. Based on previous studies it is likely that the compounds act in their triphosphate form as highly selective competitive inhibitors of HBV-DNA polymerase [6, 23].

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