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Characterizing antiviral activity of adefovir dipivoxil in transgenic mice expressing hepatitis B virus

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Abstract

Oral adefovir dipivoxil (ADV) reduced viral load in transgenic mice expressing hepatitis B virus (HBV). Liver HBV DNA was reduced to <0.1 pg of viral DNA per μg of total DNA (pg/μg) following oral ADV therapy at a dosage of 100 mg/kg/day twice daily for 10 days as compared to a mean of 3.0 pg/μg for the placebo control group. Oral ADV treatment also reduced serum HBV DNA to 3.5 $\log_{10}$ genomic equivalents (ge)/ml compared to 5.3 $\log_{10}$ ge/ml for the placebo control group. With once daily treatments, ADV antiviral activity reached near maximum viral reduction by day 10 in the liver and reached an endpoint of liver virus inhibition at 1.0 mg/kg/day. The minimum effective dose was less than 0.1 mg/kg/day using inhibition of serum virus. Lamivudine (3TC) given orally at 500 mg/kg/day using the same treatment schedule marginally reduced the serum HBV DNA by 4-fold, but did not significantly reduce HBV liver DNA. Serum titer reduction was also identified in untreated or placebo-treated animals, which may have been caused by the stress of pre-treatment bleeding and multiple oral gavage treatments. This trauma/placebo-effect may have masked the extent of viral reduction in the serum in ADV- and 3TC-treated animals. Liver HBV RNA was not reduced by oral ADV treatments. The lack of RNA reduction was expected, because the HBV transgene is stably integrated into the chromosome and ADV inhibits polymerase activity after transcription of pregenomic RNA. ADV was identified to have potent anti-HBV activity in this HBV transgenic mouse model and could serve as a suitable positive control for future drug discovery experiments.

Keywords: Adefovir dipivoxil; Hepatitis B virus (HBV); Transgenic mouse; 3TC

1. Introduction

The development of animal models for hepatitis B virus (HBV) infection is important for understanding viral replication, disease pathogenesis, and more specifically, for identifying candidate drugs for the treatment of HBV infection. However, the host-specificity of the virus has limited the availability of animal models. Chimpanzees are susceptible to HBV infection, but do not display all of the signs of disease, and are expensive and inconvenient to use as antiviral models.

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Viruses similar to HBV in woodchucks, squirrels and ducks have been very important in formulating much of our knowledge of HBV (Xiong et al., 1998). Tree shrews (*Tupaia belangeri chinensis*) have recently been found to be susceptible to infection with HBV, which is an acute self-limiting infection (Walter et al., 1996).

Limited host specificity has been partially overcome by generating transgenic animals to contain viral genomes (Morrey et al., 1991; Chisari, 1995a,b, 1996; Guidotti et al., 1995). Transgenic mice were developed to express portions of the HBV genome coding for viral proteins or the entire linear viral genome. These transgenic mice have provided information on pathogenesis, HBV immunology and virus replication that have been reviewed elsewhere (Chisari, 1997; Morrey et al., 1999c). Earlier attempts resulted in mice that expressed no, or low levels of virus in the serum. Investigators (Guidotti et al., 1995) were successful in developing transgenic mice that replicated quantifiable levels of HBV in the serum from clinically relevant liver and kidney organs.

Experiments with transgenic mice expressing HBV have demonstrated the model's utility for evaluating potential anti-HBV compounds such as interleukin-12 (Cavanaugh et al., 1997), (−)2′3′-dideoxy-3′-thiacytadine (lamivudine, 3TC) (Morrey et al., 1999a), interferon-α (Morrey et al., 1999b), and bis-POM-PMEA (Kajino et al., 1997) for treatment of HBV. This mouse model is less expensive than other hepatitis animal models, such as woodchuck, squirrel, or duck and allows for more convenient antiviral testing due to the relative ease of working with mice. The HBV transgenic mouse model does have disadvantages in that there is no immunopathogenesis and no viral replicative life-cycle involving covalently closed circular (ccc) DNA (Morrey et al., 1999c). There has, however, been detection of cccDNA in HNF1 alpha-null HBV transgenic mice, which suggests the presence of this intermediate in HBV transgenic mice (Raney et al., 2001). Another disadvantage is the inability to select drug resistant variants.

Adefovir dipivoxil (9-[2-[[bis[(pivaloyloxy)methoxy]phosphinyl]methoxy]ethyl] adenine, GS-0840, bis-POM-PMEA, ADV) acts as a chain terminator nucleotide analog and is effective against some retroviruses, herpesviruses, and hepadnaviruses including HBV. Resistance of HBV to lamivudine does not confer resistance to ADV (Xiong et al., 1998; Ono-Nita et al., 1999; Perrillo et al., 2000; Ying et al., 2000; Delaney et al., 2001; Ono et al., 2001), making this drug potentially clinically important. Natural killer cell activity and interferon production were enhanced in human patients by administration of ADV (Noble and Goa, 1999). ADV is in phase 3 of clinical trials for treatment of HBV in human patients (Gilead Sciences Inc. company website).

A previous transgenic mouse model, which expressed only low levels of HBV, was used to evaluate the antiviral activity of ADV but was inadequate to quantify the extent of virus reduction (Kajino et al., 1997). The purpose of this study was to quantify the reduction of HBV titers by ADV in a transgenic mouse model expressing quantifiable levels of HBV.

2. Materials and methods

2.1. Animals

Hepatitis B transgenic mice were obtained from Dr Francis V. Chisari (Scripps Research Institute, LaJolla, CA) which were derived from founder 1.3.32 (Guidotti et al., 1995). The mice were between 18 and 24 g. Both males and females were used in different experiments to evaluate antiviral activity. Pre-treatment serum samples were obtained to establish a base-line titer for each animal in all experiments conducted. All animal care and use was in compliance with the Utah State University Institutional Animal Care and Use Committee.

2.2. Compounds

ADV (9-[2-[[bis[(pivaloyloxy)methoxy]phosphinyl]methoxy]ethyl] adenine, GS-0840, bis-POM PMEA, ADV) is an ester prodrug of adefovir (9-(2-phosphonylmethoxyethyl)adenine, PMEA). An ester moiety was added to the parent drug, PMEA, which provided better bioavailability and uptake of the drug in human patients (Noble and Goa, 1999). Two lots of ADV were obtained from

Gilead Sciences (Foster City, CA). The older ADV lot 1 was made up in sterile saline and the newer lot 2 was dispersed in 0.4% carboxymethylcellulose (CMC) as a particulate solution or in citric acid (0.05 M, pH 4.0). CMC and citric acid were used to enhance the solubility of ADV in liquid suspension. In one experiment, a dose of 50 mg/kg of ADV was given twice daily by oral gavage to achieve a total dose of 100 mg/kg/day. In other experiments, half-log dilutions of ADV (100, 32, 10, and 3.2 mg/kg/day) were given orally once daily for the duration of the experiment (10–21 days). Lamivudine ((−)2′-deoxy-3′-thiacytidine, 3TC) was obtained from Dr Lynn Condreay of Glaxo SmithKline (Research Triangle Park, NC) as a purified powder and stored at −20 °C. Before use, 3TC was dissolved in sterile saline. Both drugs were stored at 4 °C until use.

2.3. Serum HBV DNA quantitative PCR

HBV DNA was prepared for amplification by extraction using GeneReleaser™ matrix (Bioventures, Inc., Murfreesboro, TN). Varying amounts of GeneReleaser, depending on the final volume of the reaction, were added to each 0.2 ml-well of 96-well formatted plates. Sixteen microliters of GeneReleaser™/100 μl total reaction volume were added. Serum diluted in Tris–EDTA buffer, pH 8.0, was also added to each well. Thermaseal™ (ISC Bioexpress, Ogden, Utah) was used to seal the 96-well plates, and the plates were microwaved for 6 min at 800 W according to manufacturers' instructions for GeneReleaser™. To avoid melting the 96-well plate, a beaker of double distilled water was placed in the microwave to act as a heat sink.

Total PCR reaction volumes varied between 30 and 220 μl depending on the anticipated titer of the serum. The components in a total 40 μl of PCR reaction were: 0.02 U cloned *Pfu* DNA polymerase (Stratagene, LaJolla, CA), 1X *Pfu* buffer, 200 nM of each deoxynucleotide triphosphate, and 0.2 μM of each of the forward and reverse primers. The internal control DNA was added between 10 and 450 genomic equivalents (ge)/reaction. All tubes and reagents after this point were kept on ice. The internal control consisted of a 412 bp fragment inserted into a plasmid clone of the HBV target sequence. The sizes of the internal control and the virion PCR products were 412 and 370 bp, respectively.

A 370 bp region was chosen within the core gene sequence of the HBV genome as a target for PCR amplification. The oligonucleotide primers utilized for these analyses were complementary to the HBV sequence (accession # V01460) (Galibert et al., 1979). The forward primer sequence was GATTGAGACCTTCGTCTGCGAG (position 776–797) and the reverse primer sequence was CATTGTTCACCTCACCATACTGCAC (position 1146–1122). The PCR reaction mixes were vortexed in preparation for thermal cycling. The thermal cycler was run at 94 °C for 2 min for strand separation and then run 39 times at a cycle of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for amplification of the target sequence. The amplification was followed by a final amplification at 72 °C for 5 min, after which the thermal cycler was set to hold at 22 °C. It was important to not refrigerate the samples and to store the PCR products at ambient temperature.

To prepare the samples for electrophoresis, 10 μl of a 1:2 dilution of blue dextran dye (Perkin–Elmer Corp., Foster City, CA) in 1.5 × TBE was added to each 40 μl PCR reaction. The mixture was vortexed, heated at 60 °C for 10 min, and vortexed again. The Gene Releaser™ resin was pelleted at 500 rpm for 30 s, which presumably disassociates much of the PCR product from the resin.

A 4.5% polyacrylamide gel was prepared (25 ml of 4.5% acrylamide, 20 μl TEMED, 200 μl 10% APS) in a 10 cm-long plate. After washing the wells, the gel was pre-run on a NucleoScan 2000 (NucleoTech Corp., San Mateo, CA) for 30 min at 300 V with 1 × TBE in the upper reservoir and 1.5 × TBE in the lower reservoir. The lower reservoir also contained 0.02 mg/ml of ethidium bromide. One microliter of sample was loaded in every other well and electrophoresed for 20 min at 360 V. The GelExpert™ electrophoresis analysis program (NucleoTech Corp.) was used to identify and quantify the intensity of the bands. A reference serum HBV standard (Heermann et al.,

1999) obtained from Dr K.-H. Heerman (Eurohep study group Universitatskliniken Gottingen, Kreuzbergring, Germany) was used to determine the number of genomes in the internal control. Data was transferred to a spreadsheet program for calculation of lower/upper band ratios and, finally, the concentration of the HBV DNA in the serum.

The ratio of densities between the lower and upper bands was related to the concentration of HBV DNA in the sera. The titers of the serum samples were determined by running multiple PCR reactions with each serum sample combined with different amounts of internal control with each reaction. The objective was that at least two different ratios (lower/upper bands) were to be obtained, with one greater and one less than a ratio of 1. The point at which each band was equal to the other (ratio = 1) was called the 'cross-over' point. This cross-over point was calculated by linear regression of the logarithm of the ratio versus logarithm of the concentration of the internal control added. The sera from 50 non-transgenic mice and >1000 HBV-transgenic mice were assayed by the quantitative HBV PCR assay to confirm the specificity of the assay.

Certain serum samples from mice were assayed by the National Genetics Institute (NGI, Los Angeles, CA) and by the in-house assay described above and compared for correlation. For further comparison, four human serum samples, previously tittered by the Digene II HBV DNA hybrid capture assay (Digene, Inc., Beltsville, MD), were obtained from Dr Edward R. Ashwood (University of Utah), and the Hepatitis-Retrovirus Laboratory (ARUP Laboratories, Inc., Salt Lake City, UT). The sera were serially diluted 10^{-4}, 10^{-5}, and 10^{-6}, and then assayed by the protocol described herein. The results were compared with those from ARUP Laboratories.

2.4. HBV southern blot analysis

Tissue samples were cut from the liver with a 3.5 mm biopsy punch (Baker Cummins Dermatologicals, Inc., Miami, FL) and ground with a well-fitted pestle in a microcentrifuge tube containing lysis buffer (1 mM EDTA, 10 mM Tris, 10 mM NaCl, 0.5% SDS, proteinase K) (Baginski et al., 1990). After incubation for 5–10 min at room temperature, the tubes were snap-frozen in liquid nitrogen for storage. DNA was purified using phenol/chloroform extraction and ethanol precipitation. Dried pellets were suspended in 100 μl of TE buffer (pH 8.0) and 1 μl of RNase A, diluted 1:10 in TE pH 8.0, was added to each sample.

Liver DNA cut with *Hin*dIII, which did not cut within the transgene, was fractionated by agarose gel electrophoresis and then processed for Southern blot hybridization. DNA was then transferred to BioDyne™ B positive-charged nylon membrane by alkaline transfer method (Budelier and Schorr, 1995). The membrane was baked for >30 min at 80 °C and UV-fixed using the UV Stratalinker™ 1800 (Stratagene, La Jolla, CA). Prior to hybridization, the filter was rinsed twice for 30 min in a neutralizing solution of $0.1 \times$ SSC and 0.1% SDS. Hybridization using a [^{32}P]CTP-labeled, HBV genomic probe (digested with *Hae*III) cloned into the pBluescript plasmid (gift of Dr Luca Guidotti, The Scripps Institute, LaJolla, CA) occurred overnight at 60 °C in a solution of 10% PEG-8000, 0.05 M $NaPO_4$, 0.21 mg/ml salmon sperm DNA, and 7% SDS. The membrane was washed to remove background radiation and exposed on Biomax film (Kodak, Rochester, NY) at -80 °C. The transgene and viral DNA bands on the X-ray film were measured using densitometric analysis (AlphaImager2000TM, Alpha Innotech Corporation, San Leandro, CA).

The ratio of the viral DNA bands to the transgene band was used to determine the concentration of viral DNA per host DNA. This calculation was based upon the knowledge that there were 1.3 copies of the transgene present per host cell with this line of transgenic mice (personal communication, F. Chisari, The Scripps Research Institute, LaJolla, CA). The transgene was used as an internal indicator to calculate the pg of HBV DNA per μg of cellular host DNA.

2.5. Extraction and analysis of liver RNA

Liver tissue pieces cut with a 3.5 mm biopsy punch were processed for RNA extraction using

Trizol reagent (Gibco/BRL, Gaithersburg, MD) per the manufacturer's instructions. The RNA was fractionated using agarose formaldehyde gel electrophoresis and then transferred to Bio-Dyne™ B positive-charged nylon membrane by salt transfer method of Northern blot analysis (Greenberg, 1995). The membranes were baked and cross-linked as outlined in the HBV DNA procedure. To prepare for hybridization, the membranes were wetted in $6 \times$ SSC. Hybridization, exposure of the probed membrane onto film, and analysis of the exposure was carried out as described for DNA membranes except that they were probed for both GAPDH and HBV in separate reactions, with the GAPDH serving as an internal control.

2.6. HBV antigen assays

For detection of hepatitis B core antigen (HBcAg), liver biopsies were first paraffin-embedded. The paraffin was then removed from the sections by using two 5 min treatments with xylene. Tissues were fixed with two 3 min treatments with 95% ethanol. Sections were treated with deionized water for 3 min, exposed to 3% hydrogen peroxide for 5 min, and Biotin-block (# X0590, Dako Corporation) for 5 min. The primary antibody, rabbit anti-HBcAg (1:100 dilution) (# B0586, Dako Corporation), goat anti-rabbit secondary antibody (# k684 Dako, LSAB Peroxidase Kit), strepavidin peroxidase (# K684 Dako, LSAB Peroxidase Kit), substrate–chromogen solution (3-amino-9-ethylcarbazole, AEC) were added for durations of 30, 30, 10 and 10 min, respectively. Sections were counterstained with Mayer's hematoxylin before being mounted. The total number of cells, the number of cells with stained nuclei, and the number of cells with stained cytoplasms were counted around central veins. The stained nuclei counts or the stained cytoplasm counts were divided by the total cells counts. Four central vein areas were counted with each slide sample. For the third parameter, a field, not in a central vein area, was counted for the total number of stained nuclei. One quarter of the field was counted. Four such fields were counted per liver section.

A commercial kit (ETI-EBK, DiaSorin Inc., Stillwater, MN) was used to detect hepatitis B pre-core antigen (HBeAg) in the serum as performed by the manufacturer's instructions. Standard curves were constructed using purified HBV subtype ayw (BioDesign International, MA) diluted in sterile mouse serum (Sigma, St. Louis, MO). Antigen concentration of samples at a 1/15 dilution were then determined from the equation obtained from linear regression of the standard curve.

2.7. Experimental design

2.7.1. ADV and lamivudine evaluation

Individually identified male HBV transgenic mice were divided randomly into groups of 10 animals. Lamivudine and ADV were administered by oral gavage (p.o.) twice daily (bid) for 10 days. Lamivudine was given at a total dose of 500 mg/kg/day and ADV was used at a dose of 100 mg/kg/day. Groups of mice were treated with placebo controls administered by different methods (abdominal swab, i.p. injection and oral gavage) to determine if viral titer reduction correlated with a certain method. On the last day of treatment, mice were sacrificed 2–4 h after the morning treatment to obtain tissue samples. Liver samples were snap-frozen in liquid nitrogen for storage. Blood samples were processed for collection of serum. Serum was stored at -80 °C until assayed.

In a second experiment to verify ADV antiviral activity, individually identified male transgenic mice were divided into groups of five animals and treated bid with oral ADV at 100 mg/kg/day for 10 days. A saline-treated group and an untreated group were used as controls for this experiment. Tissue samples were obtained on the last day of the experiment 2–4 h after the last treatment.

2.7.2. Time-course of ADV activity

Individual female transgenic mice were divided into groups of five animals as above. Mice were either treated per os with ADV at 100 mg/kg/day or with saline once daily until sacrifice. One group was left untreated. Five mice from each group were killed on days 2, 4, 7, 10, and 21 for collection of tissue samples.

2.7.3. ADV dose range-finding study

Two experiments were conducted because the end-point of ADV activity in the liver was not discovered in the initial experiment. Groups of five male and five female HBV transgenic mice were individually identified and randomly assigned to treatment groups consisting of half-log dilutions of ADV. The dilutions of the first experiment were prepared as particulate suspensions in 0.4% CMC. Concentrations of ADV starting at 100 and ending at 3.2 mg/kg/day were administered p.o. to the mice by oral gavage. The animals in each treatment group were treated once a day with one of the drug dilutions for a duration of 14 days. Saline-treated and untreated mice were used as controls. Transgenic HBV mice (both males and females) treated twice daily with ADV (100 mg/kg/day) were included to determine if there was any difference between mice treated once daily. In a second experiment, dilutions of oral ADV from 3.2 to 0.1 mg/kg/day in citric acid were administered once daily as above to determine the end-point of activity. Citric acid was used as a diluent to increase solubility of ADV. The results from these two experiments were combined into a single table (Table 2) without regard to gender.

3. Results

3.1. Serum HBV DNA quantification

PCR reactions of HBV transgenic mouse serum run with differing amounts of internal control (244 or 30 ge/ml) displayed lower bands when run on a polyacrylamide gel, which were indicative of virion DNA present in the serum (Fig. 1). None of the non-transgenic mice displayed the lower bands, which indicated the absence of the virus (data not shown). Upper internal control bands were displayed in all PCR reactions of non-transgenic mice, which indicated that the PCR reactions were functioning correctly. All other PCR reactions using >50 non-transgenic mice had similar negative results, whereas, transgenic mouse sera (>1000 samples) display the lower band of the virion PCR product.

The quantitative PCR was validated by comparing results of serum assayed by the procedure described above with results from assays performed at NGI, a contract laboratory assaying serum from clinical trials. The correlation was good between the two assays ($R^2 = 0.97$) (Fig. 2). The slope of the line should theoretically be equal to 1.0 and it was experimentally determined to be 1.1. Moreover, the y intercept was reasonably close to a predicted zero (-0.6). The quantitative PCR results also compared well with the DNA hybrid capture method ($R^2 = 0.94$) (data not shown).

3.2. ADV and 3TC evaluation

The bands resulting from the PCR amplification of the viral DNA and the internal control were quantified. By quantifying the ratio of the density of these signals, the titers of HBV in the serum could be calculated as described above. The ratio of PCR signals for the HBV DNA in the serum as compared to the signals for the internal control was lower than the ratios seen for the mice treated with saline (Fig. 3), i.e. the internal control signal was much higher for the ADV-treated mice as compared to the saline-treated mice. Serum titers from ADV-treated mice on the

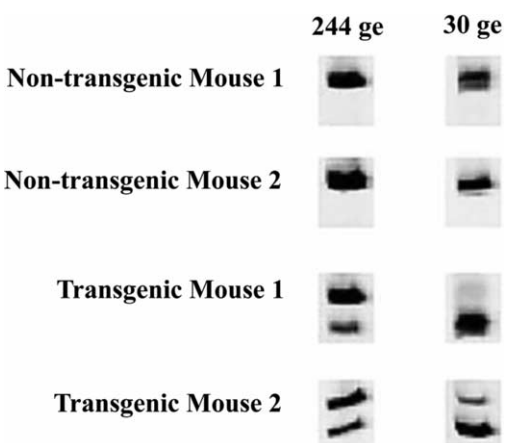

Fig. 1. Electrophoretic analysis of HBV PCR reaction products using sera from either non-transgenic or transgenic mice. Internal control containing either 244 genome equivalents (ge)/ or 30 ge/PCR tube were used for each serum sample.

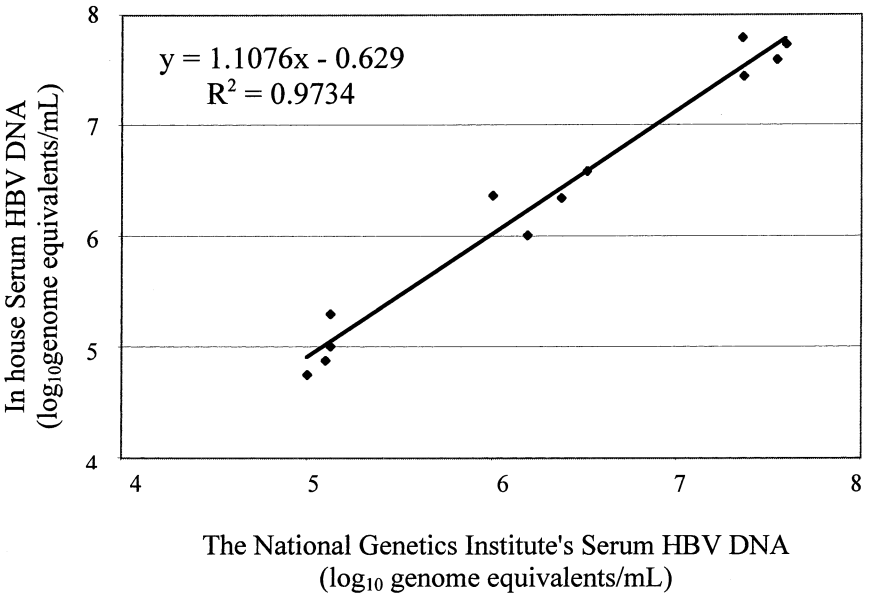

Fig. 2. Correlation of serum HBV DNA assay with National Genetic Institute's (NGI) assay.

last day of treatment (mean = 3.5 $\log_{10}$ ge/ml) were near the limits of detection (3.2 $\log_{10}$ ge/ml), but all other treatment groups were well above levels of detection (Table 1). Serum HBV DNA values from ADV-treated mice were reduced about 2 $\log_{10}$ as compared to placebo-control values ($P \leq 0.001$), whereas, 3TC-treated values were reduced by 0.6 $\log_{10}$, which was not statistically different from the placebo values. Pre-treatment serum HBV DNA titers were initially high and had means of 6.4–7.0 $\log_{10}$ ge/ml. Post-treatment serum titers were reduced in every group, including placebo and untreated controls, which we have referred to as a trauma/placebo-effect.

Oral ADV (100 mg/kg/day) administered twice daily reduced the liver HBV DNA to near the levels of detection (0.02 pg viral DNA/μg cellular DNA) ($P \leq 0.001$) as compared to the saline control values (3.0 pg/μg) and other control values (Table 1). Viral signals of the Southern blot from mice treated with ADV were generally a lower intensity, whereas, most of the higher sized viral signals were not present in the ADV-treated mice. Variability between control samples was also apparent (Table 1). No toxicity, as observed with no altered morbidity or mortality, was apparent at the highest dosage of ADV used (100 mg/kg/day). Oral 3TC administered twice daily at 500 mg/kg/day did not significantly alter the liver HBV DNA titers.

ADV had no significant effect on core antigen (HBcAg) in the liver or pre-core antigen (HBeAg) in the serum. The ratios of HBcAg-positive stained nuclei (0.3 ± 0.1) and cytoplasm (0.2 ± 0.2) in cells around central veins were also found to have no significant difference from placebo controls (data not shown). Serum HBeAg from ADV-treated groups had a mean of 4.2 ± 1.6 pg/ml and were not statistically significant from placebo control groups (data not shown). All treatments had no statistically significant effect on levels of liver HBV RNA as detected by Northern blot analysis and means from each treatment

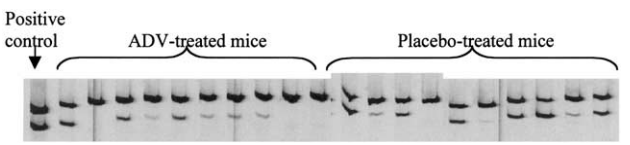

Fig. 3. Thin non-denaturing polyacrylamide gel electrophoresis of PCR products for quantitative detection of HBV DNA in serum of female transgenic mice treated orally twice a day with 100 mg/kg/day for 10 days with ADV or placebo.

Table 1
Effect of ADV, 3TC and various control treatments on liver and serum HBV DNA and liver HBV RNA in male and female transgenic mice expressing HBV

Treatment	Route/Dosage	Liver HBV DNA (mean[a] pg/μg cell DNA ± SD)		Serum HBV DNA (mean $\log_{10}$ genome equivalents/mL ± SD)		Mean relative liver HBV RNA ($\log_{10}$ ± SD)
		Post-treatment	Pre-treatment	Post-treatment	Mean Reduction	Post-treatment
ADV	Per Os, bid, 100 mg/kg/day	0.02 ± 0.01*	6.8 ± 0.8	3.5 ± 0.3*	3.3 ± 0.5	5.8 ± 2.5
3TC	Per Os, bid, 500 mg/kg/day	5.72 ± 3.3	6.7 ± 0.8	4.7 ± 0.6	2.0 ± 0.7	7.0 ± 1.4
Saline	Per Os, bid	3.0 ± 1.1	7.0 ± 0.8	5.3 ± 0.5	1.7 ± 0.7	4.7 ± 2.4
Saline	i.p., bid	2.7 ± 1.2	6.4 ± 1.1	5.3 ± 0.5	1.1 ± 0.8	4.7 ± 2.2
Saline	Swab abdomen, bid	8.2 ± 1.7	6.8 ± 1.1	5.4 ± 0.6	1.4 ± 0.9	6.2 ± 2.6
Untreated		6.0 ± 4.3	7.0 ± 0.8	5.4 ± 1.0	1.6 ± 0.9	5.4 ± 2.3

[a] $n = 10$ animals in each group (five males and five females).
* $P \leq 0.001$ when compared with placebo.

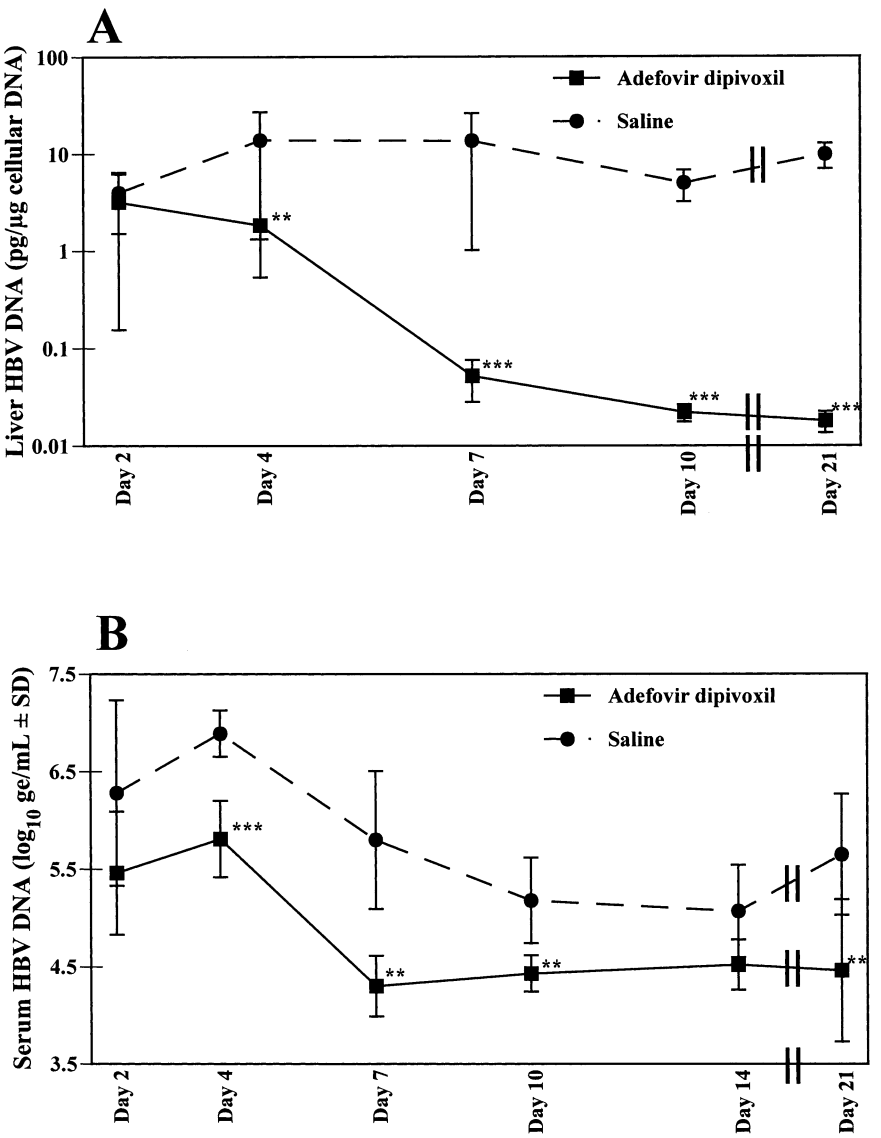

Fig. 4. Time-course inhibition of HBV DNA resulting from oral ADV treatment at 100 mg/kg/day once a day in male transgenic mice expressing HBV. (A) Liver HBV DNA results. (B) Serum HBV DNA results. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to saline treated animals.

group ranged from 4.7 to 7.0 $\log_{10}$ pg/μg with no statistical significance between groups (Table 1).

3.3. ADV time-course study

Oral ADV treatment (100 mg/kg/day) progressively reduced HBV virus load in the liver on days 2 through 21 (Fig. 4A). There was no difference in liver HBV DNA titers of ADV- or placebo-treated mice after 2 days of treatment, however, antiviral effects were observed starting at day 4 and continuing through day 21. By day 7 the virus load in the liver was strongly reduced. By day 10 virus titers were near the limits of detection in the ADV-treated group.

The antiviral effect on serum HBV DNA was not statistically significant at day 2, although an approximately 0.7 $\log_{10}$ inhibition in serum HBV DNA was seen (Fig. 4B). At days 4, 7, 10 and 21, serum HBV DNA titers were statistically lower than the values of the placebo-treated groups. The trauma/placebo-effect tended to mask the antiviral effects, as seen on day 14 when there was no statistically significant difference in serum HBV DNA between the ADV- and placebo-treated mice. There appeared to be an increase in viral titers of both ADV- and placebo-treated mice at day 4, but the difference from the day 2 values was not statistically significant, which suggests that this transient increase at day 4 may not be real or biologically significant. After day 4 the serum virus titers dropped dramatically in all animals.

3.4. ADV range-finding study

Once daily p.o. treatment with ADV at doses down to 1.0 mg/kg/day was effective in significantly lowering liver HBV DNA as compared to similarly treated placebo controls (Table 2). The antiviral effect appeared to be dose-responsive. The same ADV therapy lowered serum HBV DNA over 2 $\log_{10}$ ($P < 0.001$) at all dosages used. Although this effect did not appear to be dose-responsive (Table 2).

4. Discussion

This study demonstrated the utility of transgenic mice expressing quantitative levels of HBV for the evaluation of ADV. Oral ADV reduced HBV DNA in both serum and liver substantially greater than 3TC and also worked well at concentrations as low as 1.0 mg/kg/day. These experiments demonstrated that ADV will serve as an adequate positive control drug in future transgenic mouse studies.

A previous experiment evaluating bis-POM PMEA (ADV) in transgenic mice (Kajino et al., 1997) showed the drug to be effective in lowering HBV in the serum. This experiment, however, did not provide adequate quantification of the virus in the serum or the liver due to the low level of virus produced by the strain of transgenic mouse used. The levels of virus were reduced to below the levels of detection, and therefore the extent of antiviral activity of bis-POM PMEA was unknown in that study.

Phase I/II clinical trials showed that HBV-infected patients that received oral ADV once daily at a concentration of 125 mg for 28 days reduced HBV DNA levels by a mean of 1.8 $\log_{10}$ pg/ml (Gilson et al., 1999). One patient did have seroconversion to hepatitis B e antigen after 12 weeks of treatment, which was possibly due to treatment with ADV. Treatment with ADV has also reduced

Table 2
Dose response effect[a] of p.o. administration of ADV on liver and serum HBV DNA in transgenic mice expressing HBV

Per os adefovir dipivoxil concentration (mg/kg/day)	Treatment schedule	Liver HBV DNA mean pg/μg cell DNA ± SD (n[b])	Serum HBV DNA (mean $\log_{10}$ ge/ml ± SD) (n)
100	bid	1.1 ± 1.1 (10)*	4.3 ± 0.5 (10)*
100	qd	1.0 ± 1.2 (10)*	4.8 ± 0.2 (10)*
32.0	qd	1.0 ± 1.4 (10)*	4.3 ± 0.5 (10)*
10.0	qd	1.5 ± 1.4 (10)*	4.5 ± 0.3 (10)*
3.2	qd	2.3 ± 2.0 (15)*	4.8 ± 0.9 (10)*
1.0	qd	3.3 ± 3.3 (5)*	3.9 ± 0.3 (5)*
0.32	qd	11.0 ± 6.2 (5)	4.4 ± 0.2 (5)*
0.10	qd	8.3 ± 2.9 (5)	4.5 ± 0.5 (5)*
Placebo control	qd	10.5 ± 2.4 (5)	7.2 ± 1.1 (5)

[a] Assays performed on liver or serum samples taken 10 days after start of therapy.
[b] Number of animals/group. In the first four groups half of the mice were males and half were females. In the fifth group, 10 mice were males and five were females. The mice in the remainder of the groups were males.
*$P \leq 0.001$ when compared with placebo.

HIV viral load up to 8-fold in human patients (Noble and Goa, 1999). Phase III clinical trials are in progress to determine an effective and safe dosage for treatment of HBV infection (Gilead Sciences Inc. company website). ADV also provides anti-HBV activity against lamivudine-resistant strains of HBV, which may make the compound important in combination therapies (Xiong et al., 1998).

The transgenic mouse model used in this study could be useful in preliminary screening of antiviral compounds for use in human patients. There was a similarity in effective doses used in this mouse model with data from human clinical trials. The lowest effective dose in reducing liver HBV DNA in the mouse was 1.0 mg/kg which is calculated to be a dose of 0.083 mg/kg in man when adjusted for surface areas between the two species (Freireich et al., 1966). The dosage of 1.8 mg/kg (125 mg/70 kg person) used in human phase I/II clinical trials (Gilson et al., 1999) or the 2.1 mg/kg (150 mg/70 kg person) dosage used in another study involving human patients (Benhamou et al., 2001) were calculated to be 22 and 25 mg/kg, respectively, for dosages for the mouse when adjusted for surface area. These comparable results support the use of this transgenic mouse as a model for evaluating antiviral compounds effective against HBV.

From the data one can draw the conclusion that, at least for ADV, a 10-day duration of treatment is sufficient to achieve near-optimal antiviral activity against HBV DNA in the liver. Viral titer did not appear to drop substantially from day 10 to day 21 and an experiment duration longer than 10 days would probably not yield substantially greater reduction in HBV titer in this mouse model, but a 14 day experiment could be used to ensure a substantial reduction. The greatest difference between the serum virus titers from placebo control and ADV treated mice occurred at day 7, when antiviral activity in the liver had not yet reached maximum reduction. Liver results are probably more reliable in assaying antiviral activity than serum titers, because serum titers may be affected by confounding factors such as stress responses independent of drug treatment that contribute to the trauma/placebo-effects observed in this study. Liver HBV DNA reduction due to the trauma/placebo-effect was not observed in these experiments.

The reason why serum HBV DNA was more sensitive to ADV treatment as compared to liver HBV DNA was not known, although some hypotheses might be given. Slight modifications in liver titer undetected by Southern hybridization might result in greater measurable differences in the serum or that an additional mode of action of ADV might occur in the serum as compared to the liver. Ribavirin for example is known to modulate immune responses in addition to being a direct antiviral (Pianko and McHutchison, 2000). The parent drug of ADV, PMEA, enhances such immune function as natural killer cell activity and interferon production, and this may also occur with ADV treatment (Calio et al., 1994; Villani et al., 1994). Treatment with ADV has also been shown to inhibit the formation of NO produced by interferon-gamma and lipopolysaccharide by suppressing the mRNA transcription of inducible NO synthetase (Zidek et al., 1997, 1999). Another conceivable reason for observing a lower minimal effective dose using serum HBV DNA parameter as compared to the liver HBV DNA parameter in these mice could be related to the observation in human subjects that serum virus is cleared more rapidly in response to ADV therapy than in the liver due to the short half-life of the virus in the serum (Tsiang et al., 1999). However, this may not be the case in the transgenic mouse model, because the source of virus production is a constitutively expressing transgene as compared with reinfection and generation of supercoiled DNA in the naturally occurring infection.

It has been shown that 3TC is efficacious in human patients in the treatment of HBV (Lai and Yuen, 2000). Because of the antiviral activity seen in such human studies, it was unclear why 3TC was not very active in the mouse model in this study. Lower bioavailability, differences in metabolism, or phosphorylation activity in mouse liver as compared to human liver may be some reasons for lesser activity of 3TC in the mice. Variations in experimental design from other 3TC studies may also have contributed to a lower activity. Doses of 3TC were not prepared fresh

daily, which may have also contributed to a lower level of activity. A subsequent experiment was conducted, however, using freshly prepared 3TC before each treatment, which resulted in similar low activity (data not shown). This indicated that the low activity was probably not due to lability of the compound and may have been due to murine metabolism of 3TC.

The PCR assay described for this report was effective in quantifying serum HBV DNA for the detection of antiviral activity and for delineating placebo effects from antiviral effects of the drugs. This quantitative PCR procedure did not require expensive real-time PCR instrumentation. Specificity in the detection of HBV was also observed through the course of many experiments. Serum PCR reaction products from over 1000 mice positive for HBV run on a polyacrylamide gel ultimately resulted in the detection of viral products, whereas serum PCR products from non-transgenic mice never resulted in viral detection. All mice tested had an upper band present, which indicated that the PCR was successful in the amplification of the internal control target DNA. Sometimes neither band would show up on a gel, which indicated that further PCR reactions with varied parameters were needed to obtain data. The presence of the upper band was indicator of the functionality of the assay which avoided potential false negative results.

ADV inhibits replication of HBV through chain termination of the RNA to viral DNA (De Clercq, 1999). In this study, liver viral RNA levels were not significantly reduced by ADV and remained at normal levels. This was likely due to the continual production of viral RNA by the HBV transgene, which was then transcribed by reverse transcriptase to viral DNA. Consequently, HBV DNA in the liver would decline, but the viral RNA would remain constant. With continual production of RNA the mode of action may be observed, which may be an advantage to using the HBV transgenic mouse model. A disadvantage with this model is that supercoiled viral DNA was not the transcript for viral production as in a natural infection (Guidotti et al., 1995). When reverse transcriptase is inhibited by a nucleoside analog, many components of the cycle of infection, including the supercoiled viral DNA, are reduced with time (Peek et al., 2001). The effects of continual RNA production by the transgene were also evident in the unaltered levels of core and pre-core antigens with ADV treatment.

We observed a drop in titer of the post-treatment serum HBV DNA values as compared to the pre-treatment values in every group, including the untreated and swab-treated controls, which has been observed previously (Morrey et al., 1999c). This titer reduction was only observed to an appreciable degree in the serum and was likely due to the stress of pre-treatment serum collection and oral gavage treatment of the mice. The mechanism of this treatment response is unknown. This reduction in serum HBV titer may have been due to an acute immune or tissue repair response (Taub and Oppenheim, 1994) during pre-treatment bleeding procedures. Cytokines, antibody responses, and stress response proteins were conceivably involved with this effect. Viral RNA production by the transgene may be interrupted by the previously mentioned responses. Consequently, the liver HBV DNA titers may be more dependable in evaluating the antiviral responses of the drug treatments. Identifying the mechanism of the trauma/placebo-effect would have direct application in improving the animal model.

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References

Baginski, I., Ferrie, A., Watson, R., Mack, D., 1990. Detection of Hepatitis B Virus. Harcourt Brace & Jovanovich, San Diego.

Benhamou, Y., Bochet, M., Thibault, V., Calvez, V., Fievet, M.H., Vig, P., Gibbs, C.S., Brosgart, C., Fry, J., Namini, H., Katlama, C., Poynard, T., 2001. Safety and efficacy of adefovir dipivoxil in patients co-infected with HIV-1 and lamivudine-resistant hepatitis B virus: an open-label pilot study. Lancet 358, 718–723.

Budelier, K., Schorr, J., 1995. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smih, J.A., Struhl, K. (Eds.), Preparation and Analysis of DNA. Current Protocols in Molecular Biology, vol. 1. Wiley, USA.

Calio, R., Villani, N., Balestra, E., Sesa, F., Holy, A., Balzarini, J., De Clercq, E., Perno, C.F., Del Gobbo, V., 1994. Enhancement of natural killer activity and interferon induction by different acyclic nucleoside phosphonates. Antiviral Res. 23, 77–89.

Cavanaugh, V.J., Guidotti, L.G., Chisari, F.V., 1997. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. J. Virol. 71, 3236–3243.

Chisari, F.V., 1995a. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. 13, 29–60.

Chisari, F.V., 1995b. Hepatitis B virus transgenic mice: insights into the virus and the disease. Hepatology 22, 1316–1325.

Chisari, F.V., 1996. Hepatitis B virus transgenic mice: models of virus immunobiology and pathogenesis. Curr. Top. Microbiol. Immunol. 206, 149–173.

Chisari, F.V., 1997. Perspectives series: host/pathogen interactions. J. Clin. Invest. 99, 1472–1477.

De Clercq, E., 1999. Perspectives for the treatment of hepatitis B virus infections. Int. J. Antimicrob. Agents 12, 81–95.

Delaney, W.E., Edwards, R., Colledge, D., Shaw, T., Torresi, J., Miller, T.G., Isom, H.C., Bock, C.T., Manns, M.P., Trautwein, C., Locarnini, S., 2001. Cross-resistance testing of antihepanaviral compounds using novel recombinant baculoviruses which encode drug-resistant strains of hepatitis B virus. Antimicrob. Agents Chemother. 45, 1705–1713.

Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., Skipper, H.E., 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. Cancer Chemother. Rep. 50, 219–244.

Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., Charnay, P., 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) clone in *E. coli*. Nature 281, 646–650.

Gilson, R.J.C., Chopra, K.B., Newell, A.M., Murray-Lyon, I.M., Nelson, M.R., Rice, S.J., Tedder, R.S., Toole, J., Jaffe, H.S., Weller, I.V.D., 1999. A placebo-controlled phase I/II study of adefovir dipivoxil in patients with chronic hepatitis B infection. J. Viral Hepat. 6, 387–395.

Greenberg, M.E., 1995. Preparation and analysis of RNA. In: Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smih, J.A., Struhl, K. (Eds.), Current Protocols in Molecular Biology, vol. 1. Wiley, USA, pp. 4.9.1–4.9.8.

Guidotti, L.G., Matzke, B., Schaller, H., Chisari, F.V., 1995. High-level hepatitis B virus replication in transgenic mice. J. Virol. 69, 6158–6169.

Heermann, H.-K., Gerlich, W.H., Chudy, M., Schaefer, S., Thomssen, R., 1999. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. J. Clin. Microbiol. 37, 68–73.

Kajino, K., Kamiya, N., Yayasa, S., Takahara, T., Sakurai, J., Yamamura, K.-I., Hino, O., 1997. Evaluation of anti-hepatitis B virus (HBV) drugs using the HBV transgenic mouse: application of the semiquantitative polymerase chain reaction (PCR) for serum HBV DNA to monitor the drug efficacy. Biochem. Biophys. Res. Commun. 241, 43–48.

Lai, C.L., Yuen, M.F., 2000. Profound suppression of hepatitis B virus replication with lamivudine. J. Med. Virol. 61, 367–373.

Morrey, J.D., Bourn, S.M., Sidwell, R.W., Sisson, D.V., Bunch, T.D., 1991. Two-cell blockage of FVB/N embryo development: an artifact of cell culture. Encyclia 78, 275–284.

Morrey, J.D., Bailey, K.W., Korba, B.E., Sidwell, R.W., 1999a. Utilization of transgenic mice replicating high levels of hepatitis B virus for antiviral evaluation of lamivudine. Antiviral Res. 42, 97–108.

Morrey, J.D., Korba, B.E., Sidwell, R.W., 1999b. Transgenic mice as a chemotherapeutic model for hepatitis B virus infection. Antivir. Ther. 3 (Suppl. 3), 59–68.

Morrey, J.D., Sidwell, R.W., Korba, B.A., 1999c. Animal models for HBV infections-transgenic mice. In: Zak, O., Sande, M. (Eds.), Handbook of Animal Models of Infection. Academic Press, London, pp. 1009–1019.

Noble, S., Goa, K.L., 1999. Adefovir dipivoxil. Drugs 58, 479–487.

Ono, S.K., Kato, N., Shiratori, Y., Kato, J., Goto, T., Schinazi, R.F., Carrilho, F.J., Omata, M., 2001. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J. Clin. Invest. 107, 449–455.

Ono-Nita, S.K., Kato, N., Shiratori, Y., Lan, K.H., Yoshida, H., Carrilho, F.J., Omata, M., 1999. Susceptibility of lamivudine-resistant hepatitis B virus to other reverse transcriptase inhibitors. J. Clin. Invest. 103, 1635–1640.

Peek, S.F., Cote, P.J., Jacob, J.R., Toshkov, I.A., Hornbuckle, W.E., Baldwin, B.H., Wells, F.V., Chu, C.K., Gerin, J.L., Tennant, B.C., Korba, B.E., 2001. Antiviral activity of clevudine [L-FMAU, (1-(2-fluoro-5-methyl-beta, L-arabinofuranosyl) uracil)] against woodchuck hepatitis virus replication and gene expression in chronically infected woodchucks (*Marmota monax*). Hepatology 33, 254–266.

Perrillo, R., Schiff, E., Yoshida, E., Statler, A., Hirsch, K., Wright, T., Gutfreund, K., Lamy, P., Murray, A., 2000. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 32, 129–134.

Pianko, S., McHutchison, J.G., 2000. Treatment of hepatitis C virus with interferon and ribavirin. J. Gastroenterol. Hepatol. 15, 581–586.

Raney, A.K., Eggers, C.M., Kline, E.F., Guidotti, L.G., Pontoglio, M., Yaniv, M., McLachlan, A., 2001. Nuclear covalently closed circular viral genomic DNA in the liver of hepatocyte nuclear factor 1 alpha-null hepatitis B virus transgenic mice. J. Virol. 75, 2900–2911.

Taub, D.D., Oppenheim, J.J., 1994. Chemokines, inflamation and the immune system. Ther. Immunol. 1, 229–246.

Tsiang, M., Rooney, J.F., Toole, J.J., Gibbs, C.S., 1999. Biphasic clearance kinetics of hepatitis B virus from patients during adefovir dipivoxil therapy. Hepatology 29, 1863–1869.

Villani, N., Calio, R., Balestra, E., Balzarini, J., De Clercq, E., Fabrizi, E., Perno, C.F., Del Gobbo, V., 1994. 9-(2-Phosphonylmethoxyethyl) adenine increases the survival of influenza virus-infected mice by an enhancement of the immune system. Antiviral Res. 25, 81–89.

Walter, E., Keist, R., Niederost, B., Pult, I., Blum, H.E., 1996. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology 24, 1–5.

Xiong, X., Flores, C., Yang, H., Toole, J.J., Gibbs, C.S., 1998. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine. Hepatology 28, 1669–1673.

Ying, C., De Clercq, E., Nicholson, W., Furman, P., Neyts, J., 2000. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenovir, I-FMAU, DAPD, penciclovir and lobucavir. J. Viral Hepat. 7, 161–165.

Zidek, Z., Frankova, D., Holy, A., Boubelik, M., Draber, P., 1997. Inhibition of murine macrophage nitric oxide synthase expression by a pivoxil prodrug of antiviral acyclic nucleotide analogue 9-(2-phosphonomethoxyethyl)adenine. Biochem. Pharmacol. 54, 855–861.

Zidek, Z., Frankova, D., Holy, A., 1999. Stimulation of cytokine and nitric oxide production by acyclic nucleoside phosphonates. Nucleosides Nucleotides 18, 959–961.