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Characterization of antiviral activity of entecavir in transgenic mice expressing hepatitis B virus

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Abstract

Entecavir (ETV), a cyclopentyl guanosine nucleoside analog, was evaluated in transgenic mice expressing hepatitis B virus (HBV). ETV administered orally once daily for 10 days at a dosage of 3.2 mg/kg significantly ($P \leq 0.001$) reduced liver HBV DNA in female mice from 5.9 to <0.82 pg of HBV DNA per μg of cellular DNA, and from 8.3 to <1.1 pg/μg in male mice. To compare the efficacy of ETV with other compounds previously evaluated in this model and with ETV activities in other animal models, the efficacy of serial one-half log dilutions of ETV were evaluated in both male and female mice to determine the minimal effective dose. End-point titration experiments resulted in a statistically significant HBV DNA reduction in the liver at concentrations of 0.032 and 0.1 mg/kg per day in female and male mice, respectively. Viral liver RNA, and serum e (HBeAg), serum surface (HBsAg), and liver core antigens (HBcAg) were not affected by ETV treatment presumably because the antiviral target was viral polymerase activity and the HBV produced from the transgene was not capable of secondary rounds of infection in the mouse. ETV was well tolerated and no morbidity or mortality was observed during the 10-day study. Similar to other animal models, ETV displayed potent anti-HBV activity in this transgenic mouse model.

Keywords: Entecavir; HBV; Transgenic mouse; Hepatitis B virus

1. Introduction

Entecavir (ETV; BMS-200475) is currently in advanced clinical development as an anti-hepatitis B virus agent for treatment of chronic hepatitis B virus infection (Billich, 2001; Chang et al., 2002). ETV is a cyclopentyl guanosine nucleoside analog with potent and selective anti-hepadnaviral activity in vitro (Innaimo et al., 1997; Seifer et al., 1998) and in vivo (Colonno et al., 2001; de Man et al., 2001; Genovesi et al., 1998; Lai et al., 2001; Marion et al., 2002). After conversion to the active 5′-triphosphate form by cellular enzymes with little accumulation of mono- or di-phosphate forms (Levine et al., 2002; Yamanaka et al., 1999), ETV inhibits hepadnaviral DNA polymerase, priming, and elongation (Seifer et al., 1998). Additionally, the intracellular half-life of 15 h is considered to be relatively long (Yamanaka et al., 1999). Potent activity (EC_{50} = 3.75 nM) was observed in vitro in the HepG2 2.2.15 cell line and a CC_{50} of 30 μM, giving a selectivity index of 8000 (Innaimo et al., 1997). ETV was also effective against lamivudine-resistant HBV (Levine et al., 2002). In the woodchuck model using woodchuck hepatitis virus (WHV), ETV was effective at doses as low as 0.02 mg/kg (Genovesi et al., 1998). Treatment of chronically-infected woodchucks for up to 3 years with weekly treatments of 0.5 mg/kg/ETV resulted in viral DNA load reductions of up to 8 logs, reductions in cccDNA of >4 logs, significant decrease in the incidence of hepatocellular carcinoma and prolonged life in this animal model (Colonno et al., 2001). ETV in the duck hepatitis virus model was effective at concentrations as low as 0.01 mg/kg in reducing viral parameters in the liver and serum (Marion et al., 2002). In human clinical studies, ETV has been effective in treatment of both naive and lamivudine experienced patients, dropping viral DNA levels by >5 logs (Chang et al., 2002; Lai et al., 2001). The purpose of the present study was to compare the anti-HBV activity of ETV directly with a reference compound with known efficacy in the transgenic mouse. A transgenic mouse model expressing quantifiable levels of infectious HBV (Guidotti et al., 1995) has been used successfully in evaluating potential therapeutic substances such as interleukin-12 (Cavanaugh et al.,

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1997) and adefovir dipivoxil (Julander et al., 2002; Kajino et al., 1997). The HBV transgenic mouse model does have disadvantages in that there is no immunopathogenesis and no viral replicative life-cycle (Morrey et al., 1999). Viral RNA is produced from an integrated transgene and translated into viral proteins that are subsequently assembled into infectious virions. Covalently closed circular DNA may be present in this mouse model (Raney et al., 2001), but at negligible levels. The virions do not infect cells within the mouse, and there is no disease observed (Guidotti et al., 1995). Studies were conducted in these transgenic mice to further validate the usefulness of the model and to identify ETV's minimal effective dose for comparison with other compounds evaluated in this model and for comparisons of ETV activities in other infected species.

2. Materials and methods

2.1. Animals

Transgenic HBV mice with a genetic background of C57BL/6 were used. These mice were originally obtained from Dr. Frank Chisari (Scripps Research Institute, LaJolla, CA) and were subsequently raised in the Biosafety Level 3 (BL-3) area of the AAALAC-accredited USU Laboratory Animal Research Center (LARC). The animals were derived from founder 1.3.32 (Guidotti et al., 1995). Animal use and care was in compliance with the Utah State University Institutional Animal Care and Use Committee.

In early experiments it was observed that some animals, although theoretically genetically identical, produced lower levels of HBV in the liver and serum. This made quantitation of antiviral effect difficult. To resolve this problem, animals were divided into groups according to hepatitis B pre-core antigen (HBeAg). Titers of HBeAg were obtained for each animal and arranged from highest titer to lowest. Groups were block randomized to include animals with high to low HBeAg titers in each group. This resulted in an even distribution of HBeAg titers, which roughly correlate with HBV DNA titers in the liver.

2.2. Compounds

Entecavir (BMS-200475, CAS #142217-69-4) was obtained from Dr. Richard Colonno at Bristol-Myers Squibb Company. ETV was dissolved in physiological saline or water and stored at 4 °C during the course of the experiments. The dosages were delivered into the stomach using an oral gavage needle in a volume of 0.1 ml.

2.3. Liver HBV DNA Southern blot analysis

Liver HBV DNA was analyzed by Southern blot hybridization (Julander et al., 2002). Briefly, uniform-sized liver samples were cut with 4-mm diameter biopsy punches (Baker Cummins Dermatologicals, Inc., Miami, FL) and ground with a well-fitted pestle in a microcentrifuge tube containing lysis buffer. 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. 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 using the alkaline transfer method (Budelier and Schorr, 1995) with BioDyne™ B positive-charged nylon membranes. HBV DNA clone (gift of Dr. Luca Guidotti, The Scripps Institute, LaJolla, CA) digested with *Hae*III was used as a [^{32}P]-radiolabeled probe. 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. Southern blot analysis, instead of the PCR, was necessary to detect viral DNA in the liver because the liver also contains the HBV DNA transgene. HBV DNA was determined in the serum, but it was not included in the results because sera HBV DNA values were too variable as demonstrated in other studies using the same mouse model (Julander et al., 2002).

2.4. Extraction and analysis of liver RNA

Liver tissue pieces cut with a 4 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 BioDyne™ B positive-charged nylon membrane by salt transfer method of Northern blot analysis (Julander et al., 2002). The membranes were baked and cross-linked as outlined in the HBV DNA procedure. To prepare for hybridization, the membranes were wetted in 6× 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.

2.5. 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), and 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 amount of HBcAg was visually evaluated and given a score from 1 to 4 depending on the amount of HBcAg staining seen in cytoplasm and nuclei of hepatocytes in a given field.

An in-house assay was used to quantitate HBeAg in the serum. Anti-HBeAg antibody (cat #10-H10, clone #M2110147, Fitzgerald Industries, Inc.) was used as the capture antibody by coating black 96-well plates (cat #T3045-4, Maxi-bind, Greiner) at a concentration of 2.4 μg/ml diluted in sensitization buffer (0.05 M Tris–HCl, 0.3 M KCl, 0.002 M EDTA, pH 8) at 37 °C for 2 h. After removal of capture antibody, wells were washed three times with 300 μl of water, and then 200 μl of blocking buffer (0.3% Tween 20, 0.25% BSA in PBS) was added. The plate was incubated at room temperature for 30 min. After washing three times with water, 100 μl of transgenic mouse serum sample diluted 1:20 in blocking buffer was added and plates were incubated at 4 °C for 2 h. As with commercial kits, serum must be diluted to minimize background signal. Two negative controls (normal mouse serum diluted 1:20 in blocking buffer), and known concentrations of HBe protein (BioDesign International, MA) to make a standard curve were included. Wells were washed three times with water, incubated for 10 min with blocking buffer and washed again with water three times. Horseradish peroxidase conjugated (cat #31485, EZ-Link maleimide activated horseradish peroxidase) with tracer anti-HBeAg antibody (antibody cat #10-H10, clone #M2110146), 100 μl at a concentration of 3 μg/ml diluted in PBS, was added, and the plates were incubated at 4 °C for 2 h. After first blocking and then washing as described above, 100 μl of working solution of fluorogenic substrate (Quanta-Blu™, Pierce Biochemical) was incubated in the wells for 5 min at room temperature, at which point, 100 μl of stop solution was added. Fluorimetric development was read on a Molecular Devices fMax fluorimeter using 320/405 filter pairs. Standard curves were constructed using purified HBe protein subtype Ay (BioDesign International, MA). To validate the in-house assay, the results were correlated with a commercial assay kit (DiaSorin) ($R^2 = 0.7$) (data not shown).

The materials used to detect HBsAg in the serum were the same as the HBeAg assay already described except that the anti-HBsAg capture antibody (East Coast Biologics, cat #B04-95-33M-P), the biotinylated goat anti-HBsAg (Accurate, cat #YV51807), and the HBs protein (BioDesign International, MA) to make a standard curve were different. The capture antibody was used to coat black 96-well plates (cat #T3045-4, Maxi-bind, Greiner) at a concentration of 2.4 μg/ml diluted in sensitization buffer (0.05 M Tris–HCl, 0.3 M KCl, 0.002 M EDTA, pH 8) at 37 °C for 30 min. After removal of capture antibody, wells were washed three times with 300 μl of water, and then 200 μl of blocking buffer (0.3% Tween 20, 0.25% BSA in PBS) was added. The plate was incubated at room temperature for 2 h. After washing three times with water, 100 μl of transgenic mouse serum sample diluted 1:20 in blocking buffer was added and plates were incubated at 4 °C for 2 h. Sera were diluted to minimize background signal. Two negative controls (normal mouse serum diluted 1:20 in blocking buffer), and known concentrations of HBe protein (BioDesign International, MA) to make a standard curve were included. Wells were washed three times with water, incubated for 10 min with blocking buffer and washed again with water three times. Horseradish peroxidase conjugated (cat #31485, EZ-Link maleimide activated horseradish peroxidase) with tracer anti-HBsAg antibody, 100 μl at a 1/1000 dilution (concentration from manufacturer unknown) in PBS, was added, and the plates were incubated at ambient temperature for 1 h. After first blocking and then washing as already described, 100 μl of working solution of fluorogenic substrate (Quanta-Blu™, Pierce Biochemical) was incubated in the wells for 20 min at room temperature, at which point, 100 μl of stop solution was added. Fluorimetric development was read on a Molecular Devices fMax fluorimeter using 320/405 filter-pairs. Standard curves were constructed using purified HBe protein subtype Ay (BioDesign International, MA).

2.6. Experimental design

2.6.1. Efficacy of ETV in male and female mice

Separate experiments were performed with female mice and with male mice. The initial experiments were conducted to determine if a single, relatively high dose of ETV would be efficacious against HBV in the transgenic mice. Female age-matched mice 84–96 days old and male age-matched mice 124–154 days old were selected for the two experiments based on higher serum HBV DNA titer levels, i.e. 15% of the lowest titered mice were not entered into the experiment. The mice were block-randomized according to serum HBV DNA titers and treated by oral gavage once per day for 10 days with 3.2 mg/kg of ETV or with sterile saline placebo. Five mice were included in each group. On the 10th day at least 2 h after the last treatment, mice were euthanized and tissues were obtained and assayed for various HBV parameters.

The purpose of a second set of experiments was to determine the minimal effective dose of ETV in each gender of mice. The animals were selected based on higher serum HBV DNA or HBeAg titers in an attempt to eliminate lower-titered mice and to reduce variability. They were block-randomized according to their titers, and treated by oral gavage once per day for 10 days with one-half log dilutions of ETV (3.2, 1.0, 0.32, 0.1, 0.032, 0.010 and 0.0032 mg/kg per day) or with sterile saline placebo. The averages of liver HBV DNA titers of three separate experi-

ments were used to determine the minimal effective doses. On the 10th day samples were obtained as above.

3. Results

3.1. Effect of oral ETV

ETV administered p.o. once per day for 10 days at a dosage of 3.2 mg/kg significantly ($P \leq 0.001$) reduced liver HBV DNA in female and male mice (Table 1). Even though the male mice had higher mean liver HBV DNA titers as compared to the female mice, the ratio of reduction was about the same between the female (7.3) and the male mice (7.5), which suggested that efficacy was similar in both sexes. The exact reduction ratio could not be determined because 2/5 female and 1/5 male mice had titers below the limits of detection. Oral ETV treatment (3.2 mg/kg per day) did not affect liver HBV RNA, serum HBeAg, serum HBsAg or liver HBcAg levels (Table 2).

3.2. ETV dose range-finding study

We have previously determined (Julander et al., 2002) that amount of reduction of liver HBV DNA titers could not effectively be used to compare the efficacies of compounds, because the dynamic ranges of reduction were too small. The most accurate method for comparing efficacies between an investigational compound to that of a compound of known efficacy was to determine the lowest dosage of drug sufficient to affect viral parameters. To determine the minimal effective dose in both genders of transgenic mice, males and females were treated with half-log dilutions of ETV. In female mice, 0.032 mg/kg per day was identified statistically as the minimal effective dose, although the next one-half log dose (0.01 mg/kg per day) appeared to also reduce liver HBV DNA in a dose-responsive manner (Fig. 1). The minimal effective dose for male mice was 0.10 mg/kg per day.

4. Discussion

Oral ETV treatment for a duration of 10 days significantly reduced HBV DNA in the liver in male and female mice, but the treatment did not affect liver HBV RNA, serum HBeAg, serum HBsAg or liver HBcAg levels. The lack of effect on viral RNA and the reduction of viral DNA reflected the mode of action of ETV as an inhibitor of viral polymerase, priming, and elongation (Genovesi et al., 1998). The viral RNA was not inhibited because of its constitutive synthesis from the HBV transgene, and because multiple rounds of infection did not occur in mouse cells that would otherwise reflect the reduction of HBV DNA. The non-infectious nature of this model is clearly a disadvantage. It could be considered an advantage, however, in determining the stage at which the compound interferes with viral replication.

The calculated minimal effective dose in female mice was one-half log lower than that calculated for male mice. The biological significance of this small difference is unknown since the difference could be due to insufficient statistical power when using smaller numbers of animals. Even if the gender difference were real, the difference is small enough to probably not have clinical significance when using doses higher than the minimal effective doses.

Comparison of the activities of ETV and adefovir dipivoxil (ADV), which has been recently approved for use in HBV-infected patients, can be made because the minimal effective dose of ADV has been determined in a previous

Table 1
Effect of oral entecavir on liver HBV DNA in male and female transgenic mice

Treatment	Mean liver HBV DNA, pg viral/μg cell ± S.D. (*n*)	
	Female	Male
ETV, 3.2 mg/kg per day, p.o., once daily, 10 days	<0.8 ± 0.5 (5)[a,*]	<1.1 ± 0.9 (8)[*]
Vehicle (saline), p.o., once daily, 10 days	5.9 ± 2.7 (5)	8.3 ± 5.2 (8)

[a] The (<) symbol was used to indicate that 1/5 values of both gender were below the level of detection (0.3 pg/μg).
[*] $P \leq 001$.

Table 2
Effect of oral entecavir on liver HBV RNA, liver HBcAg, serum HBeAg and serum HBsAg in male transgenic mice[a]

Treatment	Liver HBV RNA[b] (relative value)	Liver HBcAg[c] (mean serum relative score, 0–3)	Serum HBeAg (ng/ml)	Serum HBsAg (ng/ml)
ETV, 3.2 mg/kg per day, p.o., once daily, 10 days	11.8 ± 6.5[d]	2.4 ± 0.7[d]	1146 ± 558[d]	74 ± 36[d]
Vehicle (saline), p.o., once daily, 10 days	15.5 ± 5.5	2.3 ± 0.7	1019 ± 340	64 ± 13

[a] Eight mice per group.
[b] The densitometer read of the viral RNA was divided by the reading of GAPDH housekeeping gene.
[c] Scoring: 0: no staining anywhere, 1: few red nuclei in 100× field, 2: many stained nuclei and some stained cytoplasm in 100× field, 3: 50% or greater of cells have stained cytoplasm or nuclei.
[d] No statistical differences between groups.

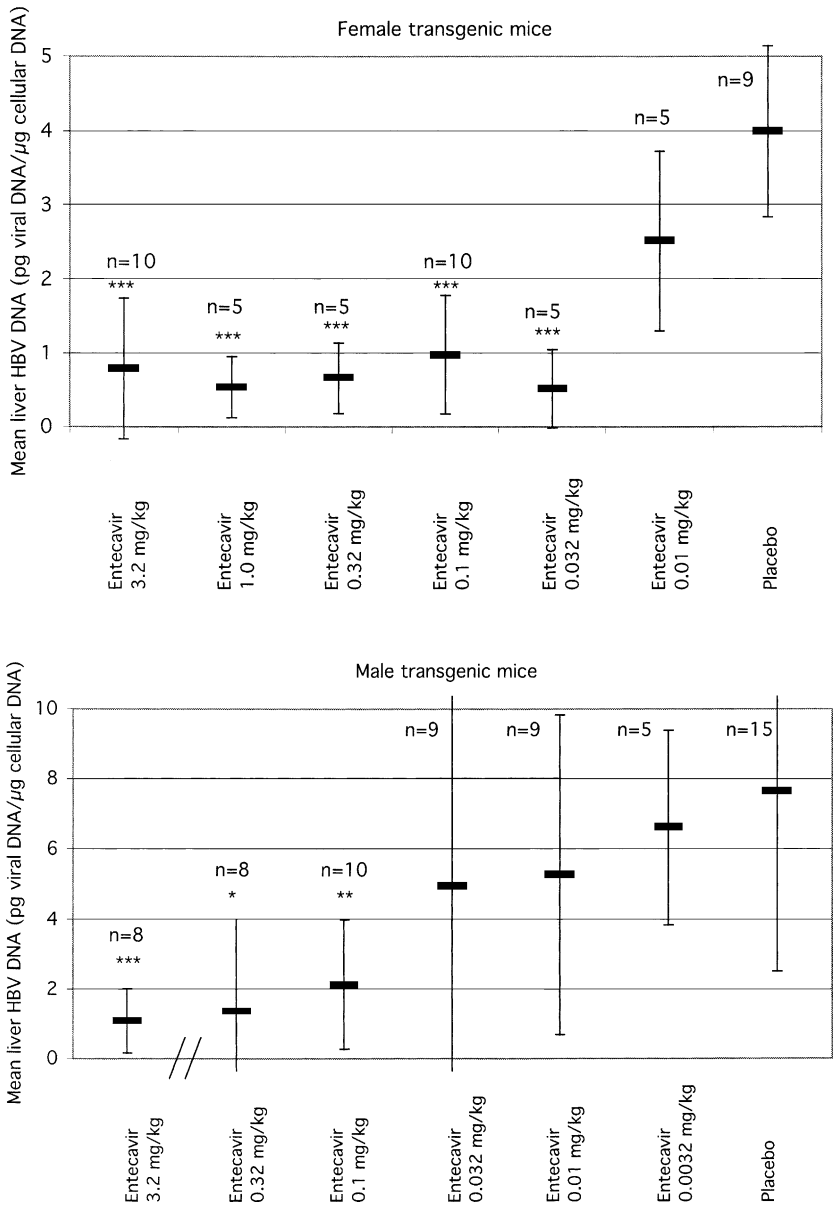

Fig. 1. End-point titration of oral entecavir on liver HBV DNA treated once daily for 10 days in female and male transgenic mice. Number of animals in each treatment group (n). $(^{*})P \leq 0.05$, $(^{**})P \leq 0.01$, $(^{***})P \leq 0.001$, compared to placebo.

study using these HBV transgenic mice (Julander et al., 2002) using the same treatment schedule, routes of administration and methods of analysis as reported for ETV in this study. Serial half-log dilutions of ADV administered daily, p.o. for 10 days was evaluated for its effect on liver HBV DNA. The minimal effective dose of ADV for male mice was 1.0 mg/kg per day, while the minimal effective doses of ETV for female and male mice were determined to be 0.032 and 0.10 mg/kg per day, respectively. It appeared, therefore, that ETV is 10-fold more efficacious than ADV in this model.

ETV has been evaluated in the woodchuck (Colonno et al., 2001; Genovesi et al., 1998) and duck (Marion et al., 2002) models for hepatitis and in human patients (Chang et al., 2002; de Man et al., 2001; Lai et al., 2001). The lowest effective doses evaluated in these other species were compared with the minimal effective doses identified in this study (Table 3). For comparison of doses in various species, the doses were converted from mg/kg to mg/m^2 of body surface area (Freireich et al., 1966). Sufficiently low doses were not used in human, duck or woodchuck studies to determine the very lowest effective dose (minimal effective dose), but some comparisons can be made with the transgenic mice. By comparing the lowest effective doses (mg/m^2) used in the human and duck studies with the transgenic mouse doses of this study (Table 3), it appeared that these other species are at least, if not more, sensitive to the effects of ETV than the transgenic mice. The lowest effective doses evaluated in human and duck studies were 0.03 and 0.05 mg/m^2,

Table 3
Comparison of entecavir activities against hepatitis viruses between various species

	Human (serum HBV) (Billich, 2001; de Man et al., 2001)	Duck (liver DHBV) (Marion et al., 2002)	Woodchuck (serum WHV) (Genovesi et al., 1998)	Transgenic female mouse (liver HBV)	Transgenic male mouse (liver HBV)
Treatment	Oral, once daily, 28 days	Oral, once daily, 21 days	Oral, once daily 28 days	Oral, once daily, 10 days	Oral, once daily, 10 days
Lowest effective dose tested (mg/kg)[a]	0.0008	0.01	0.02	0.032	0.10
Assumed body weight (kg)[b]	65	0.4	4.0	0.020	0.025
Dose (mg/m^2)	0.03	0.05	0.30	0.10	0.32

[a] These were the lowest doses evaluated and were probably not the minimal effective doses. The transgenic mouse value were the lowest doses that were effective, or the minimal effective doses.

[b] These values were assumed and used to calculate mg/m^2 body surface area (Freireich et al., 1966).

respectively, but they were clearly not the minimal effective doses. The minimal effective dose in female and male transgenic mice, however, was 0.1 and 0.3 mg/m^2, respectively, which suggested that human patients and ducks are more sensitive to the effects of ETV than transgenic mice because of the lower dose that was required to reduce HBV parameters in these species. The lowest dose used in the woodchuck model (0.30 mg/m^2) cannot be compared to the transgenic mouse, because this dosage yielded very similar results to higher doses used, and it did not appear to approach an ineffective dose. This transgenic model would not be expected to be as sensitive to the effects of ETV as these other species, because the transgenic mouse model is a non-infectious model. Conceptually, reduction of virus from ETV treatment in human, duck and woodchuck would be amplified through successive rounds of infection and replication because the virus produced in these systems infects other cells. With each round of replication in new cells, less virus is produced. Virus produced in the transgenic mouse is from integrated DNA, which is not influenced by antiviral treatment, so reduction of virus in the transgenic mouse would be detectable, but not amplified by successive rounds of replication. Therefore, potential therapies can best be compared when evaluated within the transgenic mouse model, as with the ADV and ETV described above, rather than comparisons with other species.

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References

Billich, A., 2001. Entecavir (Bristol-Myers Squibb). Curr. Opin. Investig. Drugs 2, 617–621.

Budelier, K., Schorr, J., 1995. Preparation and analysis of DNA. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smih, J.A., Struhl, K. (Eds.), Current Protocols in Molecular Biology. Wiley, USA, pp. 2.9.7–2.9.8.

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.

Chang, T.T., Hadziannis, S., Cianciara, J., Rizetto, M., Schiff, E., Pastore, G., Klesczewski, K., Denisky, G., Hindes, R., 2002. Sustained Viral Load and ALT Who Failed Lamivudine. AASLD, Boston.

Colonno, R.J., Genovesi, E.V., Medina, I., Lamb, L., Durham, S.K., Huang, M.L., Corey, L., Littlejohn, M., Locarnini, S., Tennant, B.C., Rose, B., Clark, J.M., 2001. Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. J. Infect. Dis. 184, 1236–1245.

de Man, R.A., Wolters, L.M., Nevens, F., Chua, D., Sherman, M., Lai, C.L., Gadano, A., Lee, Y., Mazzotta, F., Thomas, N., DeHertogh, D., 2001. Safety and efficacy of oral entecavir given for 28 days in patients with chronic hepatitis B virus infection. Hepatology 34, 578–582.

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.

Genovesi, E.V., Lamb, L., Medina, I., Taylor, D., Seifer, M., Innaimo, S., Colonno, R.J., Standring, D.N., Clark, J.M., 1998. Efficacy of the carbocyclic 2′-deoxyguanosine nucleoside BMS-200475 in the woodchuck model of hepatitis B virus infection. Antimicrob. Agents Chemother. 42, 3209–3217.

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.

Innaimo, S.F., Seifer, M., Bisacchi, G.S., Standring, D.N., Zahler, R., Colonno, R.J., 1997. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob. Agents Chemother. 41, 1444–1448.

Julander, J.G., Sidwell, R.W., Morrey, J.D., 2002. Characterizing antiviral activity of adefovir dipivoxil in transgenic mice expressing hepatitis B virus. Antivir. Res. 55, 27–40.

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., Rosmawati, M., Lao, J., Vlierberghe, H., Anderson, F., Thomas, N., DeHertogh, D., 2001. A phase II study of entecavir vs. lamivudine in adults with chronic hepatitis B. J. Hepatol. 34, 24.

Levine, S., Hernandez, D., Yamanaka, G., Zhang, S., Rose, R., Weinheimer, S., Colonno, R.J., 2002. Efficacies of entecavir against lamivudine-resistant hepatitis b virus replication and recombinant polymerases in vitro. Antimicrob. Agents Chemother. 46, 2525–2532.

Marion, P.L., Salazar, F.H., Winters, M.A., Colonno, R.J., 2002. Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication. Antimicrob. Agents Chemother. 46, 82–88.

Morrey, J.D., Sidwell, R.W., Korba, B.A., 1999. 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.

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.

Seifer, M., Hamatake, R.K., Colonno, R.J., Standring, D.N., 1998. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob. Agents Chemother. 42, 3200–3208.

Yamanaka, G., Wilson, T., Innaimo, S., Bisacchi, G.S., Egli, P., Rinehart, J.K., Zahler, R., Colonno, R.J., 1999. Metabolic studies on BMS-200475, a new antiviral compound active against hepatitis B virus. Antimicrob. Agents Chemother. 43, 190–193.