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Inhibition of human and duck hepatitis B virus by 2′,3′-dideoxy-3′-fluoroguanosine in vitro

Ingrid Schröder *, Birgitta Holmgren, Monica Öberg, Bengt Löfgren

Department of Medical Microbiology, University of Lund, Lund, Sweden

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Abstract

The fluorinated guanosine analog 2′,3′-dideoxy-3′-fluoroguanosine (FLG) has been shown to have an effect on duck hepatitis B virus (DHBV) in vivo and in vitro. In this study the inhibitory effect of FLG on DHBV and human hepatitis B virus (HBV) was evaluated in vitro. Cell lines transfected either with DHBV or HBV DNA and primary duck hepatocyte cell cultures were used. Virus production was analysed by PCR and a quantitative PCR was established for DHBV for determination of the inhibitory concentrations of the drug. 50% inhibition was achieved with an FLG concentration of 0.2 μg/ml (0.7 μM) and 90% inhibition was observed with an FLG concentration of 1.0 μg/ml (3.7 μM) using the DHBV transfected cell line. FLG showed an effect on DHBV production in primary duck hepatocyte cell cultures at concentrations down to 0.1 μg/ml (0.4 μM). However, the DHBV production returned to pre-treatment levels within a few days after cessation of treatment. HBV production in transfected cell lines was also inhibited by FLG. Both DHBV and HBV DNA-polymerases were inhibited by FLG triphosphate and 50% inhibition was observed at a concentration of 0.05 μg/ml (0.1 μM) for DHBV and 0.03 μg/ml (0.05 μM) for HBV. FLG is an efficient inhibitor of DHBV replication both in vivo and in vitro and of HBV in vitro which makes it a good candidate for treatment of HBV infections. However, it does not completely eliminate the virus since a relapse in virus production was observed when treatment was withdrawn. Therefore it would be interesting to evaluate FLG in combination with other types of anti-HBV drugs. © 1998 Elsevier Science B.V.

Keywords: Hepatitis B virus; Nucleoside analog; Duck hepatitis B virus; Antiviral treatment; Quantitiative PCR

1. Introduction

Infection with human hepatitis B virus (HBV) is a worldwide health problem. It is one of the major causes of acute and chronic hepatitis, and primary hepatocellular carcinoma is strongly as-

* Corresponding author. Tel.: +46 46 173274; fax: +46 46 189117.

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sociated with chronic HBV infection (Beasley et al., 1981). So far, the only generally accepted drug for specific treatment is interferon alfa, which can be used in chronic hepatitis B. Unfortunately, this treatment is effective in less than 50% of chronically infected patients (Wong et al., 1993). Therefore, there is a need for drugs that directly inhibit HBV production. Most compounds explored so far are nucleoside analogs (Sommadossi, 1994).

Since there is no convenient animal model for HBV infection, other hepadnaviruses, like woodchuck hepatitis virus (WHV) and duck hepatitis B virus (DHBV) have been used in antiviral trials (Fourel et al., 1990; Matthes et al., 1992; Shaw et al., 1994; Löfgren et al., 1996). Cell lines transfected with HBV or DHBV, have been proven useful for screening of compounds with inhibitory effects on hepadnavirus replication (Korba and Milman, 1991; Staschke et al., 1994). Primary hepatocyte cultures from duck liver have also been used (Civitico et al., 1990; Shaw et al., 1994). Such cultures can be infected with DHBV and are able to support replication of this virus (Tuttleman et al., 1986; Pugh and Summers, 1989).

In some cell culture systems, virus is produced at very low levels so that DNA amplification is needed to detect the virus. A quantification of virus production is required to express the rate of inhibition of viral replication, obtained by an antiviral compound in a cell culture system. Thus, there is an interest in evaluating quantitative DNA amplification methods.

The fluorinated guanosine analog, 2′,3′-dideoxy-3′-fluoroguanosine (FLG) is an inhibitor of the HIV reverse transcriptase (Hartmann et al., 1988). Other nucleoside analog reverse transcriptase inhibitors have been shown to be effective inhibitors of the hepadnavirus DNA-polymerase (Löfgren et al., 1989; Matthes et al., 1991). FLG has been shown to have an inhibitory effect on DHBV replication in vivo and in vitro in primary duck hepatocyte cell cultures (Hafkemeyer et al., 1996; Löfgren et al., 1996). In the present investigation, the antiviral effect of FLG on hepadnaviruses was evaluated in different in vitro systems, i.e. DHBV and HBV transfected cell lines, primary duck hepatocyte cell cultures and, as triphosphate, on the DNA-polymerase of DHBV and HBV virion preparations. A quantitative DNA amplification method for determination of rate of inhibition was established.

2. Material and methods

2.1. Compound

FLG and FLG triphosphate (FLG–TP) was obtained from Medivir AB (Huddinge, Sweden) and foscarnet from ASTRA (Astra, Södertälje, Sweden).

FLG was dissolved in dimethyl sulfoxide (DMSO) so that the final concentration of DMSO in cell culture medium was 0.1%. FLG–TP was dissolved in water and stored at −20°C until used.

2.2. Transfected cell lines

HBV transfected human hepatoma cells, the Hep G2 2.2.15 cell line (Sells et al., 1987), was a gift from Dr. I. Fourel, Lyon, France. Another Hep G2 human hepatoma cell line, transfected with DHBV, Hep G2/G3 (Galle et al., 1988) was kindly supplied by Dr. J. Colacino, Indianapolis, USA. Both cell lines secrete virus (HBV and DHBV, respectively) into cell culture medium.

Both human hepatoma cell lines (HepG2/G3 and Hep G2 2.2.15) were grown at 37° in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 40 μg/ml gentamicin and 400 μg/ml geneticin.

2.3. Primary duck hepatocytes

Cells were prepared from livers of domestic ducks according to Tuttleman et al. (1986), Pugh and Summers (1989), Offensperger et al. (1991) with minor modifications. Ten-day-old ducklings, either DHBV DNA negative or positive as determined by dot blot hybridisation on serum samples were used. The animal was sacrificed and the liver was immediately removed. It was rinsed by infusion, through the portal vein, with 100 μl of 0.5 mM EGTA (ethylene glycol-bis(β-aminoethyl

ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) in Hank's balanced salt solution (HBS) buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) pH 7.4, followed by 100 ml of 0.5 mg collagenase type 1 (Sigma) per milliliter, 2.5 mM $CaCl_2$ in buffered HBS. All solutions were kept at 37°C. A single cell suspension was prepared in Williams medium E buffered with 20 mM HEPES, pH 7.4, by gentle stirring for 30 min at 37°, followed by filtering through a fine wire-gauze mesh. The cell suspension was centrifuged at $50 \times g$ for 4 min and washed three times with the above medium. The hepatocytes were seeded at a density of 5×10^5 cells/ml in six-well tissue culture dishes (Nunc) (2.5 ml/well) using Williams medium E supplemented with 20 mM HEPES pH 7.4, 10^{-5} M hydrocortisone 21-hemisuccinate, 1 μg/ml insulin, 5% fetal bovine serum, 5 mM glutamine and 40 μg/ml gentamicin. The cells were allowed to adhere to the surface of the wells for 24 h and then the medium was changed to the above mentioned, but fetal bovine serum was replaced by 1.5% dimethyl sulfoxide (DMSO). The cells were maintained at 37° and 5% CO_2 in a humidified incubator and medium was changed every third day.

Primary duck hepatocytes isolated from DHBV negative ducks were prepared as described above. After adherence of the cells they were infected with 100 μl per well of a DHBV positive serum for 1 h and then medium with 1.5% DMSO was added. Cell culture medium was changed every third day.

Cell culture medium samples for DHBV determination were obtained every third day, centrifuged at 3000 rpm for 5 min in a microfuge and supernatants stored at −20°C until analysis.

2.4. Inhibition experiments

The transfected human hepatoma cell lines Hep G2 2.2.15 and Hep G2/G3 were seeded at a density of 5×10^5 cells/ml in 96 well microtiter plates (0.2 ml/well). Treatment started 3 days after seeding of cells, i.e. when cells had grown to confluency and continued for 10 days. FLG or foscarnet was added to cell culture medium at appropriate concentrations, in connection with medium changes every other day. 24 h after the last medium change, the microtiter plates were centrifuged for 10 min, 1000 rpm at 4°C and 100 μl of cell culture supernatant from each well were transferred to a sterile microtiter plate and stored at −20°C until analysis.

Primary duck hepatocytes were prepared as described above and treatment was started 3–5 days after seeding of the cells. Treatment was performed as for transfected cells and cell culture medium samples were collected every third day when medium was changed. 24 h after the last medium change a sample was collected and this indicates the end of treatment. Cells were then kept in FLG-free medium. This was done to determine how soon virus production regains after cessation of FLG treatment.

All cells were inspected daily by light microscopy. Levels of HBV- and DHBV-DNA were determined by polymerase chain reaction (PCR) followed by gel electrophoresis or by DIG-PCR ELISA as described below.

2.5. Toxicity measurements

Toxicity was measured in transfected cell lines by the neutral red dye method according to Korba and Gerin (1992). Cells were treated with substance as described above.

2.6. Polymerase chain reaction (PCR) analysis

For detection of DHBV DNA and HBV DNA in cell culture supernatants from Hep G2/G3 and HepG2 2.2.15, respectively, 10 μl of 50 times diluted cell culture supernatant was added to a Whatman GF/C filter and boiled for 10 min in 50 μl water (Staschke et al., 1994). For the detection of DHBV from primary duck hepatocytes a 10 μl aliquot of undiluted cell culture supernatant was applied to the filter and boiled in water as described above. A 10 μl sample of this was used as template for PCR amplification.

PCR was carried out using the thermostable DNA polymerase from Thermus aquaticus (Appligene, Illkirch, France). Oligonucleotides were obtained from Scandinavian Gene Synthesis AB (Köping, Sweden). The oligonucleotide

primers used in the DHBV PCR reaction were derived from Sprengel et al. (1985) and are as follows:

S1: 5′-TACTAGCTGGCCTAATCGGA-3′ (map position 1306–1325)

S2: 5′-GGCAGTAGTGAAGAGATGGA-3′ (map position 1654–1635)

The oligonucleotide primers used in the PCR reaction for detection of HBV DNA from Hep G2 2.2.15 cells have been published by Kaneko et al. (1989), but the map positions are according to Raney and McLachlan (1991) and are as follows:

1763: 5′-GCTTTGGGGCATGGACATTGACCCGTATAA-3′ (map position 1893–1922)

2032R: 5′-CTGACTACTAATTCCCTGGATGCTGGGTCT-3′ (map position 2162–2133)

PCR reactions were carried out in a total volume of 50 μl containing 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 0.1% Triton-X 100, 0.2 mg/ml BSA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 μl template, 1.25 units of *Taq* polymerase and 0.75 μM each of appropriate primers. For the quantitative PCR the reaction mixture contained the same as above except that the concentration of dTTP was 0.19 and 0.01 mM of digoxigenin-11-dUTP (DIG-dUTP) (Boehringer Mannheim) was included.

The amplification schedule for DHBV started with 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 53°C for 1.5 min and 72°C for 2 min. After the last cycle samples were left at 72°C for 5 min. The PCR schedule for HBV started with 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 42°C for 1 min and 72°C for 4 min.

The amplified DNA was electrophoresed through 1.7% agarose, stained with ethidiumbromide and visualised by ultraviolet illumination.

2.7. Quantitative DHBV PCR ELISA

DHBV PCR with DIG-dUTP was performed on cell culture medium from Hep G2/G3 as described above. To generate standard curves, plasmid pDHBV3 (Sprengel et al., 1984, 1985) kindly provided by Dr. H. Will was used. pDHBV3 contains a full-length genome of DHBV. This plasmid was serially diluted in water ten-fold to cover a range of 10–100 000 copies/μl before subjected to PCR as described above.

The ELISA detection assay was carried out using the Boehringer Mannheim PCR ELISA DIG-detection kit. The PCR product was used undiluted or was diluted five or ten times before 5 μl was added to 40 μl denaturing buffer (Boehringer Mannheim). After 10 min at room temperature, 455 μl hybridisation solution containing 7.5 pmol biotinylated probe per ml was added. The 5′-biotinylated probe (Scandinavian Gene Synthesis, Köping, Sweden) used is homologous to the PCR product and have the following sequence, map positions given as for DHBV PCR primer as described above.

BioS2 5′-GGAGGCTAGACTGGTGGTGGAT-3′ (map position 1372–1393)

200 μl of the denatured PCR product plus hybridisation solution was added to streptavidin coated microtiter plates (Boehringer Mannheim). The plate was incubated for 3 h at 50°C with shaking. Following incubation the wells were washed five times with washing solution (Boehringer Mannheim), before 200 μl of peroxidase conjugated antibodies against DIG (anti-DIG-POD) with a concentration of 0.01 mU/μl was added to each well. The plate was incubated for 1 h at 37°C with shaking. The anti-DIG-POD solution was discarded and the plate was washed as described above. 200 μl of peroxidase substrate, ABTS (Boehringer Mannheim) was added at a concentration of 1 mg/ml and then incubated for 30 min at 37°C before the absorbance was determined at 405 nm.

DHBV DNA contents in the original cell culture supernatants were determined from standard curves obtained with pDHBV3. The rate of inhibition was calculated by comparing the amount of DHBV DNA from cell culture supernatants from untreated cells with values obtained from FLG treated cells. Concentrations of FLG giving 50% and 90% inhibition of DHBV virion production in cell cultures were determined from inhibition curves.

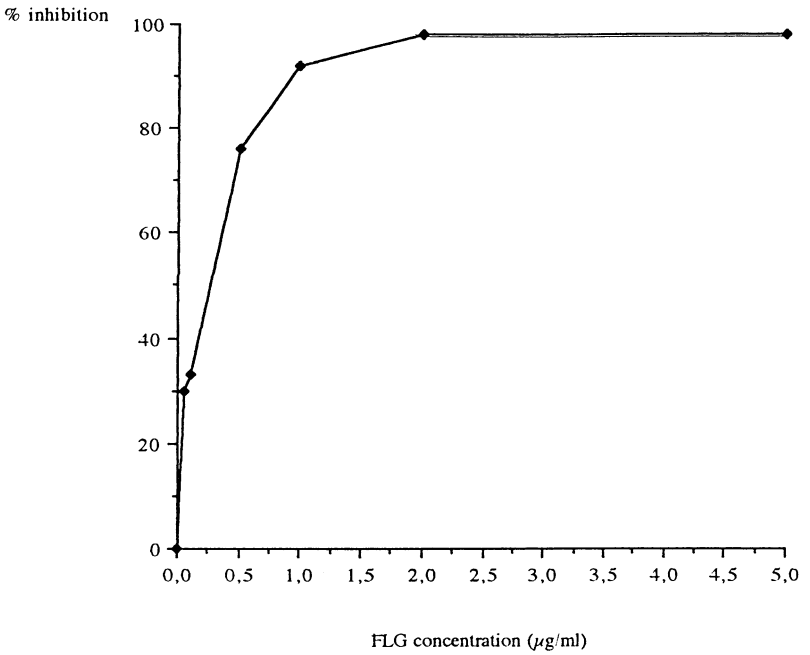

Fig. 1. Anti-DHBV activity of FLG in Hep G2/G3 cells expressed as percent inhibition. The inhibition curve was constructed from data obtained with the quantitative DHBV PCR method described in Material and Methods. The number of DHBV genomes produced from Hep G2/G3 cells treated with FLG was calculated from a standard curve obtained with pDHBV3, and compared to that of untreated cells.

2.8. DNA polymerase assay

Virions in serum from DHBV infected ducks and from patients with chronic HBV infection were used to determine inhibition of DHBV and HBV DNA polymerase activities by FLG triphosphate (FLG–TP). The DNA polymerase assays were performed as described by Löfgren et al. (1989) except that 10 μl aliquots of non-concentrated sera were used. The assays were performed with α-^{32}P-dGTP (10 mCi/ml, >400 Ci/mmol) (Amersham, UK) at a final concentration of 0.1 μM, with a reaction time of 3 h. Concentration of FLG–TP giving 50% inhibition of DNA polymerase activity (IC_{50}) was determined from inhibition curves.

3. Results

3.1. FLG inhibits production of DHBV and HBV in transfected human hepatoma cells

Concentrations of FLG tested on HepG2/G3 cells were 5, 2, 1, 0.5, 0.1 and 0.05 μg/ml (18.6, 7.4, 3.7, 1.9, 0.4 and 0.2 μM). A decrease of amplified DHBV DNA on agarose gels was observed at or above concentrations of 0.5–1 μg/ml FLG (1.9–3.7 μM) (data not shown).

To determine the rate of inhibition of virus production obtained by FLG, a quantitative PCR method was established, see Material and Methods. This method makes use of the sensitivity of PCR and the specificity of a hybridisation reaction.

The IC_{50} (0.2 μg/ml or 0.7 μM FLG) and IC_{90} (1.0 μg/ml or 3.7 μM FLG) values were determined from an inhibition curve constructed from data obtained with the DIG-PCR ELISA (Fig. 1).

By using the HBV transfected human hepatoma cell line Hep G2 2.2.15 we were able to show that FLG inhibited HBV production. As seen in Fig. 2, FLG concentrations above 0.5 μg/ml (1.9 μM) inhibited HBV production as shown by a decrease of PCR product. However, the virus production in these cells was not completely inhibited by FLG as shown in Fig. 2, i.e. even at an FLG concentration of 100 μg/ml (371 μM) HBV DNA

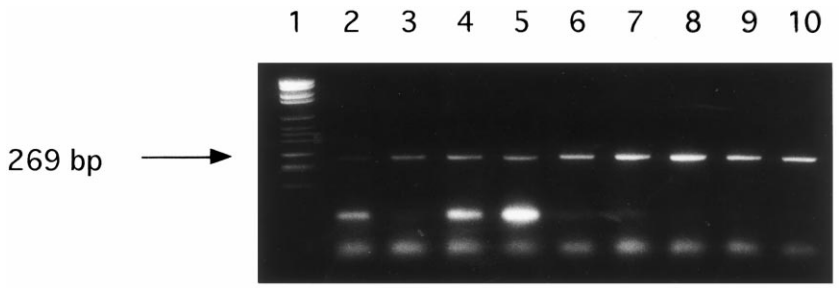

Fig. 2. Anti-HBV activity of FLG in the HBV transfected human hepatoma cell line, Hep G2 2.2.15. Cell culture supernatants were analysed by HBV PCR followed by gel electrophoresis and ethidiumbromide staining (see Material and Methods). Lane 1. DNA molecular weight marker VI (Boehringer Mannheim); 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 220 bp. Lane 2–10. 269 bp amplified HBV DNA from cell culture supernatants from Hep G2 2.2.15 cells treated with 100, 10, 5, 2.5, 1, 0.5, 0.1, 0.05 and 0 μg/ml FLG (371, 37.1, 18.6, 9.3, 3.7, 1.9, 0.4, 0.2 and 0 μM), respectively.

were detected. It was therefore difficult to establish IC_{50} and IC_{90} values, but a clear inhibitory effect was observed at concentrations above 1.0 μg/ml (3.7 μM).

As a control, inhibition experiments were also performed using the pyrophosphate analog foscarnet. We found that foscarnet inhibited DHBV in Hep G2/G3 cells at concentrations above 100 μM (data not shown). HBV was also inhibited in Hep G2 2.2.15 cells, but not to the same extent as for DHBV. A concentration above 1000 μM was required for inhibition (data not shown), which is in agreement with earlier findings by McMillan et al. (1995)

With an FLG concentration up to 100 μg/ml (371 μM), there were no signs of cell toxicity as determined by the neutral red dye method (data not shown) and by daily light microscopy examination.

3.2. DHBV production from infected primary duck hepatocytes is inhibited by FLG

Primary duck hepatocytes were prepared from either DHBV positive or negative 10-day old ducklings as described in Material and Methods. Concentrations of FLG used in the experiment with duck hepatocytes infected post-plating, were 10, 5 and 1 μg/ml (37.1, 18.6 and 3.7 μM). DHBV virion DNA in cell culture supernatants was analysed with PCR followed by analysis on ethidium bromide stained agarose gel. After 6 days of FLG treatment, i.e. two changes of medium containing FLG, DHBV DNA had decreased and no visible band on agarose gel was found for any concentration of FLG used. However, medium from control cells (untreated cells) contained DHBV DNA. In some wells DHBV DNA reappeared 5 days after change to medium without FLG (data not shown). In experiments with primary duck hepatocytes from infected livers, the concentrations of FLG used were 10, 5, 2, 1, 0.5 and 0.1 μg/ml (37.1, 18.6, 7.4, 3.7, 1.9 and 0.4 μM). After 10 days of FLG treatment the amount of DHBV DNA had decreased drastically with drug concentrations down to 0.1 μg/ml (0.4 μM) (Fig. 3). However, 6 days after the last FLG treatment, the amount of DHBV DNA had returned to the same levels as before treatment, except for cells treated with 10 μg/ml FLG (37.1 μM) (Lane 4, Fig. 3).

With the FLG concentrations used, there were no signs of cell toxicity in any of the cell experiments, as judged from daily light microscopy examination.

3.3. FLG triphosphate inhibits both DHBV and HBV DNA polymerase activities

The triphosphate of FLG inhibited the DNA-dependent DNA polymerase activities of both DHBV and HBV virion preparations. The 50% inhibitory concentration was 0.1 μM FLG–TP (0.05 μg/ml) for the DHBV DNA polymerase and

0.05 μM (0.03 μg/ml) for the HBV DNA-polymerase see (Fig. 4).

4. Discussion

Hepatitis B virus infection constitutes a world wide health problem. There is extensive research on antiviral substances that could be useful, alone or in combination therapy against chronic infection. Löfgren et al. (1996) studied the antiviral effect of FLG against DHBV on both congenitally infected and post-hatch infected ducks. The compound, given in doses down to 1 mg/kg per day, inhibited virus production. However, when treatment was withdrawn DHBV DNA levels in serum samples returned to pre-treatment levels.

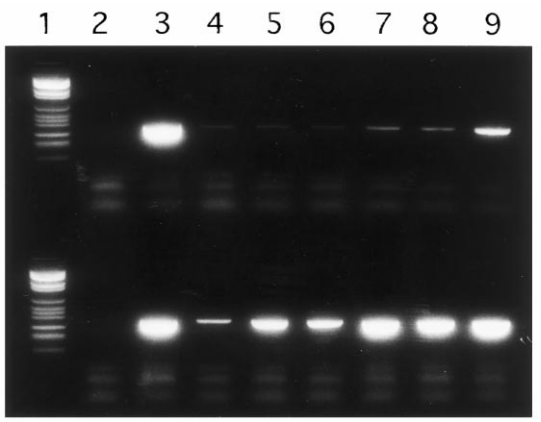

Fig. 3. Anti-DHBV activity of FLG in primary duck hepatocytes. Primary duck hepatocytes were prepared from a liver from a congenitally infected duckling as described in Material and Methods. Equal amounts of cell culture supernatants were subjected to DHBV PCR analysed by gel electrophoresis followed by ethidiumbromide staining. Top row. Lane 1. DNA molecular weight marker VI (Boehringer Mannheim); 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 220 bp. Lane 2. Water control. Lane 3–9. Amplified DHBV DNA from cell culture supernatants collected one day after the last medium change containing FLG. Lane 3. Untreated cells. Lane 4–9. Cells treated with 10, 5, 2, 1, 0.5 and 0.1 μg/ml FLG (37.1, 18.6, 7.4, 3.7, 1.9 and 0.4 μM), respectively. Bottom row. As top row except that the amplified DHBV DNA are from cell culture supernatants collected 6 days after cessation of FLG treatment, i.e. after two medium changes without FLG.

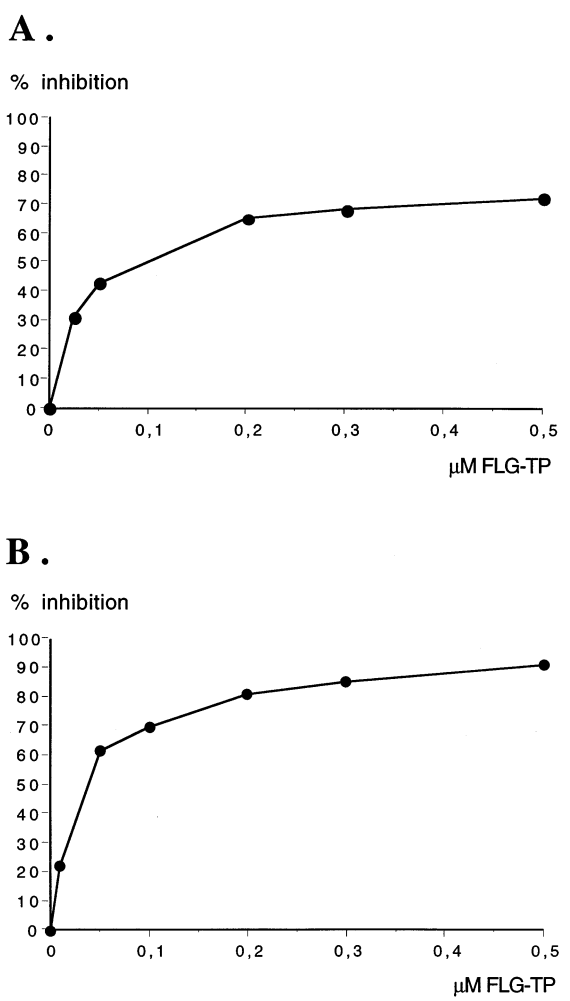

Fig. 4. Percent inhibition of A: DHBV and B: HBV DNA-polymerase activities by 2′,3′-dideoxy-3′-fluoroguanosine triphosphate. DNA-polymerase assays were performed as described in Material and Methods. Final concentration dGTP in the reaction mixture was 0.1 μM.

Another study performed by Hafkemeyer et al. (1996) has also shown an inhibitory effect of FLG on DHBV in ducks and in primary duck hepatocyte cell cultures.

In the present study we have analysed the effect of FLG on hepadnavirus production in vitro in three different cell culture systems and the effect of FLG triphosphate on both human and duck hepatitis B virus DNA-polymerase.

A quantitative method for monitoring the amount of HBV or DHBV genomes in either cell

culture media or in serum samples could be used to study the effect of different antiviral substances in in vitro or in vivo. In this study we used a quantitative PCR method for determination of DHBV virion DNA in cell culture media. We analysed, by the aid of this method, the antiviral effect of FLG on DHBV production in the transfected human hepatoma cell line, Hep G2/G3. The method consists of an amplification step using DIG-labelled dUTP in the PCR reaction, a detection step, consisting of a combination of a hybridisation reaction with a biotin-labelled DHBV-specific probe and an ELISA detection system. Using this method, it was possible to detect down to 10–100 genome copies/μl cell culture supernatant. Three or four standard curves were generated in all experiments, each with a linear range encompassing two to three logs. These results are in agreement with previously shown results (Holodniy et al., 1991; Eron et al., 1992).

Using this method, to determine DHBV virion DNA in cell culture supernatants from treated and untreated cells, a 50% inhibition of virus production was achieved at 0.2 μg/ml FLG (0.7 μM) and 90% inhibition at 1.0 μg/ml FLG (3.7 μM).

FLG was also shown to inhibit DHBV production in primary duck hepatocytes infected pre- or post-plating. The degree of inhibition was estimated from ethidumbromide stained gels of amplified virion DNA from cell culture supernatants. According to these determinations the inhibitory concentration of FLG was similar to that observed for the transfected cell line used. However, the virus production returned to pre-treatment levels 6 days after FLG was withdrawn from the cell culture medium.

The human hepatoma cell line transfected with HBV, HepG2 2.2.15 was also used to study the inhibitory effect of FLG. The results showed an inhibition of virus production as seen in Fig. 2. However, in these cells virus production was not completely inhibited by FLG, which was the case for DHBV. Even at an FLG concentration of 100 μg/ml (371 μM) there was production of virions from the cells, since HBV DNA could be detected in cell culture supernatants by PCR. FLG was not toxic at a concentration of 100 μg/ml (371 μM), as determined by neutral red dye uptake.

The inhibition of the DHBV and HBV DNA-polymerase by FLG-TP was also analysed. The results obtained using the DHBV and HBV polymerases showed a 50% inhibition at 0.1 μM (0.05 μg/ml) and 0.05 μM (0.03 μg/ml) of FLG-TP, respectively. The value for FLG inhibition of HBV polymerase is comparable with earlier findings by Matthes et al. (1991).

The inhibition data obtained for FLG in these DHBV in vitro experiments are in the same range as previously published in vivo and in vitro results (Hafkemeyer et al., 1996; Löfgren et al., 1996), indicating that these DHBV in vitro models are suitable for screening of antiviral substances. Furthermore, FLG also inhibited HBV production in the transfected human hepatoma cell line HepG2 2.2.15, but not to the same extent as for DHBV production in HepG2/G3 cells and in infected primary duck hepatocytes. It seems possible that this difference depends on the cell system used, as the inhibition data for FLG-TP indicates that the HBV polymerase is as sensitive to FLG-TP as DHBV polymerase.

The results obtained here and earlier published data show a potential use of FLG as an anti-HBV treatment. However, like for many other nucleoside analogs a regain of virus production is found when treatment is withdrawn, both in vivo (Löfgren et al., 1996) and in vitro (this study). Future studies with this substance could include different combination therapies in order to achieve a more effective inhibition and also to decrease the risk of resistance development. This can be tested utilising the various in vitro systems and in vivo trials in ducks. Combination of FLG treatment and immune modulating therapy should also be explored.

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