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Inhibitory effect of 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) on human and duck hepatitis B virus infection

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Summary

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) was evaluated for its inhibitory effect on hepadnavirus replication in three different cell systems, i.e., human hepatoma cell lines HepG2 2.2.15 and HB611 (transfected with human hepatitis B virus (HBV)) and primary cultures of duck hepatocytes infected with duck hepatitis B virus (DHBV). PMEA inhibited HBV release from HepG2 2.2.15 cells and HB611 cells at a 50% inhibitory concentration (IC_{50}) of 0.7 and 1.2 μM, respectively. Intracellular viral DNA synthesis was inhibited at concentrations equivalent to those required to inhibit virus release from the cells. DHBV secretion from duck hepatocytes was inhibited by PMEA at an IC_{50} of 0.2 μM. HBsAg secretion was inhibited by PMEA in a concentration-dependent manner in HB611 cells and DHBV-infected duck hepatocytes but not HepG2 2.2.15 cells. The 50% cytotoxic concentration, as measured by inhibition of [^{3}H-*methyl*]deoxythymidine incorporation was 150 μM for the two human hepatoma cell lines and 40 μM for the duck hepatocyte cultures. In a pilot experiment PMEA was found to reduce the amounts of DHBV DNA in the serum of Pekin ducks.

PMEA; DHBV; HBV; HepG2 2.2.15 cells; HB611 cells; Duck

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Introduction

Virus latency characterized by HBeAg seroconversion and disappearance of viral DNA from the serum holds a reduced risk of long-term complications of human hepatitis B virus (HBV) infection (i.e., the development of liver cirrhosis and hepato-cellular carcinoma). At present, interferon administration is the basic therapy for chronic active hepatitis B. However, patients with high levels of HBV DNA in serum do not seem to respond to interferon therapy (Perrillo et al., 1990). Alternative drugs have been introduced as single drug therapy (ara-A monophosphate) (Hoofnagle et al., 1984) or combination therapy (interferon combined with zidovudine, acyclovir, desciclovir, or vidarabine) (Schalm et al., 1986a,b; Garcia et al., 1987; De Man et al., 1988). The initially promising results obtained with the combination of acyclovir with interferon were not sustained (Berk et al., 1992). However, the concept of reducing the high virus load by interferon and other drugs that have a good therapeutic index should be further pursued.

Recently, various acyclic nucleoside phosphonate analogues (i.e., phosphonylmethoxyethyl (PME) and hydroxyphosphonylmethoxypropyl (HPMP) derivatives of adenine (HPMPA, PMEA), 2,6-diaminopurine (PMEDAP) and cytosine (HPMPC)) have been investigated in the treatment of human immunodeficiency virus (HIV) infections and accompanying opportunistic infections (i.e., due to herpes simplex virus type 1 (HSV-1), type 2 (HSV-2), cytomegalovirus (CMV)). The HPMP derivatives are effective against adeno, herpes and vaccinia virus infections, whereas the PME derivatives are effective against both herpes- and retroviruses (De Clercq et al., 1986, 1987; Balzarini et al., 1989, 1991a,b; De Clercq, 1990, 1991).

The replicative cycle of the hepadnaviruses resembles to some extent that of the retroviruses. The (−)DNA strand of hepatitis B virus is transcribed to full-length (+)RNA. This pregenomic RNA is transcribed to (−)DNA strand, which is then duplicated so as to give rise to double-stranded (±)DNA. In this sequence of events, the viral DNA polymerase behaves as a reverse transcriptase (RT) and may thus be expected to be inhibited by genuine RT inhibitors.

There is little information on the inhibitory effect of the phosphonylmethoxyalkylpurines on the replication of hepadnaviruses (Yokota et al., 1990, 1991; Korba and Milman, 1991). We have now evaluated 9-(2-phosphonylmethoxyethyl)adenine (PMEA) for its inhibitory effect on HBV replication and viral antigen production in two human hepatoma cell lines transfected with HBV (i.e., HepG2 2.2.15 and HB611), as well as primary duck hepatocytes infected with duck hepatitis B virus (DHBV). In addition, 3-week old Pekin ducks congenitally infected with duck hepatitis B virus were subjected to PMEA therapy.

Materials and Methods

Stock of PMEA

PMEA was synthesized as described previously (Holy and Rosenberg, 1987). A stock solution of 10 mM was prepared in distilled water.

Human hepatoma cell lines

The HepG2 2.2.15 hepatoblastoma cell line (Sells et al., 1987) was kindly provided by Dr. G. Acs (Mount Sinai Medical Center, NY); the HB611 cell line (Sherker et al., 1986) was a gift from Dr. K. Matsubara (Institute of Molecular and Cellular Biology, Osaka, Japan).

Cells were seeded in 25 cm^2 tissue culture flasks (Costar) at a density of 4×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/l L-glutamine (Flow Laboratories), garamycine (40 μg/ml), amphotericin B (2.5 μg/ml), the neomycin analogue G418 (360 μg/ml for HepG2 2.2.15 cells; 200 μg/ml for HB611 cells) and 10% fetal bovine serum (FBS, Hyclone Laboratories). Medium was changed every 3 days. When cells reached confluency at day 6, FBS concentration was reduced to 2%. Cell cultures were maintained in 5% CO_2 atmosphere at 37°C.

At day 3, the culture medium was supplemented with various concentrations of PMEA. Cell culture supernatants and cells were harvested at day 12 and subjected to HBV DNA and HBsAg analysis. The 50% cytotoxic concentration (CC_{50}) of PMEA was determined in 24-well tissue culture plates (cell density: 4×10^4 cells/cm^2) by inhibition of [^{3}H-*methyl*]dThd incorporation (Chan et al., 1989) during 24 h starting at 3 days after seeding.

Primary cultures of DHBV-infected duck hepatocytes

Uninfected Pekin ducks (Anas domesticus) were taken from a breeding stock in our laboratory. Infection was established by inoculating 1-day-old ducklings with serum containing a high DHBV titer. The DHBV strain was kindly supplied by Dr. K.N. Tsiquaye (London School of Hygiene and Tropical Medicine, University of London, London, England). Duck hepatocytes were obtained from 2-week-old DHBV DNA-positive ducklings by collagenase perfusion as will be described in detail elsewhere. Cells were seeded into 60-mm dishes (Primaria, Falcon) at a density of 1.5×10^5 cells/cm^2 in William's medium E (Flow Laboratories) containing 2 mM L-glutamine, 20 mU/ml insulin (porcine insulin, Organon, Boxtel, The Netherlands), 0.05 mg/ml gentamycine, 0.05 mM hydrocortisone and 1% (v/v) DMSO (Sigma, tissue culture grade). The cell cultures were maintained in a 5% CO_2 atmosphere at 37°C. The medium was replaced at day 1 after cell seeding and subsequently every other day. Confluent monolayers were established within 2 days. At 3 days after seeding the culture medium was supplemented with various concentrations of PMEA. Cell culture supernatants and cells were harvested at day 11 for DHBV DNA and DHBsAg analysis.

Cytotoxicity of PMEA was determined in 24-well cell culture plates (density

of 1.5×10^4 cells/cm^2). At 1 day after seeding cells were incubated with various concentrations of PMEA and the 50% cytotoxic concentration (CC_{50}) was determined 24 h later, as described for the hepatoma cells.

Analysis of HBV

For analysis of extracellular HBV DNA, the cell culture supernatant was concentrated by polyethylene glycol (PEG, 10% w/v) precipitation and the pellets were extracted with phenol, phenol/chloroform/isoamylalcohol (50:49:1), chloroform/isoamylalcohol (24:1) (phenol/chloroform extraction), and precipitated with ethanol. Samples were prepared for dot blot analysis according to Korba and Milman (1991) and applied to Hybond N+ membrane (Amersham Life Science Products) in The Convertible Filtration Manifold System (BRL, Life Technologies).

For analysis of intracellular HBV DNA, cells were collected by trypsinization and after low speed centrifugation cell pellets were resuspended in a lysis buffer consisting of 10 mM Tris-HCl (pH 8.2), 400 mM NaCl, 2 mM EDTA, 0.6 mg/ml proteinase K and 1% SDS. Samples were incubated overnight at 45°C and the procedure was continued with phenol/chloroform extraction and ethanol precipitation as described above.

After RNase treatment and *Hin*dIII digestion (*Hin*dIII does not cleave within the HBV genome) followed by phenol/chloroform extraction as described above, 10 μg DNA was electrophoresed in a 1%-agarose gel followed by Southern blotting onto Hybond N+ membrane. Before electrophoresis the amounts of cellular DNA were equalized by comparison with serial dilutions of a standard of human DNA detected by a β-globin probe (PCR product with PC03 and PC04 primers) (Saiki et al., 1985). Hybridization of HBV DNA sequences was performed by a [^{32}P]dCTP-labelled *Eco*R1 fragment of pCP10 containing the full length 3.2 kb genome of HBV (Boender et al., 1985). All labelling reactions were carried out with the Prime-a-Gene Labelling System (Promega).

HBsAg in cell culture supernatants was determined semi-quantitatively by titration in a radioimmunoassay (Ausria II, Abbott Laboratories, Chicago, Il).

Analysis of DHBV

Extracellular DHBV DNA from cell culture supernatants was prepared for dot blot analysis as described for HBV but without the PEG concentration step. For analysis of intracellular DHBV-DNA, cells were collected by scraping and DNA was extracted as described for the hepatoma cells but without *Hin*dIII digestion. After RNase treatment equal aliquots were subjected to gel electrophoresis followed by Southern blotting. After gel electrophoresis, cellular DNA was verified and standardized by ethidium bromide staining.

DHBV-DNA from serum samples was extracted as described for HBV-DNA (Heijtink et al, 1987). For hybridization a [^{32}P]dCTP-labelled *Eco*RI fragment of the plasmid pBR322 containing the full length (3.0 kb) genome of DHBV was used. The plasmid was kindly provided by Dr. H.E. Blum (Freiburg,

Germany). All labelling reactions were carried out with the Prime-a-Gene Labelling System (Promega). DHBsAg from cell culture supernatants was analyzed by an immuno disc assay (IDA) (as will be described elsewhere in detail) after PEG (12%, w/v) precipitation. Briefly, serial dilutions of samples were added to nitrocellulose (NC) membrane discs placed onto the wells of a flat bottom 96-well cell culture plate. After removal of non-adsorbed material the discs were incubated with rabbit anti-DHBsAg (adsorbed with normal duck serum and normal duck hepatocytes) and subsequently with horse anti-rabbit IgG (Sigma) conjugated with horse radish peroxidase (HRP). As a substrate, α-chloro-1-naphthol (HRP Color Development Reagent, Bio-Rad Laboratories) was used. For analyzing DHBsAg in serum samples the same procedure was used as described for the cell culture specimens but without PEG precipitation.

IC_{50} determination

For the calculation of the 50% inhibitory concentrations (IC_{50}) from the autoradiograms of dot spot hybridization a LKB 222-020 Ultra Scan XL laser Densitometer was used.

In vivo evaluation of PMEA in ducklings

Four 3-week-old ducklings from a congenitally DHBV-infected breeding stock were treated with PMEA by intraperitoneal injection. Two of these ducklings received a weight-adapted PMEA dose of 30 mg/kg and the other two ducklings received a PMEA dose of 15 mg/kg. Injections started at day 21 after hatching. PMEA was given every other day for 3 weeks. Four animals of similar age and weight served as controls but received no treatment. Blood samples were obtained at regular intervals.

Results

Anti-HBV activity of PMEA in human hepatoma cell lines

Both the HepG2 2.2.15 cells and HB611 cells secreted Dane particles in amounts increasing with time after seeding. The secreted HBV DNA, measured at 3-day intervals over a period of 21 days, increased up to day 12 when a plateau was reached. HBsAg still increased at least up to day 21 (data not shown). HBV DNA as well as HBsAg secretion from HB611 cells was 4-fold lower than from HepG2 2.2.15 cells.

The influence of the continuous presence of various concentrations of PMEA, ranging from 0.05 μM to 25 μM, on the secretion of HBV DNA and HBsAg was determined at 12 days after seeding. The 50% inhibitory concentration (IC_{50}) of PMEA, required to inhibit HBV DNA secretion in the cell culture supernatants, was 0.7 μM for the HepG2 2.2.15 cells and 1.2 μM for the HB611 cells (Fig. 1). Intracellular episomal HBV-DNA production, as revealed by Southern blotting (RC and SS bands in Fig. 2), was reduced by PMEA resulting in an IC_{50} of 1.0–2.5 μM in the two cell lines. In contrast with

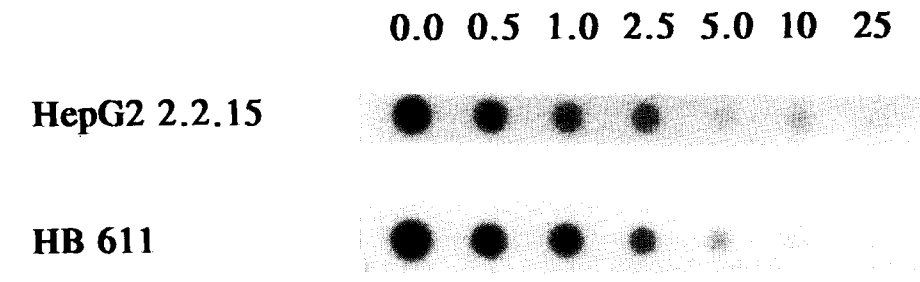

Fig. 1. HBV DNA detection by dot blot hybridization from the culture supernatants of HepG2 2.2.15 and HB611 cells in the continuous presence of the indicated concentrations of PMEA (μM). Cells were incubated with PMEA from the 3rd day after seeding. Culture medium was changed every 3rd day. The medium was harvested at day 12 for determination of HBV content.

the integrated HBV DNA (I band), the relaxed circular (RC) and single-stranded (SS) DNA bands disappeared at the higher PMEA concentrations (Fig. 2).

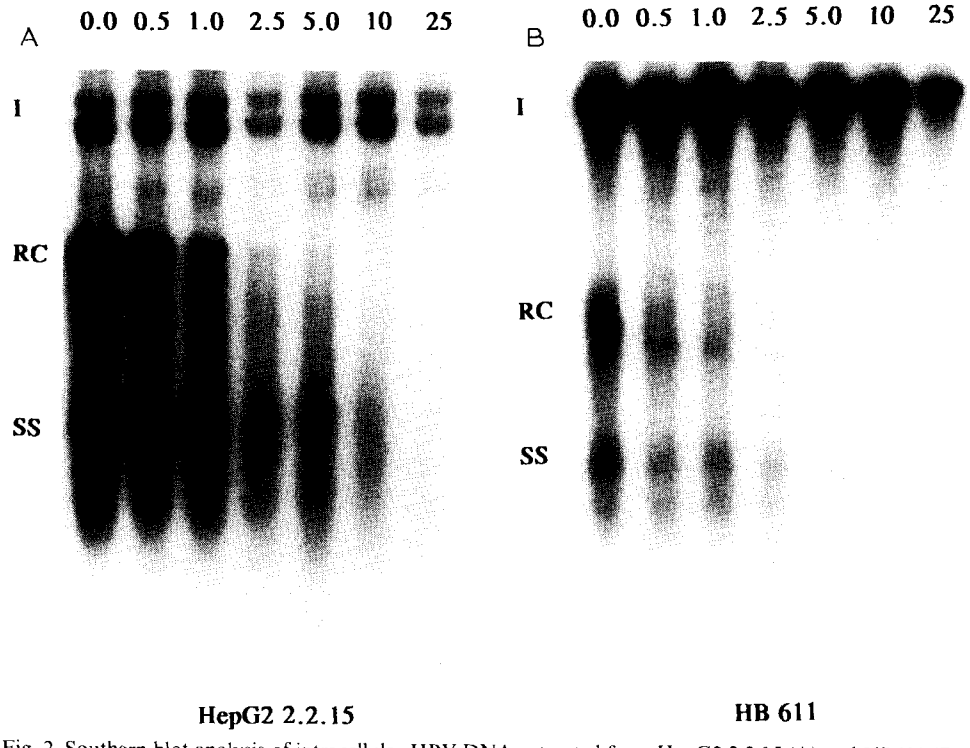

Fig. 2. Southern blot analysis of intracellular HBV DNA extracted from HepG2 2.2.15 (A) and HB611 (B) cells after continuous exposure to the indicated concentrations of PMEA (μM) from day 3 to 12 after seeding. Cellular DNA was digested with *Hin*dIII which does not cleave the HBV genome. HBV DNA molecular forms are indicated: I, integrated; intracellular episomal HBV DNA: RC, relaxed circular, SS, single-stranded.

HBsAg secretion from HB611 cells was inhibited by 30% at a PMEA concentration of 1.0–2.5 μM. This reduction gradually increased to 70% when the PMEA concentration was increased to 25 μM. In HepG2 2.2.15 cells, HBsAg secretion was not markedly inhibited by PMEA (data not shown).

The CC_{50} of PMEA as measured by [^{3}H-*methyl*]dThd incorporation was 150 μM in both cell lines.

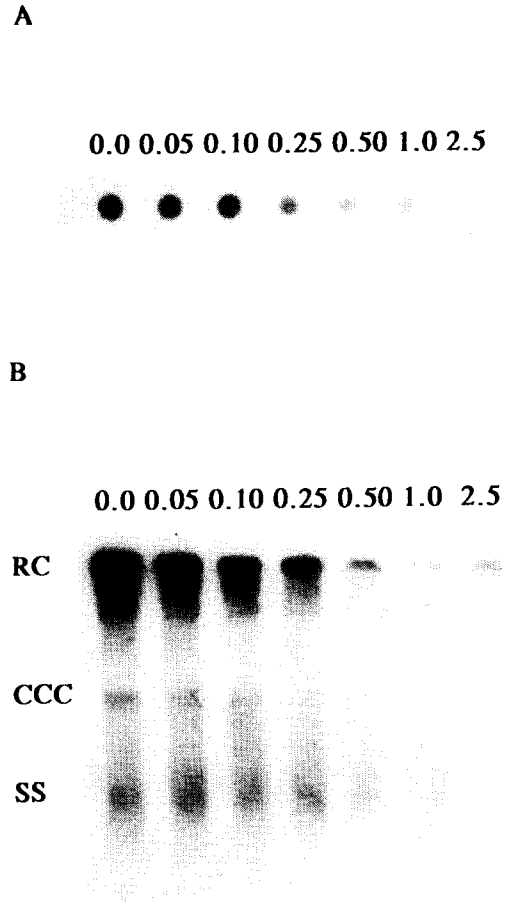

Fig. 3. (A) DHBV DNA detection by dot blot hybridization from supernatant of primary cultures of DHBV-infected duck hepatocytes at day 11 after continuous exposure to indicated concentrations of PMEA (μM). Cells were incubated with PMEA from the 3rd day after seeding. Culture medium was changed every other day. The medium was harvested at day 11 for determination of DHBV content. (B) Southern blot analysis of intracellular DHBV DNA in primary cultures of DHBV-infected duck hepatocytes after continuous exposure (days 3–11) to indicated concentrations of PMEA. DHBV DNA molecular forms are indicated: RC, relaxed circular; CC, covalently closed circular; SS, single-stranded.

Anti-DHBV activity of PMEA in primary cultures of DHBV-infected duck hepatocytes

Duck hepatocytes were cultured for 11 days. The secretion of DHBV DNA was stable with time from day 3 to day 11 (data not shown). The inhibitory effect of varying PMEA concentrations, ranging from 0.05 μM to 2.5 μM, on DHBV DNA secretion was examined by dot blot hybridization at day 11 (Fig. 3A). The IC_{50} of PMEA was 0.2 μM. A similar IC_{50} (0.25 μM) was found for intracellular DHBV DNA (Fig. 3B). This is about 4-fold lower than that observed in the two human hepatoma cell lines. DHBsAg production gradually decreased with increasing concentrations of PMEA to about 25% of that of untreated cells (Fig. 4). The CC_{50} of PMEA for [^{3}H-*methyl*]dThd incorporation in the duck hepatocytes was 40 μM.

Anti-DHBV activity of PMEA in DHBV-infected ducklings

Serum samples collected before, during and after PMEA treatment of the DHBV-infected ducklings were investigated for DHBV DNA and DHBsAg content. During therapy, a marked reduction of DHBV DNA was observed with the higher dose of PMEA but not with the lower dose (Fig. 5).

DHBsAg titers in the serum of the DHBV-infected ducks tended to decline after 40–50 days (data not shown). No marked differences were observed in the serum DHBsAg titers among PMEA-treated and untreated animals. At day 98 all animals were sacrificed, and the liver was investigated for DHBsAg and DHBcAg by immunofluorescence (rabbit anti-DHBsAg and rabbit anti-DHBcAg was kindly provided by Dr. W. Gerlich). No differences were observed between PMEA-treated and control animals.

During the whole period of the experiment, no toxic side effects that may have been related to PMEA were observed.

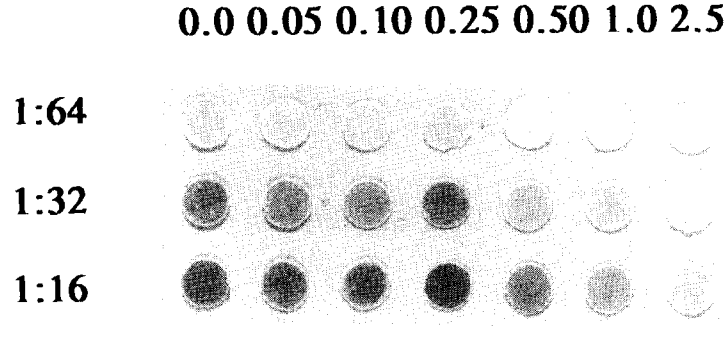

Fig. 4. DHBsAg evaluation by immuno disc assay (IDA) of culture supernatants harvested at day 11 after continuous exposure to indicated concentrations of PMEA (μM) starting on day 3 after seeding. Rows give results for sample dilutions with indicated concentrations of PMEA.

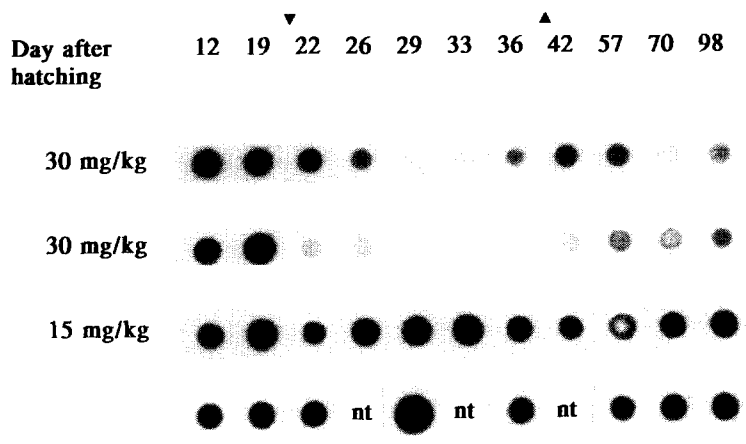

Fig. 5. Evaluation of DHBV DNA by dot blot hybridization in consecutive serum samples from congenitally DHBV-infected Pekin ducks following PMEA treatment at either 30 mg/kg or 15 mg/kg every other day, or no treatment (control). Treatment started at day 21 (▼) and ended at day 39 (▲). nt = not tested.

Discussion

In this study we described the inhibitory effect of PMEA on intra- and extracellular (D)HBV-DNA production and (D)HBsAg secretion by 2 human hepatoma cell lines and DHBV-infected duck hepatocytes. The IC_{50} of PMEA for extracellular viral DNA production from DHBV-infected duck hepatocytes was somewhat lower than for the 2 human hepatoma cell lines (0.2 μM vs. 0.7–1.2 μM). Similar results were noted for intracellular episomal DNA. The CC_{50} of PMEA for the duck hepatocytes was about 4-fold lower (40 μM) than for the human hepatoma cells (150 μM).

Korba and Milman (1991) reported that PMEA inhibits intracellular DNA synthesis from day 9 and 19 in HepG2 2.2.15 cells at an IC_{50} of <10 μM. Cytotoxicity, as determined by cell growth inhibition, was seen at a PMEA concentration greater than 10 μM. Yokota et al. (1991) found that in HB611 cells PMEA inhibits intracellular DNA production from days 2–12 at an IC_{50} of about 0.2 μM. Its CC_{50}, based on [^{3}H-*methyl*]dThd incorporation was 48 μM.

For 2′,3′-dideoxyadenosine (ddA) the IC_{50} ranges from 0.12 and 4.2 μM in duck hepatocytes to 100 μM in HB611 cells (Susuki et al., 1988; Lee et al., 1989; Ueda et al., 1989). The IC_{50} for 2′,3′-dideoxycytidine (ddC) varies from 1.6 μM in HB611 cells (Ueda et al., 1989) to 2.8 μM and 100 μM in HepG2 2.2.15 cells (Doong et al., 1991; Lampertico et al., 1991) and >10 μM in duck hepatocytes (Susuki et al., 1988; Lee et al., 1989; Yokota et al., 1990). For 2′,3′-dideoxyguanosine (ddG) and 2′,3′-dideoxy-2,6-diaminopurine riboside (ddDAPR) the IC_{50} is 0.2 μM in duck hepatocytes (Lee et al., 1989) and 10 μM in HepG2 cells (Korba and Milman, 1991). Similar IC_{50} were reported for

HPMPA in duck hepatocytes and HepG2 2.2.15 cells (1.2 μM), whereas for PMEDAP the IC_{50} values greatly differed from one cell type to the other (2.4 μM and 0.06 μM, respectively) (Yokota et al., 1990, 1991).

These results clearly illustrate that evaluation of anti (D)HBV activity in only one cell culture system may not be representative for the antiviral activity of the drug. The reason for the observed differences between the different cell systems may be related to metabolic peculiarities, as documented for the HepG2 2.2.15 cells (Aoki-Sei et al., 1991; Kitos et al., 1991). Also, the genomic organization of the hepadnavirus DNA seems to differ from one cell system to another: no integration is observed for DHBV, whereas in HB611 and HepG2 2.2.15 cells HBV DNA shows one and two integration bands, respectively. It is unknown whether this differential genomic organization influences the susceptibility of viral DNA synthesis to the antiviral drugs.

Part of the discrepancies noted between the results of different investigations may also be ascribed to the experimental design (time, duration of drug administration), the choice of the drug concentrations and the method of IC_{50} determination. For a better comparison of the results standardized techniques should be used whenever possible.

In addition to the evaluation of the inhibitory effect of PMEA on (D)HBV DNA secretion we investigated (D)HBsAg production during PMEA treatment. DHBsAg secretion from DHBV-infected duck hepatocytes showed a PMEA concentration-dependent inhibition. This is not unexpected since DHBV DNA occurs in not-integrated form, and the immuno dot blot assay used is capable of detecting both pre-S and S protein. However, a PMEA concentration-dependent inhibition was also observed for HBsAg secreted from HB611 (but not HepG2 2.2.15) cells. The behavior of HB611 cells in this aspect is unexpected since HBsAg production is thought to be controlled by the cellular genome. Similar observations have been reported by Matthes et al. (1990) for HepG2 2.2.15 cells using several 2′,3′-dideoxynucleoside analogues.

Our experiments with PMEA (30 mg/kg/day, every other day) in ducklings point to a suppressive action of PMEA on DHBV replication in vivo. This is further proven by the relapse of DHBV DNA synthesis after cessation of therapy. Various antiviral agents (i.e., foscarnet, ara-A 5′-monophosphate (ara-AMP), acyclovir, suramin, ddA, ddC, zidovudine (AZT) and other 2′,3′-dideoxynucleoside analogues) have been studied in Pekin ducks (Fourel et al., 1992; Haritina et al., 1989; Kassianides et al., 1989; Lee et al., 1989; Matthes et al., 1992; Susuki et al., 1988; Zuckerman et al., 1987; Sherker et al., 1986) after prescreening on duck hepatocytes or HepG2 2.2.15 cells. Except for AZT and ara-AMP, all test compounds caused an inhibitory effect on serum and/or liver DHBV DNA production in the ducks. Phosphonylmethoxyalkylpurine derivatives had so far not been evaluated in Pekin ducks. The preliminary in vivo results presented here for PMEA which are in keeping with the in vitro evidence for anti-(D)HBV activity indicate that the drug may be effective in the treatment of hepatitis B.

PMEA has antiretroviral as well as antiherpetic properties. It has been

shown to act as a DNA chain terminator (Balzarini et al., 1991a), and, in this capacity, PMEA may inhibit both the reverse transcriptase reaction and the DNA-dependent DNA polymerase reaction associated with the hepatitis B virus replicative cycle.

PMEA has distinct qualities that makes it an attractive candidate for the treatment of hepatitis B infection. It has a long intracellular half-life and a relatively low toxicity (Balzarini et al., 1991a). It is antivirally effective when given infrequently (i.e., once or twice a week) (Balzarini et al., 1990; Naesens et al., 1991). It also increases the activity of natural killer (NK) cells (Del Gobbo et al., 1991) and it has an immunostimulatory effect which may at least partially be mediated by interferon production (Del Gobbo et al., 1991). These properties, together with the anti-(D)HBV activity found in vitro and in vivo, make PMEA a prime candidate compound for further evaluation in patients with HBV infection.

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