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The in vitro anti-hepatitis B virus activity of FIAU [1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-iodo)uracil] is selective, reversible, and determined, at least in part, by the host cell

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Summary

A human hepatoblastoma cell line was stably transfected with a head-to-tail dimer of the Hepatitis B virus (HBV), subtype *adw*, genome to generate a cell line which produces HBV. FIAU [1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-iodo)uracil] inhibited viral replication in these cells with an IC_{50} of 0.90 μM, as determined by PCR analysis of extracellular Dane particle DNA, and displayed a 50% cytotoxic concentration (TC_{50}) of 344.3 μM, as determined using the MTT assay. The selectivity index of FIAU (TC_{50}/IC_{50}) was 382.6. In cells incubated for 10 days with FIAU (100 μM) and then incubated with drug-free media with daily media changes for 7 days, viral DNA replication was markedly inhibited but resumed within 24 h after drug removal, demonstrating that the in vitro anti-HBV activity of FIAU is reversible. Both the antiviral activity and cytotoxicity of FIAU were reversed by the addition of equimolar to 10-fold excess molar concentrations of thymidine. The de-iodinated metabolite of FIAU, FAU, had only marginal anti-HBV activity at 100 μM, indicating that this metabolite does not contribute significantly to the activity of FIAU. The examination of intracellular viral DNA replicative intermediates revealed that FIAU was 2000-fold more active against duck HBV DNA replication in human hepatoma cells (IC_{50} = 0.075 μM) than against this same virus in chicken liver cells (IC_{50} = 156 μM). FIAU was anabolized to a 25-fold greater extent in human hepatoma cells than in chicken cells, indicating that the anti-HBV activity of this nucleoside analog is dependent, in part, on its phosphorylation by the host cell.

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Hepatitis B virus; FIAU; Nucleoside analog; Antiviral agent; Polymerase chain reaction

Introduction

Human hepatitis B virus (HBV) is a small, partially double stranded, DNA virus belonging to the *hepadenaviridae*, which includes woodchuck hepatitis virus (Summers et al., 1987), ground squirrel hepatitis virus (Marion, et al., 1980; Seeger et al., 1984) and duck hepatitis B virus (DHBV; Mason et al., 1980). These viruses have the unique feature of replicating their DNA genome by reverse transcription of an RNA intermediate (Summers and Mason, 1982). HBV is a causative agent of both acute and chronic hepatitis (Hoofnagle et al., 1987) and is causally related to the development of hepatocellular carcinoma (Gerber et al., 1983). Despite the development of vaccines, which effectively eliminate infection by HBV (Purcell and Gerin, 1978), hepatitis B virus infections remain a world-wide health problem, infecting approximately 5% of the world's population (Gerin, 1991; Szmuness, 1978).

The development of useful therapies for treating HBV infections has been hindered due to the lack of an in vitro tissue culture system which allows the direct isolation and propagation of hepatitis B virus. Recently, major advances have been made in this area, whereby cloned HBV DNA co-transfected with a selectable marker (antibiotic resistance) into established cell lines enables the constitutive production of both replication intermediates and infectious virus (Sureau et al., 1986; Tsurimoto et al., 1987; Yaginuma et al., 1987; 1988; Sells et al., 1987, 1988; Acs et al., 1987). A cell line designated 2.2.15, derived by co-transfection of HBV genomes into human hepatoblastoma HepG2 cells, continuously produces mature Dane particles (virions), replicative DNA intermediates, and large amounts of all structural viral polypeptides (Sells et al., 1987). Cell lines which constitutively replicate HBV are useful for the demonstration of the inhibition of HBV DNA replication by potential anti-HBV therapeutic agents (Ueda et al., 1989; Matthes et al., 1990; Korba and Milman, 1991; Lampertico et al., 1991; Korba and Gerin, 1992).

Several classes of nucleoside analogs have shown promise as selective inhibitors of HBV replication. Some examples include 2′,3′-dideoxycytidine (Kassianides et al., 1989; Ueda et al., 1989; Yokota et al., 1991), the 2′,3′-dideoxy-3′-halogenated pyrimidine nucleosides (Matthes et al., 1990; Matthes et al., 1992), the diaminopurine nucleosides, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine and 9-(phosphonylmethoxyethyl)adenine (Yokota et al., 1991), carbocyclic nucleosides such as the carbocyclic analog of 2′-deoxyguanosine (Price et al., 1989), and the oxathiolane nucleosides such as 2′,3′-dideoxy-3′-thiacytidine (3TC; Doong et al., 1991) and 5-fluoro-2′,3′-thiacytidine (FTC; Furman et al., 1992).

The 2′-fluoro-substituted arabinosylpyrimidine nucleosides, have been

shown to possess potent in vitro and in vivo activity against medically important herpes viruses (Lopez et al., 1980; Colacino and Lopez, 1983; Lin et al., 1983; Chiou and Cheng, 1985; Schinazi et al., 1986; Chou et al., 1987; Fox et al., 1988). FIAC [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine] and FIAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil] are preferentially phosphorylated in herpes simplex type 1 infected cells via the virus encoded thymidine kinase (Kreis et al., 1982; Colacino and Lopez, 1983). Consequently, these compounds are more effective as inhibitors of herpes viruses which are TK^+ than against viruses which are deficient in TK activity (Lopez et al., 1980). Furthermore, resistance to FIAC is correlated by an impaired ability of the virus to phosphorylate this nucleoside analog (Colacino et al., 1986).

The analogs, FIAC, FMAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil] and FEAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil] have been shown to inhibit woodchuck hepatitis virus replication in the chronically infected woodchuck (Fourel et al., 1990). To a lesser extent, FMAU and FIAC demonstrated activity against DHBV in chronically infected ducks (Fourel et al., 1992). Recently, FIAU has been shown to decrease the levels of HBV DNA in patients with chronic hepatitis B (Fried et al., 1992). The mechanism of action underlying the anti-HBV activity of these compounds is not well characterized, since HBV does not appear to encode a pyrimidine nucleoside kinase. However, the triphosphate of FIAC has been shown to inhibit endogenous HBV DNA polymerase activity (Hantz et al., 1984; Fourel et al., 1987). This observation suggests that the anti-HBV activity of this class of compounds may be mediated, in part, at the level of viral DNA polymerase.

In this report we describe the tissue culture activity of FIAU using a PCR-based assay to monitor the replication of human hepatitis B virus. Also, we show that the differential activity of this nucleoside analog against duck hepatitis B virus in human hepatoma cells versus the same virus in chicken liver cells can be explained, in part, by an increased phosphorylation of FIAU in the human cells. Additionally, we show that thymidine is able to reverse the anti-HBV activity and cytotoxicity of FIAU.

Materials and Methods

Plasmid construction

Plasmids were constructed using standard recombinant DNA techniques (Sambrook et al., 1989). Plasmid pTHBV (obtained from C.-H. Lee, Indiana University School of Medicine, Indianapolis, IN) contains a head-to-tail dimer of the human hepatitis B virus genome, subtype adw, cloned into the *Eco*RI site of pUC9. Plasmid pTHBVneo was constructed by first excising the neomycin resistance gene under the control of the SV40 early promoter with *Bam*HI from LTR-CAT-neo (obtained from S. Richard Jaskunas, Lilly Research Laboratories, Indianapolis, IN) and subcloned into the *Sal*I site of pTHBV after a

two-base fill-in reaction with Klenow enzyme.

Cells and transfections

HepG2 cells (human hepatoblastoma) were obtained from the American Type Tissue Collection (HB 8065) and were routinely passaged in growth medium consisting of Earle's minimal essential media supplemented with sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hep10A cells were derived from HepG2 cells. Briefly, HepG2 cells were seeded into 60-mm culture dishes in growth medium, and when approximately 50% confluent, were transfected with pTHBVneo using the calcium phosphate precipitation method of Graham and van der Eb (1973). 4 h post-transfection, the cells were shocked with 10% DMSO and 20% glucose for 5 min, after which they were allowed to recover for 24 h. The transfected cells were passaged and reseeded in the presence of growth medium containing 800 μg/ml geneticin (Sigma Chemical Co., St. Louis, MO). The cells were monitored for the appearance of geneticin-resistant cells. A geneticin-resistant cell line which produces HBV was identified by PCR analysis of cell culture fluid and routinely passaged in growth medium containing 400 μg/ml geneticin. This cell line will be referred to as Hep10A.

Hep10A cells and HepG2/G3, a human hepatoma cell line which constitutively replicates duck hepatitis B virus (Galle et al., 1988; obtained from W.S. Mason, Fox Chase Cancer Center, Philadelphia, PA) were routinely passaged in growth medium consisting of Earle's minimal essential media supplemented with sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml geneticin (Sigma). D2 cells, a chicken liver cell line which constitutively replicates duck hepatitis B virus (Condreay et al., 1990) were obtained from W.S. Mason and were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium/F-12 Nutrient Mixture (50:50) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml geneticin (Sigma).

Antiviral compounds

FIAU was obtained from Oclassen Pharmaceuticals, Inc., San Rafael, CA. [2-^{14}C]FIAU (53.3 μCi/mg, 99.4% radiochemical purity) was prepared by Amersham Corporation, Arlington Heights, IL from FIAU supplied by us. Bromovinyldeoxyuridine (BVdU), the monophosphate of adenine arabinoside (vidarabine monophosphate; ara-AMP), and acyclovir (9-(2-hydroxyethoxymethyl)guanine; ACV) were obtained from Sigma.

Synthesis of FAU

The de-iodinated metabolite of FIAU [1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)uracil](FAU) was synthesized for evaluation of its anti-HBV activity. To eliminate the possibility that trace quantities of active FIAU would influence the biological assay results, FAU was prepared by coupling bis(trimethylsilyl)uracil and 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofur-

anosyl bromide (Tann et al., 1985) in dichloroethane at 70°C. The β-anomer was purified by recrystallization (EtoAc) and deprotected with sodium methoxide in methanol to give FAU. The compound displayed identical physical properties to those described previously (Sterzycki et al., 1990).

Polymerase chain reaction (PCR) analysis

A 10 μl aliquot of cell culture supernatant was spotted onto a Whatman GF/A filter and boiled in 50 μl of water for 10 min. A 10 μl sample of this was then used as template for PCR amplification. PCR analysis was carried out using the thermostable DNA polymerase from *Thermus aquaticus* (Perkin Elmer Cetus, Norwalk, CT). Oligonucleotides were synthesized on an Applied Biosystems 391 PCR-Mate DNA synthesizer. The oligonucleotide primers used in the PCR reaction were derived from Kaneko et al. (1989), and are as follows:

S238L: 5′-ATACCACAGAGTCTAGACTCGTGGTGGACT-3′
S714R: 5′-AAGCCCTACGAACCACTGAACAAATGGCAC-3′

PCR reactions were carried out in a total volume of 50 μl containing 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 0.001% gelatin; 200 μM each of dATP, dCTP, dGTP, dTTP; 1.0 μM primers; 10 μl template, and 1.25 units of *Taq* polymerase. Samples were subjected to 30 cycles of amplification in the GeneAmp™ PCR System 9600 as follows:

Cycle 1–29: 15 s at 94°C, 15 s at 60°C, 30 s at 72°C
Cycle 30: 15 s at 94°C, 15 s at 60°C, 2 min at 72°C
End: Storage at 4°C until used

The 477 base-pair PCR amplified product was electrophoresed through 1.2% agarose, stained with ethidium bromide, and visualized under ultraviolet illumination.

In vitro antiviral treatments and Southern blot analysis

Hep10A cells were seeded into 96-well microtiter plates at a density of approximately 50000 cells per well, allowed to grow to confluence, and maintained at confluence for 2–4 days to allow HBV DNA levels to stabilize. Similar to 2.2.15 cells (Sells et al., 1987; Korba and Milman, 1991), virus production in this cell line increases after the cells have reached confluence (data not shown). At this time, cells were treated with FIAU, FAU, acyclovir, BVdU, or ara-AMP for 10 days, with daily changes of media containing fresh drug. On day 10, culture supernatant samples were collected and stored at 4°C until needed. PCR analysis was performed on supernatant samples as described above. Following electrophoresis through agarose gels, the DNA was transferred to nylon membranes (Optiblot, IBI, New Haven, CT) by capillary action (Southern, 1975) using 1× SSC (1× SSC = 0.15 M NaCl, 0.015 M

sodium citrate). After baking in vacuo for 30 min, the blots were hybridized for 15–18 h to 1 × 10^6 cpm/ml of ^{32}P-labeled HBV specific probes. Hybridization probes were labeled by the method of random priming (Feinberg and Vogelstein, 1983). Hybridization was carried out at 42°C using 50% formamide, 6× SSPE, 10× Denhardt's solution, 0.5% SDS, and 50 μg/ml salmon sperm DNA (1× SSPE = 0.18 M NaCl; 10 mM NaH_2PO_4, pH 7.4; 1 mM EDTA; 1× Denhardt's solution = 0.02% Ficoll; 0.02% PVP-360; 0.02% BSA). The hybridized blots were washed as follows: twice in 2× SSC/0.1% SDS at room temperature for 1 h, once in 0.1× SSC/0.1% SDS at room temperature for 30 min, and once in 0.1× SSC/0.1% SDS at 65°C for 15 min. The filters were exposed to X-ray film and quantified by laser densitometry.

Cytotoxicity assay

Hep10A cells were seeded into 96-well microtiter plates at a concentration of approximately 12–15 000 cells per well and incubated for 24 h at 37°C. At this time, cells were treated with FIAU for 5 days with daily changes of media containing fresh drug. On day 5, the FIAU containing media was aspirated, 100 μl of growth media containing 500 μg/ml [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Sigma) was added, and the cells were incubated at 37 °C for 3–4 h (Mosmann, 1983). The formazan product was solubilized and the cells lysed by the addition of 100 μl of acidified DMSO containing 1% Triton X-100. Cell viability was quantified by reading the plates in a Dynatech MR7000 plate reader at 550 nm.

Intact cell electrophoresis (ICE)

Intact duck hepatitis B virus (DHBV) producing cells were lysed, digested, and electrophoresed in situ following the protocol of Gardella et al. (1984) with modifications. A two-part gel was used for electrophoresis. The first part of the gel consisted of 0.75% agarose which was poured into a gel tray without a well-forming comb. The gel was allowed to solidify and a 5-cm section was cut out. The well-forming comb was placed into the gel tray and 0.8% agarose containing 1% SDS and 100 mg of proteinase K per ml was poured into the remaining area of the trough, forming the top part of the gel. The gel was kept at room temperature until solid, after which time the comb was carefully removed.

DHBV-producing cells were prepared by washing the monolayer with phosphate buffered saline (PBS) and trypsinizing using 0.25% trypsin. The cells were collected by centrifugation, resuspended in PBS, and counted using a Coulter counter. Between 100 000 and 200 000 cells were centrifuged in a microcentrifuge for 3 min at room temperature. The cells were then resuspended in 20 μl of 15% Ficoll in electrophoresis buffer and added directly to a well of the two-part gel described above. A gel-loading/lysis buffer, consisting of bromophenol blue, xylene cyanol, 15% Ficoll, 1% SDS, and 1 mg of proteinase K per ml, was carefully layered on top of the cells. Electrophoresis (3 h at 0.8 V/cm and 24 h at 4.5 V/cm) at room temperature

was started immediately to avoid SDS diffusion. After electrophoresis, the gel was washed three times for 30 min in 500 ml of 10 mM sodium phosphate (pH 7.0) with gentle rocking. The gel was then stained with ethidium bromide (1 μg/ml) prior to photography, blotting, and hybridization.

Using the above electrophoretic system, the virus-producing cells are lysed and protein is digested away from the released intracellular DNA within the agarose gel (Gardella et al., 1984). High-molecular-weight chromosomal DNA remains in the top portion of the gel while DHBV DNA replicative intermediates are resolved in the bottom portion of the gel. Viral DNA replicative intermediates are detectable by Southern blot analysis using DHBV-specific probes.

Anabolism of FIAU

Chicken liver cells producing DHBV (D2 cells) and human hepatoma cells producing the same virus (HepG2/G3 cells) were evaluated for their ability to phosphorylate FIAU. Cells were seeded into 24-well tissue culture plates at an initial density of 300 000 cells per well and allowed to incubate overnight. The media was removed and replaced with fresh media containing 2 μg of [^{14}C]FIAU (53.3 μCi/mg) per ml. At 0, 2, 6, 24, 48, and 72 h, the cells were washed three times with PBS, and once with 0.25% trypsin and incubated for 30 min at 37°C after which time the cells were dispersed in 1 ml of media and counted using a Coulter counter. The cells were then centrifuged and the pellet was washed once more with PBS, centrifuged, and lysed by resuspending them in 400 μl of distilled water and keeping on ice for at least 5 min. Extraction of nucleotides from cell lysates was done with perchloric acid (PCA; Kreis et al., 1977; Uchida and Kreis, 1969). PCA-soluble extracts of these cells were prepared as previously described (Colacino et al., 1993). PCA (600 μl of 7 N) was added to the cells in distilled water and the lysates were kept on ice for at least 5 min. The PCA insoluble material was removed by centrifugation, resuspended in 500 μl of distilled water and evaluated for radioactive content by liquid scintillation counting using 10 ml of Aquassure (NEN Dupont). KOH (80 μl of 10 N) was added to the PCA-soluble fraction which was then kept on ice for at least 5 min. The insoluble potassium perchlorate was removed by centrifugation and 500 μl of the soluble extract was assayed for radioactivity by liquid scintillation counting using 10 ml of Aquassure (NEN Dupont). Counts per min in the PCA-soluble fraction were converted to picomoles of FIAU phosphorylated per 1×10^6 cells at the indicated times of incubation.

Results

FIAU selectively inhibits the replication of HBV in human hepatoma cells

A representative experiment in which HBV-producing human hepatoma cells were incubated in various concentrations of FIAU and the cell supernatant medium was examined for the presence of HBV virion DNA by

PCR analysis, as described in Materials and Methods, is shown in Fig. 1. Between the concentrations of 0.01 and 10 μg of FIAU per ml, a concentration-dependent inhibition of HBV replication was observed. FIAU inhibited the replication of HBV in these cells with a 50% inhibitory concentration (IC_{50}) of 0.90 μM and a 50% toxic concentration (TC_{50}) of 382.6 μM, as determined by linear regression analysis. The selective index (TC_{50}/IC_{50}) was 382.6.

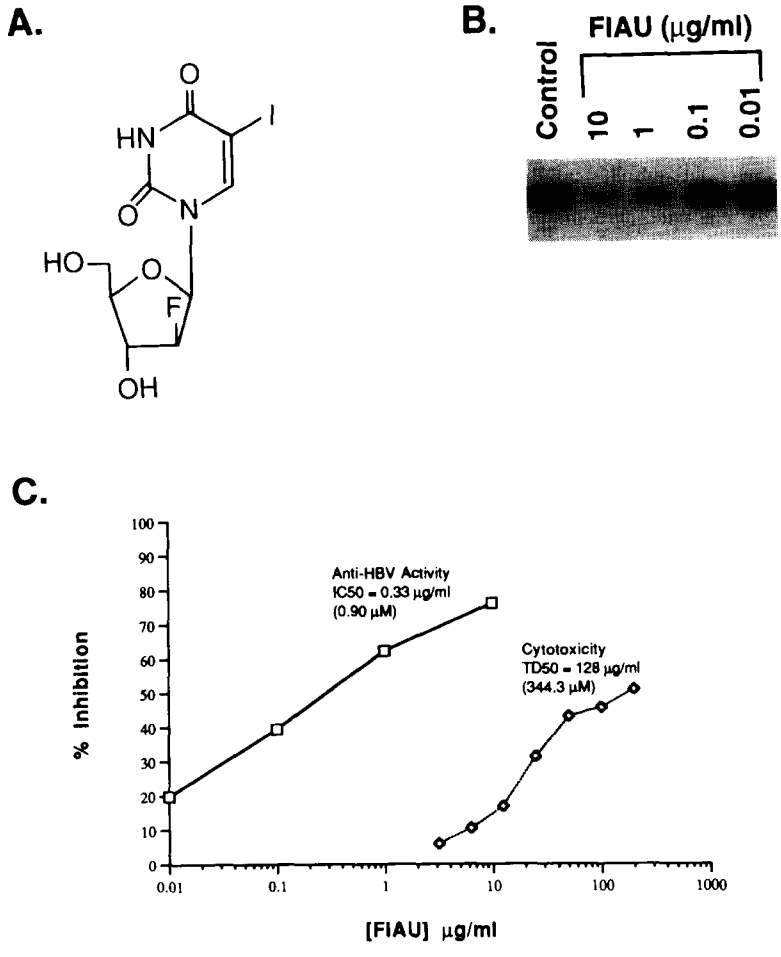

Fig. 1. Antiviral activity and toxicity of FIAU. (A) Structure of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU). (B) PCR and Southern blot analysis of Hep10A culture supernatants following 10 days of treatment with FIAU as described in the Materials and Methods. A reduction in the level of the expected 477 bp PCR product is indicative of a decline in HBV replication. (C) The autoradiogram in B was scanned by laser densitometry, the percent inhibition of PCR product formation calculated and plotted versus drug concentration. For comparison, FIAU induced toxicity to cycling Hep10A cells as determined by MTT assay is also shown in C. The IC_{50} and TD_{50} values were determined by linear regression analysis. By dividing the TD_{50} by the IC_{50}, a selective index of 382.6 was obtained.

FIAU is active against DHBV in human cells but not in chicken cells

The ability of FIAU to inhibit the formation of DHBV relaxed circular (RC) DNA intermediates in chicken liver (D2) and in human hepatoma cells (HepG2/G3) was evaluated by Southern blot analysis of intracellular DNA electrophoresed as described in Materials and Methods. In HepG2/G3 cells, FIAU was potent against DHBV replication with an IC_{50} of 0.028 μg/ml (0.075 μM) (Fig. 2). In contrast, FIAU was relatively inactive against DHBV

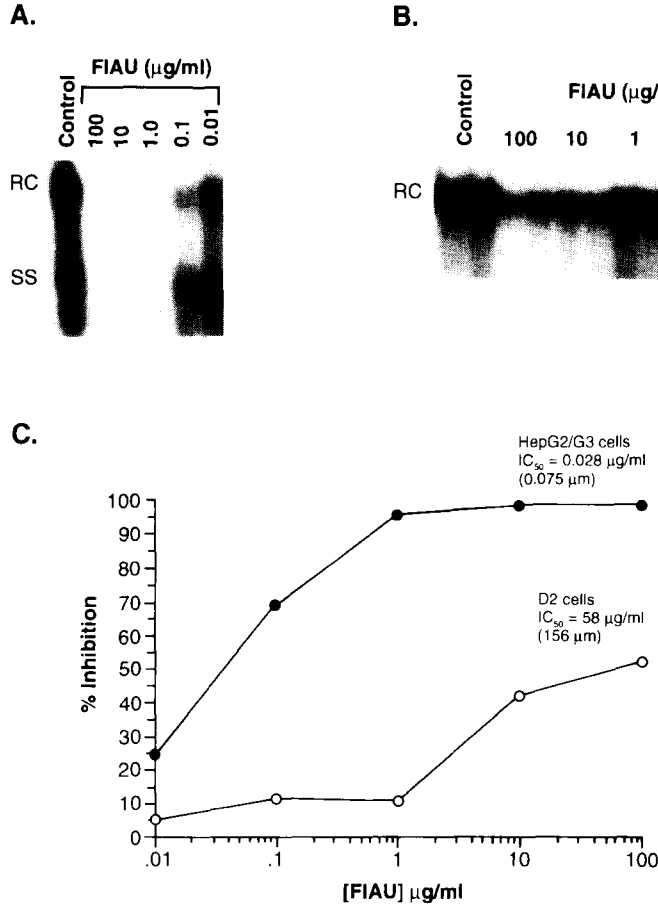

Fig. 2. Anti-DHBV activity of FIAU in HepG2/G3 and D2 cells. The DHBV-producing cells, HepG2/G3 (human) and D2 (chicken), were seeded into 24-well plates and allowed to grow to confluence. The cells were then incubated for 8 days without or with the indicated concentrations of FIAU with daily changes of medium. Equivalent numbers of cells were analyzed by intact cell electrophoresis (ICE) as described in Materials and Methods. Southern blot analysis was conducted using a DHBV-specific DNA probe labeled with ^{32}P by random priming according to standard protocols. (A) ICE and Southern blot analysis of HepG2/G3 cells (100 000 cells per lane). (B) ICE and Southern blot analysis of D2 cells (200 000 cells per lane). (C) The autoradiograms in A and B were scanned by laser densitometry, the percent inhibition calculated and plotted versus FIAU concentration.

replication in D2 cells with an IC_{50} of approximately 58 μg/ml (156 μM), i.e., FIAU was 2000-fold less active in chicken cells than in human cells. These data indicate that the anti-HBV activity of FIAU is mediated, in part, by host-cell activation (most likely phosphorylation to the mononucleotide form). Therefore, it was of interest to study the anabolism of FIAU in HepG2/G3 versus D2 cells.

Anabolism in HepG2/G3 cells and in D2 cells

PCA-soluble fractions of HepG2/G3 and D2 cells which were incubated in [^{14}C]FIAU were prepared for the quantification of phosphorylated cellular anabolites of FIAU. As shown in Fig. 3, phosphorylation of FIAU in HepG2/G3 cells over background levels was detectable at 24 h of incubation and increased with time of incubation reaching a level of approximately 160 pmol of FIAU phosphorylated per 1×10^6 cells at 72 h. In contrast, FIAU was phosphorylated to a much lesser extent in D2 cells and at 72 h was approximately 6.3 pmol per 1×10^6 cells. Phosphorylation of FIAU in D2 cells was detectable but, on a per cell basis, did not increase with time of incubation. Radioactivity was not present at a level greater than background in PCA insoluble fractions of either D2 or HepG2/G3 cells (data not shown).

The cytotoxicity and anti-HBV activity of FIAU are reversed by thymidine

Subconfluent Hep10A cells were treated with 50 μg of FIAU per ml and/or deoxycytidine or thymidine at 0-, 0.1-, 1.0-, or 10-fold molar concentrations and cell viability was determined using the MTT assay as described in Materials and Methods. Thymidine reversed the cytotoxicity of FIAU in a dose-dependent manner; at an equimolar concentration of thymidine, cytotoxicity

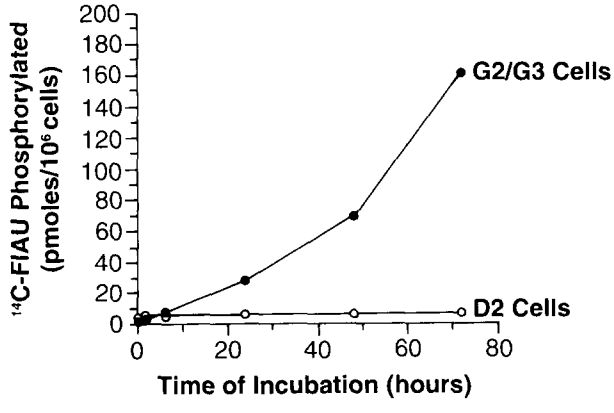

Fig. 3. Anabolism of FIAU. HepG2/G3 or D2 cells were seeded in 24-well plates at an initial density of 300 000 cells and incubated for 24 h. The media was removed and replaced with fresh media containing [^{14}C]FIAU (2 μg/ml, 53.3 μCi/mg). At the indicated times of incubation, cells were counted and PCA-soluble fractions were prepared from duplicate cultures as described in Materials and Methods and assayed for radioactivity representing pmol of phosphorylated FIAU.

was reduced by approximately 70% (Fig. 4A). With a 10-fold excess of thymidine (1.34 mM), FIAU displayed a greater cytotoxicity than with an equimolar amount of the natural nucleoside. This may be attributable to the relative insolubility of thymidine at high concentrations in aqueous media. In contrast to thymidine, an equimolar concentration of deoxycytidine reversed the cytotoxicity of FIAU by less than 10% (data not shown).

HBV-producing human hepatoma cells were incubated in 10 μg of FIAU per ml (26.9 μM) and a 0-, 0.1-, 1-, or 10-fold molar concentration of thymidine or deoxycytidine (Fig. 4B). Thymidine reversed the anti-HBV activity of FIAU in a concentration-dependent manner. At a 10-fold molar excess of thymidine, the anti-HBV activity of FIAU was reversed by approximately 70%. Deoxycytidine was also able to reverse the anti-HBV activity of FIAU, but at a 10-fold molar excess, it was much less effective than thymidine (data not shown). This observation indicates that the anti-HBV activity of FIAU is due, at least in part, to its ability to act as an analog of thymidine.

The antiviral activity of FIAU is reversible in culture

To test whether the antiviral activity of FIAU is irreversible in culture, HBV-producing cells were incubated with FIAU (100 μM) for 10 days with daily changes of medium. On day 10, an aliquot of the cell supernatant was obtained to determine antiviral effect by PCR analysis. The media containing FIAU was then removed and replaced with fresh medium containing no FIAU. Prior to each daily change of cell culture medium, an aliquot of culture fluid was taken

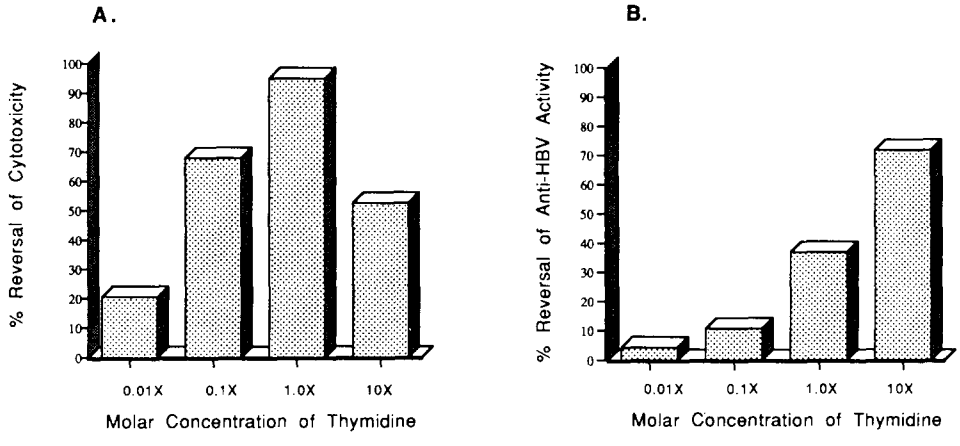

Fig. 4. (A) Reversal of FIAU-induced toxicity. Subconfluent Hep10A cells were treated with 50 μg/ml (134.5 μM) FIAU and thymidine at the indicated molar concentrations for 5 days. Cell growth was then monitored by MTT assay as described in Materials and Methods. The percent inhibition of cellular replication at each FIAU and thymidine concentration was determined. (B) Reversal of Anti-HBV activity of FIAU. Hep10A cells were treated with 10 μg/ml (26.9 μM) FIAU and thymidine at the indicated molar concentration for 10 days. Following PCR and Southern blot analysis of cell culture supernatants and laser densitometry of the autoradiogram as described in Materials and Methods, the percent inhibition of HBV replication at each FIAU and thymidine concentration was determined.

for PCR analysis of released virion DNA. For comparative purposes, the same experiment was done with acyclovir (500 μM) or ara-AMP (vidarabine monophosphate; 100 μM). At a concentration of 100 μM FIAU, inhibition of HBV replication was nearly complete after 10 days of incubation. However, the resumption of viral DNA replication could be detected 24 h after the removal of FIAU and by 4 days after the removal of compound, viral replication was equivalent to that in cells incubated for 10–17 days in medium alone. Similar results were obtained for acyclovir (500 μM) and ara-AMP (100 μM).

The antiviral activity of FAU

The principal metabolite of FIAU identified in plasma and urine of mice, rats, dogs, and man is FAU (Feinberg et al., 1985; Philips et al., 1983). Therefore, it was of interest to determine whether FAU itself had anti-HBV activity. This compound was synthesized by a route which rigorously excludes any FIAU and the absence of FIAU was confirmed by assay using HPLC (data not shown). The antiviral activity of FAU was evaluated as described for FIAU using concentrations of 100, 10, 1, and 0.1 μM. At 100 μM, FAU displayed a slight but reproducible inhibition of HBV replication. No inhibition was observed at the lesser concentrations.

Discussion

FIAU was originally developed for the treatment of infections caused by medically important herpesviruses (Lopez et al., 1980; Colacino and Lopez, 1983; Chiou and Cheng, 1985; Schinazi et al., 1986; Chou et al., 1987; Fox et al., 1988). This nucleoside analog is more active against herpes simplex viruses encoding an active thymidine kinase than against viruses deficient in thymidine kinase (Lopez et al., 1980). Furthermore, FIAU is phosphorylated efficiently by herpes simplex virus thymidine kinase (Colacino and Lopez, 1983).

Recently, FIAC, FMAU, and FEAU have shown in vivo anti-HBV activity in the woodchuck (Fourel et al., 1990) and duck (Fourel et al., 1992) models of hepatitis B virus infection. FIAU has demonstrated activity against hepatitis B virus replication in vitro and in HBV-infected patients (Oclassen Pharmaceuticals, Inc., San Rafael, CA, data on file). FIAC and FIAU have been shown to inhibit HBV replication in 2.2.15 cells by 90% at concentrations of 34 $\pm$ 7 μM and 24 $\pm$ 4 μM, respectively, as determined by blot hybridization (Korba and Gerin, 1992). In this report, FIAU was shown to be a selective inhibitor of HBV DNA replication in vitro, using a PCR-based assay. In an HBV-producing hepatoblastoma cell line, FIAU inhibited the replication of HBV by 50% (IC_{50}) at a concentration of 0.9 μM and displayed a 50% cytotoxic concentration (TC_{50}) of 344.3 μM, yielding a selective index (TC_{50}/IC_{50}) of 382.6. However, we have observed that the TC_{50} of FIAU decreases with decreasing initial cell plating density (data not shown). This observation is consistent with the notion that FIAU and similar nucleoside analogs are more

toxic to rapidly dividing cells. Furthermore, in this report, the evaluation of FIAU toxicitiy was limited to hepatoma cells and the results obtained here may not be representative of the toxicity of FIAU in normal or HBV-infected hepatocytes.

The anti-HBV activity of FIAU (10 μM) was reversed 62% by a 10-fold molar excess of thymidine and the cytotoxicity of FIAU was reversed 70% by an equimolar amount of thymidine, suggesting that FIAU behaves as an analog of thymidine. FIAU was 2000-fold more active against duck hepatitis B virus in human hepatoblastoma cells than against this virus in chicken liver cells. This observation indicates that the anti-HBV activity of FIAU is determined, at least in part, by the host cell. Indeed, our results show that the anti-HBV activity of FIAU is dependent, in part, on its phosphorylation by the host cell. Phosphorylation of FIAU was time-dependent and was 25-fold greater in extent in human cells than in chicken cells. These results do not preclude the possibilities that FIAU is inefficiently transported into chicken liver cells or that phosphorylated anabolites of FIAU are not able to accumulate in these cells. Non-hepatic mouse L cells which are deficient in thymidine kinase (LTK^-) have been transfected with a dimer of hepatitis B virus and cloned LTK^- cells which secrete HBsAg, HBeAg, and HBV DNA have been described (Seifer et al., 1990). It would be of interest to extend the results reported here by determining whether FIAU is able to inhibit the replication of HBV in LTK^- cells.

In culture, the resumption of viral DNA replication could be detected 24 h after the removal of FIAU (100 μM) and by 4 days after the removal of compound, viral replication was equivalent to that in cells incubated for 10 to 17 days in medium alone. Similar results were obtained for vidarabine monophosphate. These results must be interpreted carefully, however, since unlike an in vitro infection where virus is added to an uninfected culture and not every cell is infected, stably transfected cells contain integrated copies of the HBV genome which are passed to daughter cells during cell replication. Therefore it would be necessary to clear each cell of the integrated viral DNA to achieve a permanent in vitro antiviral effect. A nucleoside analog such as FIAU would not be expected to act in this manner.

The principal metabolite of FIAU identified in plasma and urine of mice, rats, dogs, and man is FAU (Feinberg et al., 1985; Philips et al., 1983). This compound was synthesized, checked for purity by HPLC, and evaluated for activity against hepatitis B virus. At 100 μM, FAU displayed a slight but reproducible inhibition of HBV replication. No inhibition of HBV replication was observed at the lesser concentrations of FAU. FAU may not be able to enter cells as efficiently as FIAU or, alternatively, FAU may be a poor substrate for cellular pyrimidine nucleoside kinase. Furthermore, the possibility exists that the triphosphate of FAU, generated subsequent to the reductive de-iodination of FIAU-monophosphate by thymidylate synthetase (Garrett et al., 1979; Braun et al., 1982) accounts for all or part of the anti-HBV activity of FIAU.

The mechanism of action underlying the selective anti-HBV activity of FIAU is not completely understood, since HBV does not appear to encode a pyrimidine nucleoside kinase. The antiviral and cytotoxic activities of FIAU could be reversed by the addition of exogenous thymidine, indicating that FIAU acts as a thymidine analog against HBV replication. The triphosphate of FIAC, the aminated metabolic precursor of FIAU, has been synthesized and has been shown to inhibit endogenous HBV DNA polymerase activity (Hantz et al., 1984). Considered together with the results described here, these observations indicate that the anti-HBV activity of FIAU may be the result of its intracellular anabolism, as a thymidine analog, to the mono-, di- and triphosphate nucleotides. Accordingly, experiments are underway to assess the ability of FIAU-triphosphate to inhibit endogenous HBV DNA polymerase.

References

Acs, G., Sells, M.A., Purcell, R.H., Price, P., Engle, R., Shapiro, M. and Popper, H. (1987) Hepatitis B virus produced by transfected Hep G2 cells causes hepatitis in chimpanzees. Proc. Natl. Acad. Sci. USA 84, 4641–4644.

Braun, T.J., Feinberg, A., Chou, T.-C., Watanabe, K.A., Matsuda, A., Fox, J.J., and Philips, F.S. (1982) Proc. Am. Assoc. Cancer Res. 23, 839.

Chiou, J.-F. and Cheng, Y.-C. (1985) Interaction of Epstein-Barr virus DNA polymerase and 5'-triphosphates of several antiviral nucleoside analogs. Antimicrob. Agents Chemother. 27, 416–418.

Chou, T.-C., Kong, X.-B., Fanucchi, M.P., Cheng, Y.-C., Takahashi, K., Watanabe, K.A. and Fox, J.J. (1987) Synthesis and biological effects of 2'-fluoro-5-ethyl-1-β-D-arabinofuranosyluracil. Antimicrob. Agents Chemother. 31, 1355–1358.

Colacino, J. and Lopez, C. (1983) Efficacy and selectivity of some nucleoside analogs as anti-human cytomegalovirus agents. Antimicrob. Agents Chemother. 24, 505–508.

Colacino, J.M., Brownridge, E., Greenberg, N. and Lopez, C. (1986) Enzymology and pathogenicity in mice of herpes simplex virus type 1 mutant resistant to 2'-fluoro-2'-deoxy-1-β-d-arabinofuranosyl-5-iodocytosine. Antimicrob. Agents Chemother. 29, 877–882.

Colacino, J.M., Birch, G.M. and Tang, C.J. (1993) Cellular metabolism and anti-influenza activity of 1,3,4-thiadiazol-2-ylcyanamide (LY217896). Antiviral Chem. Chemother., in press.

Condreay, L., Aldrich, C.E., Coates, L., Mason, W.S. and Wu, T.-T. (1990) Efficient duck hepatitis B virus production by an avian liver tumor cell line. J. Virol. 64, 3249–3258.

Doong, S.-L., Tsai, C.- H., Schinazi, R.F., Liotta, D.C. and Cheng, Y.- C. (1991) Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy-3'-thiacytidine and related compounds. Proc. Natl. Acad. Sci. USA 88, 8495–8499.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132,6–13.

Feinberg, A., Leyland-Jones, B., Fanucchi, M.P., Hancock, C., Fox, J.J., Watanabe, K.A., Vidal, P.M., Young, C.W. and Philips, F.S. (1985) Biotransformation and elimination of [2-^{14}C]-1-(2-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine in immunosuppressed patients with herpesvirus infections. Antimicrob. Agents Chemother. 27, 733–738.

Fourel, I., Hantz, O., Cova, L., Allaudeen, H.S. and Trepo, C. (1987) Main properties of duck hepatitis B virus DNA polymerase: comparison with the human and woodchuck hepatitis B virus DNA polymerases. Antiviral Res. 81, 89–199.

Fourel, I., Hantz, O., Watanabe, K.A., Jacquet, C., Chomel, B., Fox, J. and Trepo, C. (1990) Inhibitory effects of 2'-fluoro arabinosyl-pyrimidine nucleosides on woodchuck hepatitis virus replication in chronically infected woodchucks. Antimicrob. Agents Chemother. 34, 473–475.

Fourel, I., Li, J., Hantz, O., Jacquet, C., Fox, J.J. and Trepo, C. (1992) Effects of 2'-fluorinated arabinosyl-pyrimidine nucleosides on duck hepatitis B virus DNA level in serum and liver of chronically infected ducks. J. Med. Virol. 37, 122–126.

Fox, J.J., Watanabe, K.A., Chou, T.-C., Schinazi, R.F., Soike, K.F., Fourel, I., Hantz, O. and Trepo, C. (1988) Antiviral activities of 2'-fluorinated arabinosyl-pyrimidine nucleosides. In: Taylor, F.N. (Ed.), Fluorinated Carbohydrates: Chemical and Biochemical Aspects, pp. 176–190, American Chemical Society, Washington, DC.

Fried, M.W., Di Bisceglie, A.M., Straus, S.E., Savarese, B., Beames, M.P. and Hoofnagle, J.H. (1992) Hepatology 16, 127A.

Furman, P.A., Davis, M., Liotta, D.C., Paff, M., Frick, L.W., Nelson, D.J., Dornsife, R.E., Wurster, J.A., Wilson, L.J., Fyfe, J.A., Tuttle, J.V., Miller, W.H., Condreay, L., Averett, D.R., Schinazi, R.F. and Painter, G.R. (1992) The anti-hepatitis B virus activities, cytotoxicities and anabolic profiles of the (−) and (+) enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. 36, 2686–2692.

Galle, P.R., Schlicht, H.J., Fischer, M. and Schaller, H. (1988) Production of infectious duck hepatitis B virus in a human hepatoma cell line. J. Virol. 62, 1736–1740.

Gardella, T., Medveczky, P., Sairenji, T. and Mulder, C. (1984) Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. 50, 248–254.

Garrett, C., Wataya, Y. and Santi, D.V. (1979) Thymidylate synthetase. Catalysis of dehalogenation of 5-bromo- and 5-iodo-2'-deoxyuridylate. Biochemistry 18, 2798–2804.

Gerber, M.A., Thung, S.N. and Popper, H. (1983) Viral hepatitis and hepatocelluar carcinoma. In: F. Deinhard and J. Deinhard (Eds.), Viral Hepatitis, pp. 327–341. Marcel Dekker, Inc., New York.

Gerin, J.L.(1991) Antiviral agents for hepatitis B. Hepatology 14, 198–199.

Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

Hantz, O., Allaudeen, H.S., Ooka, T., De Clercq, E. and Trepo, C. (1984) Inhibition of human and woodchuck hepatitis virus DNA polymerase by the triphosphates of acyclovir, 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine and E-5-(2-bromovinyl)-2'-deoxyuridine. Antiviral Res. 4187–199.

Hoofnagle, J.H., Shafritz, D.A. and Popper, H. (1987) Chronic type B hepatitis and the healthy HBsAg carrier state. Hepatology 7758–763.

Kaneko, S., Feinstone, S.M. and Miller, R.H. (1989) Rapid and sensitive method for the detection of hepatitis B virus DNA using the polymerase chain reaction technique. J. Clini. Microbiol. 27, 1930–1933.

Kassianides, C., Hoofnagle, J.H., Miller, R.H., Doo, E., Ford, H., Broder, S. and Mitsuya, H. (1989) Inhibition of duck hepatitis B virus replication by 2',3'-dideoxycytidine: A potent inhibitor of reverse transcriptase. Gastroenterology 97, 1275–1280.

Korba, B.E. and Gerin, J.L. (1992) Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. Antiviral Res. 19, 55–70.

Korba, B.E. and Milman, G. (1991) A cell culture assay for compounds which inhibit hepatitis B virus replication. Antiviral Res. 15, 217–228.

Kreis, W., Damin, L., Colacino, J. and Lopez, C. (1982) In vitro metabolism of 1-β-D-arabinofuranosylcytosine and 1-β-D-2'-fluoroarabino-5-iodocytosine in normal and herpes simplex type 1 infected cells. Biochem. Pharm. 31, 767–773.

Kreis, W., Gordon, C. and Woodcock, T. (1977) Extraction and analytic procedures for cytosine arabinoside and 1-β-D-arabinofuranosyluracil and their 5'-mono-, di- and tri-phosphates. Cancer Treatm. Rep. 61, 643–646.

Lampertico, P., Malter, J.S. and Gerber, M.A. (1991) Development and application of an in vitro model for screening anti-hepatitis B virus therapeutics. Hepatology 13, 422–426.

Lin, J.-C., Smith, M.C. and Cheng, Y.-C. (1983) Epstein-Barr virus: Inhibition of replication by three new drugs. Science 221, 578–579.

Lopez, C., Watanabe, K.A. and Fox, J.J. (1980) 2'-fluoro-5-iodoaracytosine, a potent and selective anti-herpesvirus agent. Antimicrob. Agents Chemother. 17, 803–806.

Marion, P.L., Oshiro, L.S., Regnery, D.C., Scullard, G.H. and Robinson, W.S. (1980) A virus in Beechey ground squirrels which is related to hepatitis B virus of man. Proc. Natl. Acad. Sci. USA 77, 2941–2945.

Mason, W.S., Seal, G. and Summers, J. (1980) Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. 36, 829–836.

Matthes, E., Langen, P., von Janta-Lipinski, M., Will, H., Schröder, H.C., Merz, H., Weiler, B.E. and Müller, W.E.G. (1990) Potent inhibition of hepatitis B virus production in vitro by modified pyrimidine nucleosides. Antimicrob. Agents Chemother. 34, 1986–1990.

Matthes, E., M. von Janta-Lipinski, Will, H., Schröder, H.C., Merz, H., Steffen, R. and Müller, W.E.G. (1992) Inhibition of hepatitis B virus production by modified 2',3'-dideoxy-thymidine and 2',3'-dideoxy-5-methylcytidine derivatives. Biochem. Pharm. 43, 1571–1577.

Mosmann, T.(1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Philips, F.S., Feinberg, A., Chou, T.-C., Vidal, P.M., Su, T.-L., Watanabe, K.A. and Fox, J.J. (1983) Distribution, metabolism and excretion of 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)thymine and 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine.Cancer Res. 43, 3619–3627.

Price, P.M., Banerjee, R. and Acs, G. (1989) Inhibition of the replication of hepatitis B virus by the carbocyclic analogue of 2'-deoxyguanosine. Proc. Natl. Acad. Sci. USA 86, 8541–8544.

Purcell, R.H. and Gerin, J.L. (1978) Hepatitis B vaccine: on the threshold. Am. J. Clin. Pathol. 70S, 159–169.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Seeger, C., Ganem, D. and Varmus, H.E. (1984) Nucleotide sequence of an infectious molecularly cloned genome of ground squirrel hepatitis virus. J. Virol. 51, 367–375.

Seifer, M., Heermann, K.H. and Gerlich, W.H. (1990) Replication of hepatitis B virus in transfected nonhepatic cells. Virology 179, 300–311.

Sells, M.A., Chen, M.-L. and Acs, G. (1987) Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B DNA. Proc. Natl. Acad. Sci. USA 84, 1005–1009.

Sells, M.A., Zelent, A.Z., Shvartsman, M. and Acs, G. (1988) Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. J. Virol. 62, 2836–2844.

Schinazi, R.F., Fox, J.J., Watanabe, K.A. and Nahmias, A.J. (1986) Activities of 1-(2-deoxy-2-fluoro-Beta-D-arabinofuranosyl)-5-iodocytosine and its metabolites against herpes simplex virus types 1 and 2 in cell culture and in mice intracerebrally with herpes simplex virus type 2. Antimicrob. Agents Chemother. 29, 77–84.

Southern, E.M.(1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Sterzycki, R.Z., Ghazzouli, I., Brankovan, V., Martin, J.C. and Mansuri, M.M. (1990) Synthesis and anti-HIV activity of several 2'-fluoro-containing pyrimidine nucleosides. J. Med. Chem. 33, 2150–2157.

Summers, J. and Mason, W.S. (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29, 403–415.

Summers, J., Smolec, J. and Snyder, R. (1987) A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA 75, 4533–4537.

Sureau, C., Romet-Lemonne, J.-L., Mullins, J.I. and Essex, M. (1986) Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell 47, 37–47.

Szmuness, W. (1978) Hepatocellular carcinoma and the hepatitis B virus: evidence for a causal association. Prog. Med. Virol. 24, 40–69.

Tann, C.H., Brodfuehrer, P.R., Brundidge, S.P., Sapino, C. and Howell, H.G.(1985) Fluorocarbohydrates in synthesis. An efficient synthesis of 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil (β-FIAU) and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)thymine(β-FMAU). J. Org. Chem. 50, 3644–3647.

Tsurimoto, T., Fujiyama, A. and Matsubara, K. (1987) Stable expression and replication of

hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. Proc. Natl. Acad. Sci. USA 84, 444–448.

Uchida, K. and Kreis, W. (1969) Studies on drug resistance. I. Distribution of 1-β-d-arabinofuranosylcytosine, cytidine and deoxycytidine in mice bearing sensitive and resistant P815 neoplasms. Biochem. Pharmacol.18, 1115–1128.

Ueda, K., Tsurimoto, T., Nagahata, T., Chisaka, O. and Matsubara, K. (1989) An in vitro system for screening anti-hepatitis B virus drugs. Virology 169, 213–216.

Yaginuma, K., Shirakata, Y., Kobayashi, M. and Koike, K. (1987) Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. Proc. Natl. Acad. Sci. USA 84, 2678–2682.

Yokota, T., Mochizuki, S., Konno, K., Mori, S., Shigeta, S. and De Clercq, E. (1991) Inhibitory effects of selected antiviral compounds on human hepatitis B virus DNA synthesis. Antimicrob. Agents Chemother. 35, 394–39.