Long-Term Entecavir Treatment Results in Sustained Antiviral Efficacy and Prolonged Life Span in the Woodchuck Model of Chronic Hepatitis Infection

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Entecavir (ETV) is a guanosine nucleoside analogue with potent antiviral efficacy in woodchucks chronically infected with woodchuck hepatitis virus. To explore the consequences of prolonged virus suppression, woodchucks received ETV orally for 8 weeks and then weekly for 12 months. Of the 6 animals withdrawn from therapy and monitored for an additional 28 months, 3 had a sustained antiviral response and had no evidence of hepatocellular carcinoma (HCC). Of the 6 animals that continued on a weekly ETV regimen for an additional 22 months, 4 exhibited serum viral DNA levels near the lower limit of detection for >2 years and had no evidence of HCC. Viral antigens and covalently closed circular DNA levels in liver samples were significantly reduced in all animals. ETV was well tolerated, and there was no evidence of resistant variants. On the basis of historical data, long-term ETV treatment appeared to significantly prolong the life of treated animals and delay the emergence of HCC.

Human hepatitis B virus (HBV) infection is widespread and is a major cause of human liver disease [1]. Although primary HBV infections in adults are usually self-limiting, 6%–10% of infected individuals become chronically infected carriers and are at significant risk for the development of liver cirrhosis and hepatocellular carcinoma (HCC). Worldwide, there are nearly 400 million chronic carriers [2, 3], and treatment options are limited to lamivudine (3TC) and α-interferon. 3TC is the only approved antiviral for HBV infections, and, although it is clinically effective, sustained responses are observed in only a limited number of treated patients, because of the emergence of drug-resistant variants over time [4–7]. In addition, there is no evidence that 3TC can eliminate the reservoir of long-lived covalently closed circular DNA (cccDNA), the key replicative intermediate thought to be responsible for sustaining a chronic viral infection [8]. Thus, additional agents are needed to treat HBV infections.

The Eastern woodchuck, Marmota monax, chronically infected with the hepadnavirus woodchuck hepatitis virus (WHV), is an established animal model for studies of the pathogenesis of and therapy for HBV infection [9–11]. In most immunologically immature neonatal woodchucks, WHV infection causes a life-long WHV-carrier state with chronic viremia. A feature of the WHV-carrier state that is common to the HBV-carrier state of humans is the progressive development of primary HCC [12]. It is a particularly challenging animal model in which to observe a prolonged antiviral response, because 96% of these WHV chronically infected carrier woodchucks usually die by the age of 4 years, and all eventually die as a result of HCC [13].

Entecavir (ETV; BMS-200475, Bristol-Myers Squibb) is a cyclopentyl 2′-deoxyguanosine nucleoside analogue (figure 1) with excellent potency against hepadnaviruses, displaying an EC50 of 4 nM against human HBV replication in HepG2.2.15 cells and an EC50 of 0.13 nM in duck HBV (DHBV)—infected duck hepatocytes [14–16]. ETV is a highly selective inhibitor with only modest activity against a panel of 6 unrelated RNA and DNA viruses (EC50 range, 10–80 μM) [15] and has been shown previously to significantly reduce WHV DNA serum levels in WHV chronically infected woodchucks at dose levels as low as 0.02 mg/kg per day [17]. ETV at doses as low as 0.1 mg/kg per day is highly effective in inhibiting the replication of DHBV in infected ducklings [16]. ETV is readily phosphorylated to its active triphosphate form by cellular enzymes [18]. Mechanism-of-action studies confirmed [19] that ETV triphosphate directly inhibits all 3 functions of hepadnaviral polymerases and reduces cccDNA levels in cell culture and animal models [17]. Ongoing clinical studies in chronically infected human subjects have confirmed the potent antiviral activity of ETV that has been observed in animal models [20].

The objectives of the current study were to evaluate the long-term antiviral efficacy and tolerance of ETV in WHV chroni-
cally infected woodchucks over a prolonged treatment period, to determine whether suppression of viral replication could be maintained using a once-a-week dosing regimen. Such a sustained treatment period would allow us to monitor for the emergence of resistant variants over time, to explore the relationship between viral replication and development of HCC, and to study the impact of a highly effective antiviral on the life span of chronically infected animals.

Materials and Methods

Animals and treatment protocol. Hepatitis-infected woodchucks were obtained from Marmotech. Nineteen animals were used, and all had detectable serum WHV DNA levels at age 6 months and were considered to be chronically infected when the study began.

Treatment was initiated in 17 animals at age 8 months and in 2 at age 20 months. ETV was dissolved in sterile distilled water at 1 mg/mL, and the appropriate amount was given orally with 5 mL of Liquid Woodchuck Diet (Dyets). At weekly or other selected intervals, animals were anesthetized (50 mg/kg ketamine with 5 mg/kg xylazine), blood samples were obtained, and the animals were weighed. Serum samples were stored at −20°C. Animal behavior, serum chemistry panels, and hematology profiles were monitored throughout the study and at necropsy, to monitor drug tolerance and safety. The serum chemistry panel consisted of glucose, urea nitrogen, creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, cholesterol, calcium, phosphorous, sodium, potassium, chloride, albumin:globulin ratio, urea nitrogen:creatinine ratio, globulin, lipase, amylase, triglycerides, creatine phosphokinase, γ-glutamyl transpeptidase, and calculated osmolality. The complete blood cell count panel consisted of hemoglobin, hematocrit, white and red blood cells, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet, and leukocyte differential.

Serum WHV DNA levels. WHV DNA was initially quantitated by use of a solid-phase dot-blot hybridization method [21], in which WHV DNA in serum samples was hybridized to a whole genomic WHV DNA probe consisting of randomly radiolabeled [32P]-linear DNA. Signals were quantitated with a Storm 860 PhosphorImager (Molecular Dynamics). A calibration curve was prepared using 2-fold dilutions of a WHV plasmid DNA reference standard in clarified WHV-negative woodchuck serum. The lower limit of detection of WHV DNA by this method was 30 pg/mL. Results are shown as the mean of duplicate assays in picograms per milliliter.

A real-time fluorescent probe–based polymerase chain reaction (PCR; TaqMan) method was used to quantify WHV in the plasma [22]. DNA was extracted from 400 μL of plasma. One-tenth of the recovered DNA was used for each PCR. The primers WHV-F (TCCGTTGTGCTTTGCCGAGCGA) and WHV-R (GATTTGCTAGTCCACGAGGACA) and probe WHVtaq (TGCGAAATCATGATTTCCACCAGTG) were used in the TaqMan assay. WHVtaq was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine (Synamegen). The PCR conditions were as described elsewhere [23]. To ensure that negative results were not due to nonspecific inhibition of the PCR, each PCR was spiked with 50 copies of internal control jellyfish gene DNA (EXO), two 30 nM primers (EXO186F and EXO314R), and a 50 nM probe (PiMP-242T), as described elsewhere [24]. All negative PCR results required detection of EXO DNA to be considered valid.

Liver WHV DNA and cccDNA determinations. DNA was extracted from liver samples by use of a tissue extraction kit (Qiagen). Oligonucleotide primers were supplied by GeneWorks. Primers to selectively amplify WHV cccDNA were modified from those described by Coffin and Michalak [25]. These primers span the minus strand gap region: ccc1 5′-TGGTGTGTCWCTGTGTTTCTGACGC-3′, ccc2 5′-CCGGGAAAGTGGAGGAAATGCTGGC-3′, and ccc4 5′-GCCCACAGGTRTCATCGACA-3′. The WHV relaxed circular DNA nonselective primers amplify a region encompassing the catalytic domain of the polymerase gene: WHV568 5′-CTTGTTGGGTTCTCCTGGGACTGG-3′ and WHV1005 5′-GAATGTARATGATTGCCACCC-3′. The fatty acid binding protein (FABP) primers FABPfor (5′-ACCTCTGGGGAGAGATGGTGAAG-3′) and FABPrev (5′-CAGGATACCATATTACGACAC-3′) amplify a 178-bp fragment of the FABP gene.

Before cccDNA-specific PCR, DNA was incubated with 1 mM ATP, 1× Plasmid-Safe buffer (33 mM Tris acetate, 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM DTT), and 10 U of Plasmid-Safe ATD Dependent DNase (Epicerb Technologies) for 30 min at 37°C. The DNase was subsequently inactivated by heating at 70°C for 30 min.

For quantitative PCR, using the LightCycler (Roche Diagnostics), reactions were prepared according to the manufacturer’s instructions, using the SYBR Green I kit (Roche Diagnostics), including TaqStart antibody (Clontech). Each reaction contained 5 mM MgCl2, and each primer at 0.3 μM. PCR cycling conditions were 1 min at 95°C, followed by 40 cycles of 0 s at 95°C, 10 s at 55°C, and 30 s at 72°C, and 2 s at 82°C (fluorescence acquired). 78°C, or 86°C for total WHV PCR, FABP PCR, and WHV cccDNA PCR, respectively. Melting-curve analysis was done to analyze the specificity of the reactions, followed by quantification analysis, using the LightCycler Software (Roche Diagnostics).
For semiquantitation by hybridization, total WHV DNA was amplified, using each primer at 0.12 μM, 4.5 mM MgCl₂, and 1.5 U Taq DNA polymerase (Qiagen). Cycling conditions were 3 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1 min at 72°C, followed by an extension step of 7 min at 72°C. The first round of the heminested PCR conditions for WHV cccDNA used primers ccc1 and ccc2. The second round used primers ccc1 and ccc4. DNA was amplified, using the same cycling conditions as above, with annealing at 50°C with 4.5 mM MgCl₂. The PCR products were transferred to nylon membrane and were probed with a WHV probe prepared by PCR, using the relaxed circular DNA primers, and a plasmid containing a single copy of the WHV genome in pGEM (provided by John Newbold, University of North Carolina, Chapel Hill) was used as template, incorporating DIG-II–UTP into the PCR product. The DIG-labeled probe was detected using the manufacturer’s protocol (Roche Diagnostics.)

Viral antigens in serum and liver. Serum woodchuck hepatitis surface antigen (WHsAg), anti-woodchuck hepatitis core antigen (WHcAg), and anti-WHsAg were measured by use of WHV-specific ELISAs [26]. Formalin-fixed liver tissue sections were stained with hematoxylin-eosin for conventional light microscopic examination. Liver biopsy samples were immunostained by use of indirect immunoperoxidase streptavidin-biotin detection of WHV core antigen in formalin-fixed tissue [26].

Genotypic analysis for resistance. WHV DNA was extracted from serum samples, using either a QIAamp Viral RNA Mini Kit (Qiagen) or a NucleiSens Isolation Kit (Organon Teknika), according to the manufacturer’s instructions. For pretreatment samples, 140 μL of serum was used for DNA extraction, and one-sixth of the DNA sample was used for PCR. For samples with low virus loads, 1 mL of serum was used for DNA extraction, and the entire DNA sample was used for PCR.

The WHV polymerase domain was PCR-amplified using primers BRWHV-1 (5′-GGATCCCTCGAGCTCGACGACTGGGGAC-CTTGCGC-3′) and BRWHV-2 (5′-CCTAGGGAATATCCACGGGATTGTCACTGCCCACA-3′) and Platinum Taq DNA Polymerase High Fidelity (Life Technologies), according to the manufacturer’s instructions. The PCR products then were purified by use of a PCR purification kit (Qiagen). If samples were negative, as determined by agarose gel electrophoresis, after the first PCR, half of the first product was used for a nested PCR, using primers BRWHV-3 (5′-TCTGAGCTCGAGGGAGATGTCACCATCAAGTCT-3′) and BRWHV-4 (5′-GAGCTCGATATCCCGGAAAGGAGTTGACAGGT-3′) and Platinum Taq DNA Polymerase High Fidelity. The PCR products were purified as before and were sequenced directly by use of a dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystems) and 2 internal WHV polymerase sequencing primers, BRWHV-FS1 (5′-ACCCCTCTTACTGTGTTG-3′) and BRWHV-RS1 (5′-AGTTAAGAATACCAGTCAATC-3′). The samples were run on an ABI 373 automated sequencer (PE Applied Biosystems), and the data were analyzed by use of Sequencher software (Gene Codes). For each sample, the polymerase domain from aa 594–671 was sequenced directly and was analyzed for changes during treatment. Use of these conditions should detect a mutation if it is present in 25% of the population.

Results

Nineteen chronically infected woodchucks were dosed daily with ETV (0.5 mg/kg orally) for 8 weeks during the induction phase of the study. In all but 2 of these animals, treatment was initiated at age 8 months; the remaining 2 animals were a year older, and treatment for them was initiated at age 20 months. Serum WHV DNA levels were initially monitored by dot-blot analysis (limit of detection, 30 pg/mL), which showed serum viral DNA levels in all 19 animals reduced to undetectable levels (averaging >4 log₁₀ reduction) by the fifth week of daily therapy (table 1). The treatment period required to achieve undetectable levels did not appear to correlate with initial baseline serum viral DNA levels. After completion of the 8-week daily dosing period, 6 of the 19 animals were withdrawn from therapy and were monitored for an additional 28 weeks. All 6 animals exhibited a rebound in their serum WHV DNA levels within 1–8 weeks after completing the daily dosing phase of the study (table 1). Again, there was no obvious correlation between initial baseline DNA levels, time needed to achieve undetectable levels by dot-blot analysis, and the time until a rebound was observed. This group of animals served as controls, illustrating that the initial decreases in WHV DNA levels had been directly attributable to ETV treatment.

The 13 remaining animals, including the 2 older animals, were included in the maintenance phase of the study, in which daily oral treatment was replaced by a regimen of 0.5 mg/kg of ETV administered orally once a week for an additional 12 months. In addition to the dot-blot method, DNA levels were subsequently quantitated by PCR (limit of detection, 200 copies/mL). As shown in figures 2 and 3, viral DNA levels decreased in all animals with continued ETV treatment. One animal (no. 96-043) died of HCC before completion of the 12-month treatment (during study week 39), despite experiencing a 4-log₁₀ drop in viral DNA levels during the 10 months of ETV treatment. The remaining 12 animals showed 5–8 log₁₀ reductions in their viral DNA levels during the weekly dosing period. Two woodchucks (nos. 95-012 and 96-043) showed transient increases in viral DNA levels shortly after the switch to weekly treatment, although serum WHV DNA levels continued to decrease with continued weekly therapy. One of these animals (no. 96-043) was the one that died at week 39 after developing HCC.

Viral surface antigen levels, which were also monitored during treatment, showed a concomitant reduction in parallel with viral DNA reductions in all animals for whom this was measured (figures 2 and 3). Despite treatment, there was no measurable anti-WHsAg detected, and the levels of anti-WHcAg remained unchanged. After completion of the maintenance phase (total of 14 months of therapy), liver biopsy specimens were obtained from 10 selected animals and were examined for WHV core antigen. Compared with findings in biopsy samples from untreated animals, viral core antigen was undetectable by immunostaining in the hepatocytes from 9 of the 10 animals and was detected at
Evidence of HCC. Two other animals (nos. 96-025 and 96-152) had biopsy results for this woodchuck showing no histopathologic changes at that level, despite the discontinuation of ETV treatment. Liver viral DNA was undetectable levels (by PCR) within 6 months and maintained this status throughout the study.

An illustrative micrograph of cells from woodchuck 96-101, compared with cells from an untreated animal. This animal achieved a nearly 7-log reduction in viral DNA and surface antigen levels when treatment was stopped, but the levels again became undetectable without additional therapy.

For >2 years, all animals in the 36-month treatment group had serum viral DNA levels near or below the limit of PCR detection (figure 3). One (no. 96-101) of the 5 animals died at week 149 of therapy (43 months of age) and had evidence of HCC at necropsy, despite having undetectable serum WHV DNA levels by PCR. The remaining 4 animals survived until the end of the study—5 months after being withdrawn from therapy. Three of the 4 animals had undetectable serum WHV DNA levels by PCR, and 1 (no. 96-162) had a return to pretreatment serum WHV DNA levels 12 weeks after withdrawal of therapy. WHV surface antigen levels paralleled serum viral DNA levels in each of these animals except woodchuck 96-101, in which there was an unexplained increase during the last 18 months of treatment. None of the 4 surviving animals had gross evidence of HCC at necropsy.

At study end, the 6 surviving animals in both treatment groups were ~4 years old. Liver biopsy material from these 6 animals (nos. 96-025, 96-152, 96-012, 96-023, 96-131, and 96-162) was again stained for the presence of viral core antigen: 5 remained negative for viral core antigen, whereas the 1 animal (no. 96-162) that exhibited a rebound in viral DNA levels was also positive for viral core antigen in hepatocytes (data not shown). The livers from these 6 animals were also evaluated

### Table 1. Serum woodchuck hepatitis virus (WHV) DNA levels during induction treatment and recovery periods.

<table>
<thead>
<tr>
<th>Woodchuck identification no.</th>
<th>WHV DNA level at baseline, pg/mL</th>
<th>Week when WHV DNA was undetectable (&lt;30 pg/mL)</th>
<th>Fold reduction in WHV DNA level</th>
<th>Week of posttreatment rebound</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-042</td>
<td>34.626</td>
<td>1</td>
<td>&gt;1.1 × 10^3</td>
<td>7</td>
</tr>
<tr>
<td>96-091</td>
<td>644.320</td>
<td>2</td>
<td>&gt;2.1 × 10^4</td>
<td>1</td>
</tr>
<tr>
<td>96-021</td>
<td>102.400</td>
<td>2</td>
<td>&gt;3.4 × 10^4</td>
<td>5</td>
</tr>
<tr>
<td>96-165</td>
<td>32.300</td>
<td>5</td>
<td>&gt;1.1 × 10^4</td>
<td>4</td>
</tr>
<tr>
<td>96-115</td>
<td>336.640</td>
<td>5</td>
<td>&gt;1.1 × 10^4</td>
<td>8</td>
</tr>
<tr>
<td>96-122</td>
<td>924.120</td>
<td>3</td>
<td>&gt;3.1 × 10^4</td>
<td>3</td>
</tr>
<tr>
<td>96-025</td>
<td>210.440</td>
<td>4</td>
<td>&gt;7.0 × 10^3</td>
<td>NA</td>
</tr>
<tr>
<td>96-044</td>
<td>924.120</td>
<td>4</td>
<td>&gt;3.1 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-061</td>
<td>74.020</td>
<td>3</td>
<td>&gt;2.5 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-152</td>
<td>135.080</td>
<td>3</td>
<td>&gt;4.5 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-161</td>
<td>127.848</td>
<td>2</td>
<td>&gt;4.3 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-043</td>
<td>82.480</td>
<td>4</td>
<td>&gt;2.7 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>95-012</td>
<td>499.480</td>
<td>5</td>
<td>&gt;1.7 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-012</td>
<td>1,424.560</td>
<td>4</td>
<td>&gt;4.7 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-023</td>
<td>9460</td>
<td>1</td>
<td>&gt;3.2 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-101</td>
<td>518.960</td>
<td>3</td>
<td>&gt;1.7 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-131</td>
<td>35.980</td>
<td>1</td>
<td>&gt;1.2 × 10^4</td>
<td>NA</td>
</tr>
<tr>
<td>96-162</td>
<td>68.860</td>
<td>2</td>
<td>&gt;2.3 × 10^3</td>
<td>NA</td>
</tr>
<tr>
<td>95-323</td>
<td>637.380</td>
<td>4</td>
<td>&gt;2.1 × 10^4</td>
<td>NA</td>
</tr>
</tbody>
</table>

**NOTE.** WHV DNA levels were determined by dot-blot analysis. NA, not applicable.
Viral DNA and woodchuck hepatitis virus (WHV) surface antigen (WHsAg) levels for individual woodchucks in a 14-month entecavir treatment group. Animal identification nos. are shown in the upper right corner of each panel. The narrow, darker gray areas indicate 8 weeks of initial daily dosing at 0.5 mg/kg. The wide, lighter gray areas indicate 12 months of subsequent weekly dosing at 0.5 mg/kg. ●, Serum viral DNA levels (copies/mL), as determined by polymerase chain reaction (PCR); △, serum viral surface antigen levels (% of control).

For the presence of viral surface antigen: results for animals 96-012, 96-131, and 96-152 were negative. Livers from animals 96-023 and 96-025 were also negative for membrane-associated antigens, but cytoplasmic staining was marginally positive (<1 positive cell/1000). Membrane-associated antigens and cytoplasmic staining of the liver sample from woodchuck 96-162 were positive for WHsAg.

Because cccDNA is thought to play a key role in sustaining chronic HBV infections, the presence of viral DNA and cccDNA in liver samples also was determined by use of a newly developed PCR methodology, as described in Materials and Methods. After completion of the maintenance phase, all 9 woodchuck liver samples examined had achieved at least a 4-log₁₀ reduction in cccDNA levels, with 8 below the level of...
Figure 3. Viral DNA and woodchuck hepatitis virus (WHV) surface antigen (WHsAg) levels for individual animals in a 36-month entecavir treatment group. Animal identification nos. are shown in the upper right corner of each panel. The narrow, darker gray areas indicate 8 weeks of initial daily dosing at 0.5 mg/kg. The wide, lighter gray areas indicate 34 months of subsequent weekly dosing at 0.5 mg/kg. ●, Serum viral DNA levels (copies/mL), as determined by polymerase chain reaction (PCR); △, serum viral surface antigen levels (% of control).

detection (table 2). Similar reductions were also observed for WHV DNA levels in analyzed livers (table 2). Liver samples obtained at the conclusion of the study were very similar, with the only increase in cccDNA levels observed in the woodchuck (no. 96-162) who exhibited a rebound in serum WHV DNA to pretreatment levels.

Despite such a long treatment period, there was no evidence of the emergence of resistant WHV variants. To confirm this observation, the sequences of the viral polymerase gene from several of the treated woodchucks were examined at the end of the treatment period. The WHV polymerase domain extends from aa 418–731, with the B domain extending from aa 544–568 and the C domain (YMDD) extending from aa 585–591. Mutations associated with resistance to 3TC occur within or near the conserved B and C domains of the polymerase. PCR amplification of the viral polymerase gene was attempted for 10 woodchucks at time points near the end of their ETV treatment: number 96-044 at week 74; 96-061, 96-025, and 96-161 at week 59; 96-152 at week 66; 96-101, 96-023, and 96-131 at week 144; and 96-012 and 96-162 at week 172. Because of the unexpected rebound in viral DNA levels after 3 years of treatment, the WHV polymerase gene of woodchuck 96-162 also was sequenced at week 178. Compared with the sequence of the parent virus, no nucleotide changes in the polymerase gene were identified in any of the 7 animals where a PCR product could be recovered, suggesting that replication may have been reduced to levels too low to generate resistant variants (data not shown).

ETV treatment was well tolerated in this study: no compound related effects were noted during a 3-year treatment period for some animals. Compared with results in a historic woodchuck study [13], the clinical chemistry and hematology profile results showed no variations from normal values in treated animals. The expected increases in γ-glutamyl transpeptidase were observed in animals that developed HCC.
Figure 4. Micrograph of liver cells stained for woodchuck hepatitis virus core antigen. A, Immunostaining of hepatocytes from an untreated woodchuck. B, Immunostaining of hepatocytes from woodchuck 96-101 after treatment with entecavir for 14 months.

Discussion

Woodchucks chronically infected with WHV are a reliable and predictive model for evaluating the effectiveness and toxicity of antiviral agents [27, 28]. These animals closely mimic the human carrier state in regard to viral pathogenesis and represent a particularly challenging model, since nearly all the infected animals will eventually die of HCC within 3–4 years after infection [13]. Because progression to HCC is accelerated in these animals, they provide an excellent opportunity to explore the relationship between WHV replication and the development of HCC. ETV is an ideal antiviral for this purpose, because it exhibits excellent potency and pharmacokinetics enabling weekly dosing and is very well tolerated. There were several objectives for the current study, including evaluation of the long-term antiviral efficacy and safety, resistance development, effect on the development of HCC, and overall survival resulting from prolonged therapy with ETV.

As reported elsewhere [17], oral dosing of ETV at concentrations as little as 20 $\mu$g/kg daily can reduce serum viral DNA levels by several logs. Because of the favorable pharmacokinetic properties of ETV and the relatively long intracellular half-life of ETV triphosphate [18], a strategy of using a weekly dosing regimen was undertaken to enable a long-term study to be more easily conducted. Although not an optimal dosing regimen, a weekly regimen had been efficacious in earlier experiments. All the animals in the current study displayed multilog decreases in serum viral DNA levels that were sustained during the initial 12 months of weekly treatment with ETV, confirming the effectiveness of a weekly treatment regimen. Viral DNA and cccDNA levels in the liver were all significantly reduced during ETV treatment, and all treated animals demonstrated a 4-log$_{10}$ reduction in intrahepatic cccDNA levels relative to levels in controls. Furthermore, intrahepatic expression of viral core and surface proteins during therapy mirrored viral DNA levels. Three of the animals who were withdrawn from treatment after 14 months showed a sustained response during the 2-year post-treatment period, and all 5 of the younger animals in the 36-month treatment group had nearly undetectable viral DNA for >2 years.

It is believed that effective suppression or elimination of cccDNA may be required for sustained virus suppression and clearance of virus, since this key replicative intermediate serves as template for the transcription of all viral RNAs [29]. Normally present at 10–50 copies per infected hepatocyte, cccDNA serves to sustain chronic HBV infection [30–32]. It is widely viewed as being refractory to nucleoside therapy and, hence, could pose a major barrier to the eradication of HBV infection [33]. Treatment with 2 other effective inhibitors of HBV replication, 3TC and adefovir, failed to reduce cccDNA levels [34, 35]. In contrast, ETV treatment reduced cccDNA levels in both DHBV-infected ducks and WHV-infected woodchucks [16, 17]. Examination of liver biopsy samples from ETV-treated animals...
showed a significant decrease and long-term suppression of this key target species. This suppression likely contributes to the large decreases in viral DNA levels and the sustained response observed in several animals when treatment was withdrawn (figures 2 and 3).

As recently reviewed by Bre´chot et al. [36], it has been well established that HBV plays a role in liver cell carcinogenesis. HBV DNA sequences are integrated into cellular DNA in ~90% of liver tumor samples from hepatitis B surface antigen–positive patients, although integration is not always necessary. Although there does not appear to be a preferential integration site, integration of viral sequences can cause chromosomal DNA instability and insertional mutagenesis, with insertional activation of oncogenes frequently observed in woodchucks [37]. In addition, these integrations usually maintain a functional X open-reading frame, a known transactivator of a wide variety of cellular genes.

A number of animals in this study (4/11) died of HCC, despite the apparent effectiveness of ETV treatment in significantly reducing viral DNA and surface antigen levels. These results appear to support earlier models that propose that random integration events during viral replication will undoubtedly lead to a critical integration event that results in cellular transformation and progression down a pathway to HCC development. Such a model appears to be supported by the relationships observed between HCC development, viral DNA levels, and age of the animal when treatment was initiated. Both of the older woodchucks (infected for 20 months before treatment) died of HCC at about age 3 years, despite having suppressed viral DNA levels. Woodchuck 96-101 developed HCC, despite having undetectable viral DNA levels, as determined by PCR, during several months of treatment. These results suggest an uncoupling of viral replication and progression to HCC and support the concept that integration is a random consequence of viral DNA replication and that an early integration event can result in progression to HCC, regardless of subsequent intervention of viral replication.

Selection of lamivudine-resistant strains, which may lead to a failure of antiviral therapy in individuals with chronic HBV infections, occurs in ~16%–43% of patients within 1 year, appearing as early as 6 months and increasing with duration of treatment [4–7]. Lamivudine-resistant variants also were observed in the woodchuck after 6 months of treatment, with genotypic resistance appearing months before phenotypic resistance was observed [38]. The apparent absence of any resistance development during the prolonged ETV treatment period is yet another measure of the potent inhibition achieved. Since resistant viruses usually arise through a selection process of advantaged variants containing randomly generated mutations, a minimum threshold level of viral DNA polymerase activity must remain if a sufficient number of mutations are to be generated for selection. ETV, which inhibits all 3 viral polymerase functions (priming, RNA-dependent DNA synthesis, and DNA-dependent DNA synthesis), appears to suppress replication rates below this threshold level. Indeed, viral DNA levels in many of the treated animals were below the PCR cutoff of 200 copies/mL. Supporting this conclusion was the absence of any nucleotide changes from the baseline sequence when viral DNA was isolated and the WHV polymerase genes were sequenced.

The finding that several of the animals had sustained responses and survived to the age of 4 years likely is a reflection of the potent inhibitory activity of ETV. The finding of 1 animal who showed a rebound in viral DNA levels, despite 3 years of significant suppression by ETV treatment, was surprising but not unexpected. This animal displayed viral DNA just above PCR-detectable levels for most of the treatment period, suggesting that low-level viral replication was continuing during treatment. In hindsight, we might have achieved even better antiviral efficacy had we used a higher concentration of ETV or a daily dosing regimen. Nonetheless, it is interesting to consider how the virus managed to persist at low levels during a 36-month treatment period. Earlier studies by Michalak [39] demonstrated a life-long occult persistence of pathogenic virus in woodchucks who had appeared to clear acute WHV infection.

Of particular importance was the finding that the long-term survival rates in the absence of HCC of the 11 younger animals used in the 14- and 36-month treatment groups were 50% and 80%, respectively, compared with only 4% for historic controls [13]. Each of the ETV-treated groups was compared with the uninfected and infected/untreated groups [13] by use of Fischer’s exact test [40]. Because repeated testing increases the probability of false results, the 2 *P* values were corrected by use of Bonferroni’s method [41]. The results were statistically significant...
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The results from the current study show that ETV is a very potent inhibitor of WHV, as evidenced by the multilog decrease in viral DNA levels, cccDNA, and viral surface and core antigen expression. ETV was well tolerated, and there was no evidence of viral resistance, despite a treatment period of up to 3 years in some animals. ETV treatment appeared to prolong life in several animals and delay the onset of HCC. These results support the continued human clinical evaluation of ETV in the treatment of chronic HBV infections.

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