Antiviral Effect of Lamivudine, Emtricitabine, Adefovir Dipivoxil, and Tenofovir Disoproxil Fumarate, Administered Orally Alone and in Combination, to Woodchucks with Chronic Woodchuck Hepatitis Virus Infection

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Running title: 3TC, FTC, ADV, and TDF in chronic WHV infection

Key words: Woodchuck hepatitis virus, antiviral therapy, adefovir dipivoxil, emtricitabine, lamivudine, tenofovir disoproxil fumarate.

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Adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) are nucleotide analogs that inhibit replication of wild-type hepatitis B virus (HBV) and lamivudine-resistant (3TC) resistant virus in HBV-infected patients, including those who are co-infected with the human immunodeficiency virus. Combination of ADV or TDF with other nucleoside analogs is a proposed strategy for managing antiviral drug resistance during treatment of chronic HBV infection. The antiviral effect of oral ADV or TDF, alone or in combination with 3TC or emtricitabine (FTC), against chronic woodchuck hepatitis virus (WHV) infection was evaluated in a placebo-controlled study in the woodchuck, an established and predictive model for antiviral therapy. Once-daily treatment for 48 weeks with ADV + 3TC or TDF + FTC significantly reduced serum WHV viremia from pretreatment level by 6.2 and 6.1 log_{10}, respectively, followed by TDF + 3TC (5.6 log_{10}), ADV alone (4.8 log_{10}), ADV + FTC (one survivor, 4.4 log_{10}), TDF alone (2.9 log_{10}), 3TC alone (2.7 log_{10}), and FTC alone (2.0 log_{10}). Individual woodchucks across all treatment groups also demonstrated pronounced declines in serum WHV surface antigen, characteristically, accompanied by declines in hepatic WHV replication and hepatic expression of WHV antigens. Most woodchucks had prompt recrudescence of WHV replication after drug withdrawal, but individual woodchucks across treatment groups had sustained effects. No signs of toxicity were observed for any of the drugs or drug combinations administered. In conclusion, oral administration of 3TC, FTC, ADV, and TDF, alone and in combination, was safe and effective in the woodchuck model of HBV infection.
INTRODUCTION

Chronic infection with the hepatitis B virus (HBV) is a major public health problem and is responsible for 1.2 million deaths per year worldwide (64). It is estimated that more than 2 billion people have serological evidence of previous or current HBV infection, and over 350 million are chronic carriers of HBV (64). Carriers of HBV are at high risk of developing chronic hepatitis, hepatic cirrhosis, and hepatocellular carcinoma (HCC). Although safe and effective prophylactic vaccines against HBV are available, improvements in drug and/or immunotherapeutic strategies for the treatment of chronic HBV infection are still needed. Therapy with interferon-alpha and nucleoside analogs, alone or in combination, can be effective against HBV; however, side effects of interferon and the emergence of nucleoside-resistant mutants often limit treatment outcomes (34).

Lamivudine (3TC) was the first nucleoside analog licensed for treatment of chronic HBV infection. Although 3TC is safe and effective, its therapeutic value is limited by the time-dependent development of drug-resistant HBV mutants (32); therefore, various combination therapies have long been proposed to counter drug resistance in HBV infection. More recently, the nucleotide analog adefovir dipivoxil (ADV) was licensed for treatment of HBV infection and was shown to inhibit the replication of 3TC-resistant virus mutants in patients also treated with 3TC (1, 3, 4, 16, 37, 47, 66). In fact in chronic HBV carriers, even monotherapy with ADV for up to 5 years had a high degree of safety and efficacy, and resistant mutants developed less frequently than in parallel studies with 3TC alone (2, 19, 35, 48, 65). Treatment for 48 weeks with two different doses of ADV reduced viremia by 3.5 to 4.8 log\textsubscript{10} in patients with chronic HBV infection (35). A similar
decrease in serum HBV DNA of 3.5 and 3.9 log_{10} was demonstrated in two other studies after 48 weeks of treatment with ADV (20, 51). Tenofovir disoproxil fumarate (TDF), a nucleotide analog approved for therapy of infection with the human immunodeficiency virus (HIV), was also effective in HBV-infected patients who developed 3TC resistance (5, 7, 43, 45, 50, 59, 61, 63). Treatment with TDF for 24 to 71 weeks in HIV-co-infected patients demonstrated that HBV DNA concentrations decreased by approximately 4 to 5 log_{10} on average (5, 18, 31, 43, 45, 50, 62, 63). Furthermore, TDF treatment for 12 months of patients infected with 3TC-resistant HBV mutants led to average reductions in HBV DNA concentrations of 4.5 to 5.5 logs, which are similar to those observed in HBV/HIV co-infected patients (30, 62, 63). Because ADV and TDF effectively inhibit the replication of 3TC-resistant HBV mutants in HBV-infected patients, it has been hypothesized that co-administration of these drugs in combination with 3TC from the onset of treatment would prevent or significantly delay the emergence of 3TC-resistant HBV mutants. In fact, in tissue culture studies, combination of ADV with 3TC, emtricitabine (FTC), and other nucleoside and nucleotide derivatives resulted in additive or synergistic interactions and with no statistically significant antagonism (17, 52). Furthermore, combination therapy with TDF and 3TC for at least 12 months reduced HBV DNA concentration by 4.5 log_{10} in HBV/HIV-co-infected patients (25). Combination therapy with ADV and 3TC for up to 2 years in patients with chronic HBV infection reduced viremia by more than 3 log_{10} (47, 49). In another study in patients with chronic HBV infection, 24 weeks of treatment with ADV in combination with 3TC reduced HBV DNA concentrations by 3.6 log_{10}. 


Woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (Marmota monax), represent a well-characterized mammalian model for research on HBV including the pathogenesis of acute and chronic HBV infection, as well as for preclinical evaluation of the safety and efficacy of candidate antiviral drugs and therapeutic immunomodulators for the treatment of chronic HBV infection (39, 56) and prevention of HCC (58). In particular, the results of drug efficacy studies in the woodchuck have been predictive of responses in patients chronically infected with HBV (27).

In pharmacodynamic studies of 3TC in chronic WHV carrier woodchucks, 3TC-resistant mutations in the B domain of the polymerase gene developed after treatment for 9 to 12 months, and there was an associated recrudescence of serum WHV DNA to pretreatment levels (22, 24, 27, 36, 55). Treatment of chronic WHV carriers with FTC for 4 weeks produced a short-term antiviral profile similar to that of 3TC (23, 29). The antiviral effects of ADV and TDF in chronic WHV carrier woodchucks also were tested, and moderate but significant antiviral activity was demonstrated after 12 and 4 weeks of treatment, respectively (14, 40).

In the present placebo-controlled study, antiviral activity in the woodchuck model was determined for 48 weeks of treatment with 3TC, FTC, ADV, and TDF, alone and in combination. The results demonstrate that the combination of 3TC and ADV, and that of FTC and TDF were most effective in suppressing viral replication during chronic infection with wild-type WHV. The observed antiviral activity and favorable safety profile in woodchucks following drug treatment with doses comparable or higher than
those used in humans support the continued clinical development of combination
treatment regimens with ADV or TDF for long-term treatment of chronic HBV infection.

MATERIALS AND METHODS

Woodchucks. All experimental procedures involving woodchucks were performed
under protocols approved by the Cornell University Institutional Animal Care and Use
Committee. Woodchucks were born to WHV-negative females and reared in
environmentally controlled laboratory animal facilities at Cornell University.
Woodchucks were inoculated at 3 days of age with 5 million woodchuck infectious doses
of standardized WHV inoculums (WHV7P1 or cWHV7P2) (12). Woodchucks were
selected as chronic WHV carriers on the basis of the persistent detection of WHV surface
antigen (WHsAg) and WHV DNA in serum prior to initiation of treatments. All animals
were free of HCC at the beginning of the study as determined by hepatic ultrasound
examination and normal serum activity of \( \gamma \)-glutamyl-transferase (GGT).

Drug. FTC, ADV, and TDF were provided by Gilead Sciences, Inc. (Durham, NC).
3TC (Epivir®) was purchased from GlaxoSmithKline, Inc. (Philadelphia, PA). 3TC,
FTC, ADV, and TDF, alone and in combination, were administered orally to
woodchucks, once daily, for 48 weeks, by dose syringe (57), with one exception: TDF
was not administered to the TDF monotherapy group or to the TDF + 3TC and TDF +
FTC combination therapy groups during week 43 of treatment for a total of 6 days
because of a delay in drug supply. 3TC, ADV, and TDF were weighed and dissolved in
isotonic saline; FTC was obtained as a solution in isotonic saline. Immediately prior to
administration, all drugs were suspended in a semi-synthetic liquid diet formulated for
woodchucks (Dyets Inc., Bethlehem, PA). The liquid diet alone was administered daily as placebo to control woodchucks.

**Antiviral study.** Forty-five adult woodchucks, all chronically infected with WHV, were stratified equally by age, sex, body weight, serum viral load, and serum GGT activity into nine treatment groups of five animals each. Woodchucks were treated daily with oral doses of 3TC (15 mg/kg per day), FTC (15 mg/kg per day), ADV (15 mg/kg per day), TDF (15 mg/kg per day), ADV + 3TC (15 mg/kg per day each), ADV + FTC (15 mg/kg per day each), TDF + 3TC (15 mg/kg per day each), TDF + FTC (15 mg/kg per day each), or vehicle alone as placebo. The woodchucks were treated for 48 weeks (exceptions noted above) and were observed for an additional 12 weeks following cessation of treatment with drugs and placebo.

Dosages used in the present study for treatment of chronic WHV carrier woodchucks have been shown previously to be effective and safe in other studies in woodchucks (14, 23, 24, 26, 36, 40, 55, 70, 71). On the basis of metabolic body size, the per kilogram dose for woodchucks would be approximately three times that of the human dose. The standard therapeutic dose of 3TC for humans with chronic HBV infection is 100 mg per day or 1.43 mg/kg for a 70 kg patient. The scaled equivalent woodchuck dose would be 4.29 mg/kg, and the 15 mg/kg dose of 3TC used in the present study is approximately 3.5-fold higher compared to the clinical treatment dose in humans. The standard therapeutic dose of FTC for treatment of chronic HBV infection in humans is 200 mg or 2.86 mg/kg for a 70 kg patient and when scaled for woodchucks, the equivalent dose would be 8.58 mg/kg. The 15 mg/kg dose of FTC used in the present study is approximately 1.7-fold higher compared to the clinical treatment dose in humans. The
standard therapeutic dose of ADV for humans with chronic HBV infection is 10 mg per
day or 0.14 mg/kg for a 70 kg patient and when scaled for woodchucks, the equivalent
dose would be 0.43 mg/kg. The 15 mg/kg dose of ADV used in the present study is
approximately 35-fold higher compared to the clinical treatment dose in humans. TDF
has been shown to be safe and effective in humans with chronic HBV infection at
therapeutic doses ranging from 75 to 300 mg/kg or 1.07 to 3.21 mg/kg for a 70 kg patient.
The scaled equivalent woodchuck doses would be 3.21 to 12.86 mg/kg, and the 15 mg/kg
dose of TDF used in the present study is approximately 1.2 to 4.7-fold higher compared
to the clinical treatment dose in humans.

Blood samples were obtained for WHV DNA analysis and serological testing while
animals were under general anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg
intramuscularly). Samples were taken prior to drug administration on the first day of
treatment ("week 0"); at 1, 2, 3, 5, and 7 days of drug treatment; at 2, 3, 4, 6, and 8 weeks
of drug treatment; and then monthly until the end of drug treatment at week 48.

Thereafter, samples were obtained at 1, 2, 4, 6, 8, and 12 weeks following termination of
treatment. The woodchucks were weighed each time they were anesthetized and bled, and
drug dosages for individual woodchucks were based on the most recent body weight.

Serum WHV DNA was measured quantitatively by two different methods, depending
on concentration: either by dot blot hybridization (assay sensitivity, $\geq 1.0 \times 10^7$ WHV
genome equivalents per ml [WHVge/ml]) or by real time PCR (assay sensitivity, $\geq 1.0 \times
10^3$ WHVge/ml), as previously described (38). Serum WHsAg, antibodies to WHV core
antigen (anti-WHc), and WHV surface antigen (anti-WHs) were determined with WHV-
specific enzyme immunoassays (13) at the intervals described above.
Serum biochemical measurements were performed 2 weeks prior to drug administration, on the first day of treatment ("week 0") prior to drug administration, every other week through week 12 of drug treatment, and then monthly until the end of drug treatment at week 48. Thereafter, measurements were performed at 2, 4, 6, 8, and 12 weeks following termination of drug treatment. Complete blood counts were performed 2 weeks prior to drug administration; at 12, 36, and 48 weeks of drug treatment; and then at 12 weeks following termination of drug treatment. Serum chemistry measurements included serum GGT, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), total bilirubin, albumin, blood urea nitrogen (BUN), creatinine, Na⁺, K⁺, Cl⁻, bicarbonate, total serum iron, iron binding capacity, and percent iron saturation (57). Serum activities of AST, ALT, and SDH are markers of hepatocellular injury in woodchucks.

Liver biopsies were obtained prior to drug administration; at 12, 36, and 48 weeks of drug treatment; and then at 12 weeks following drug withdrawal. Biopsies were performed while the animals were under general anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg intramuscularly) with 16-gauge Bard Biopty-Cut (C.R. Bard Inc., Covington, GA) disposable biopsy needles directed by ultrasound imaging (46, 57). Aliquots of biopsy specimens were fixed in phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological analysis (i.e., portal hepatitis, lobular hepatitis, bile duct proliferation, steatosis, and liver cell dysplasia (46, 57). According to their severity, the specific lesions were graded on a scale of 0 to 4 (representing absent lesion to the most severe lesion, respectively). Sections of these tissues also were stained for intrahepatic WHV core antigen (WHcAg) and WHsAg.
detection using immunohistochemical methods (46, 57). Specimens stained for
cytoplasmic WHsAg were scored on a scale of 0 to 4 for determining the frequency of
hepatocytes expressing this antigen (1 = staining of up to 1% of hepatocytes, 2 = staining
of up to 2% of hepatocytes, 3= staining of up to 5% of hepatocytes, and 4= staining of
10% or more hepatocytes). A second aliquot of liver was placed immediately in liquid
nitrogen and stored at -70ºC until nucleic acid analyses were performed. Levels of
hepatic WHV DNA and WHV RNA were measured quantitatively by Southern or
northern blot hybridization as previously described (27, 57).

**Statistical analyses.** The antiviral effects of oral administration of 3TC, FTC, ADV,
and TDF, alone and in combination, were analyzed by comparing the log transformed
serum WHV DNA values during drug treatment (weeks 1-48) and following termination
of drug treatment (weeks 49-60), with the respective pretreatment (week 0) and placebo
control values. The antiviral effect induced by 3TC, FTC, ADV, and TDF, alone and in
combination, was assessed further by comparing the mean serum WHsAg levels for log
transformed values, the mean hepatic WHV nucleic acid values, the mean percentages or
scores of hepatocytes expressing WHV antigens, and the mean scores of portal and
lobular hepatitis during and following drug treatment with the pretreatment and placebo
control values. Statistical comparisons were performed using Student’s *t*-test (2-tailed),
Wilcoxon signed rank test, or Mann-Whitney *U* test (2-tailed, exact value,) with the SPSS
program 13.0 (SSPS Inc., Chicago, IL). Differences with *P*-values < 0.05 were
considered statistically significant.
RESULTS

Serum WHV DNA. The results on serum WHV DNA of individual woodchucks from all treatment groups are shown in Figure 1A-I and the mean serum WHV DNA values for the groups are shown in Figure 2A-D. No changes in serum WHV DNA were observed in four of five placebo-treated control woodchucks throughout the drug treatment period (Figures 1A, 2A). One of these four (M6539), however, had a remarkable decrease in serum WHV DNA late, at week 54 of the study. In the fifth woodchuck of this group (F3073), chronic WHV infection appeared to resolve spontaneously early on, as indicated by the stable reduction of serum WHV DNA from week 4 onward, with parallel reduction in serum WHsAg and development of anti-WHs antibody. Overall, throughout the study, the mean serum WHV DNA values in this group were not significantly different from the mean pretreatment value ($P > 0.05$).

Monotherapy with 3TC produced a gradual reduction in serum WHV DNA averaging 1.9 log$_{10}$ after the initial 12 weeks of treatment, with recrudescence to pretreatment levels shortly thereafter (Figures 1B, 2A&B). In three woodchucks (F3121, M3304, F7036), a later and further reduction in serum WHV DNA of 4-5 log$_{10}$ was observed beginning at 36 weeks of treatment which continued until the end of treatment. One woodchuck (F7033) had a marked and sustained reduction in serum WHV DNA nearly throughout the study. Mean serum WHV DNA levels in woodchucks treated with 3TC were significantly reduced from the pretreatment level at days 1-5 and between weeks 1 and 8 ($P < 0.05$), and were significantly lower than those in the placebo-control group at days 2-5 and at weeks 1 and 2 of treatment ($P < 0.05$). By the end of treatment at week 48, serum WHV DNA was reduced by an average of 2.7 log$_{10}$ from the pretreatment level.
After drug withdrawal, recrudescence of viral replication was observed in all woodchucks treated with 3TC.

Monotherapy with FTC induced a more rapid decline in serum WHV DNA than treatment with 3TC. Reductions in WHV DNA levels averaged $2.5 \log_{10}$ during the initial 6 to 8 weeks of treatment, with recrudescence in four of five woodchucks beginning at week 12 (Figures 1C, 2C&D). Mean serum WHV DNA levels were significantly reduced compared with the pretreatment level at days 2-5, between weeks 1 and 12, and again between weeks 28 and 52 of the study ($P < 0.05$), and were significantly lower than those in the placebo-control group at days 2-5 and at weeks 1 and 2 of treatment ($P < 0.05$).

The timing of the antiviral response varied across individual woodchucks. By the end of treatment at week 48, serum WHV DNA was reduced by an average of $2.0 \log_{10}$. Recrudescence of viral replication after treatment ended was immediate in all woodchucks.

Monotherapy with ADV resulted in a prompt reduction in serum WHV DNA, averaging $3.4 \log_{10}$ during the initial 12 weeks of treatment (Figures 1D, 2A&C). The antiviral responses in this group showed little individual variation during this time period. This initial reduction in serum WHV DNA was followed by recrudescence in all woodchucks starting between weeks 12 and 16 of treatment, and lasting until week 24. Serum WHV DNA became reduced again later between weeks 28 and 32 of treatment. Woodchuck F6554 was found dead during week 47 of treatment. The actual cause of death was not determined but giant cell hepatopathy was present. By the end of treatment, serum WHV DNA was reduced by an average of $4.8 \log_{10}$. After drug withdrawal, recrudescence of viral replication was observed immediately in three woodchucks and...
later, at week 52, in the fourth (M6556). Mean serum WHV DNA levels in this group were significantly reduced compared with the pretreatment level at days 1-5 and between weeks 1 and 44 of treatment ($P < 0.05$). Serum WHV DNA also was significantly less than that in the placebo control group at days 2-5, and between weeks 1 and 3 of treatment ($P < 0.05$).

Monotherapy with TDF produced a prompt decline in mean serum WHV DNA levels, averaging 3.6 log$_{10}$ after the initial 12 weeks of treatment, with a high degree of individual variation in the antiviral responses (Figures 1G, 2B&D). Pronounced and sustained reductions in serum WHV DNA were observed in three woodchucks (M3303, F6526, F7030). A less robust antiviral response was observed in a fourth woodchuck (F6525) during initial treatment, but by the end of the first 12 weeks, serum WHV DNA had returned to the pretreatment level, only to decline again at week 36. The fifth woodchuck (M3084) did not respond to TDF. Two woodchucks (F7030, M3303) were euthanized at week 41 and 44, respectively, because of seizures that were recognized clinically as a pre-terminal sign of HCC. By the end of treatment, serum WHV DNA was reduced by an average of 2.9 log$_{10}$. In one (F6525) of two woodchucks surviving after drug withdrawal, there was immediate recrudescence of viral replication, whereas in the second (F6526), the antiviral effect was sustained until the end of the study. Mean serum WHV DNA levels were significantly reduced compared with the mean pretreatment level at days 2-5, between weeks 1 and 6, and again between weeks 28 and 36 of treatment ($P < 0.05$). Serum WHV DNA levels also were significantly less than those in the placebo control group at days 2-5, and between weeks 1 and 3 of treatment ($P < 0.05$).
Combination therapy with ADV and 3TC resulted in a prompt reduction in mean serum WHV DNA levels, with an average $4.7 \log_{10}$ decrease after 12 weeks (Figures 1E, 2A). The initial antiviral responses of this group showed little individual variation. Recrudescence of viral replication during treatment was then observed in four woodchucks (F3221, M6521, F7034, M7039) beginning at week 16. Serum WHV DNA in these four woodchucks decreased again after 36 weeks of treatment. Woodchuck M7039 was euthanized at week 36 because of signs of advanced HCC. A sustained reduction in serum WHV DNA was observed in the fifth woodchuck (F7011). By the end of treatment, mean serum WHV DNA was reduced by an average of $6.2 \log_{10}$. After drug withdrawal, recrudescence of viral replication was observed in three woodchucks starting between weeks 49 and 52 of the study (M6521, F7011, F7034), and was delayed slightly longer in the fourth surviving woodchuck (F3221). Mean serum WHV DNA levels in this group were significantly reduced compared to the mean pretreatment level at days 1-5, and between weeks 1 and 36 of treatment ($P < 0.05$). Mean serum WHV DNA levels also were significantly lower than those in the placebo control group at days 1-5, and between weeks 1 and 3 of treatment ($P < 0.05$).

Combination therapy with ADV and FTC also resulted in a prompt reduction in mean serum WHV DNA, averaging $3.1 \log_{10}$ during the initial 12 weeks of treatment (Figures 1F, 2C). Beginning at week 16, recrudescence of viral replication was observed in four of five woodchucks (M3306, M6501, F6536, F7007). One woodchuck (M3306) was euthanized because of advanced HCC at week 22. Serum WHV DNA levels decreased again in the other three woodchucks (M6501, F6536, F7007) between weeks 24 and 28, and remained below pretreatment levels in two (M6501, F7007) until the time of death or
euthanasia related to HCC In the other woodchuck (F6536), serum WHV DNA remained below the pretreatment level until drug treatment ended, followed by immediate recrudescence. The fifth woodchuck in this group (F3122) had a more sustained antiviral response with recrudescence of viral replication prior to death during week 24 of treatment. Death was sudden and attributed to cecal torsion with infarction and peritonitis. Myocardial and skeletal muscle degeneration and necrosis, and mild vacuolar hepatopathy were present. However, no prodromal signs suggestive of nucleoside-induced mitochondrial toxicity had been observed. By the end of treatment, serum WHV DNA in the single surviving woodchuck (F6536) was reduced by 4.4 $\log_{10}$. Mean serum WHV DNA levels in this group were significantly reduced compared with the pretreatment level at days 2-5, and between weeks 1 and 24 of treatment ($P < 0.05$). Mean serum WHV DNA levels were also significantly less than those in the placebo control group at days 2-5, and between weeks 1 and 3 of treatment ($P < 0.05$).

Combination therapy with TDF and 3TC resulted in a prompt reduction in serum WHV DNA in four of the woodchucks (F3143, F6508, M7010, F7026), but in the fifth (M3082), no initial antiviral response was observed (Figures 1H, 2B). Woodchuck F3143 was found partially paralyzed during week nine of treatment and was euthanized. After 12 weeks of treatment, serum WHV DNA was decreased by an average of 3.7 $\log_{10}$. The antiviral response of one woodchuck (M7010) was sustained during treatment and following drug withdrawal. Two other woodchucks (F6508, F7026) showed recrudescence of viral replication starting at weeks 16 and 24 of treatment, respectively. Reduction in serum WHV DNA was observed again in both woodchucks between weeks 24 and 28, respectively. In the fifth woodchuck of this group (M3082), serum WHV
DNA started to decline at week 28 and decreased progressively until the end of treatment. By the end of treatment, serum WHV DNA was reduced on average by 5.6 log_10. After drug withdrawal, recrudescence of viral replication was observed in three surviving woodchucks (M3082, F6508, F7026), while the antiviral effect was sustained in the fourth woodchuck (M7010). Mean serum WHV DNA levels in this group were significantly reduced compared with the pretreatment level at days 2-5, and between weeks 1 and 8 of treatment (P < 0.05). Mean serum WHV DNA levels also were significantly less than those in the placebo control group at days 2-5, and between weeks 1 and 3 of treatment (P < 0.05).

Combination therapy with TDF and FTC induced a prompt decline in serum WHV DNA, averaging 4.4 log_10 during the initial 8 weeks of treatment, with individual variation in the antiviral response (Figures 1I, 2D). This was followed by recrudescence of viral replication in four woodchucks (M3305, F6524, F6531, F7004). The fifth woodchuck (M7040) had a sustained reduction in serum WHV DNA lasting until the time of death during week 59. Death was attributed to rupture of an aortic aneurysm, atherosclerosis and glomerulopathy, all consistent with the presence of hypertension. Advanced HCC was also present. In the other four woodchucks, serum WHV DNA started to decrease again between weeks 24 and 36. Woodchuck F7004 was euthanized during week 32 of treatment because of signs of advanced HCC. WHV DNA remained below pretreatment levels until the end of treatment in the three surviving woodchucks (M3305, F6524, and F6531). By the end of treatment, serum WHV DNA was reduced by an average of 6.1 log_10. After drug withdrawal, recrudescence of viral replication was observed in three woodchucks (M3305, F6524 and F6531), but at the end of the study,
serum WHV DNA in two (F6524 and F6531) again decreased remarkably. Mean serum
WHV DNA levels in this group were significantly reduced compared with the
pretreatment level at days 1-5, and between weeks 1 and 32 of treatment ($P < 0.05$).
Mean serum WHV DNA levels also were significantly less than those in the placebo
control group at days 2-5, and between weeks 1 and 3 of treatment ($P < 0.05$).

**Serum WHsAg.** Remarkable reductions were observed in the serum WHsAg of
individual woodchucks across all groups (Figure 3). One woodchuck each in the groups
receiving ADV (M6556), ADV + 3TC (F7011), and ADV + FTC (F3122), and two
woodchucks each in the groups receiving 3TC (F3121, F7033) and FTC (M3074, F7035),
had pronounced reductions in serum WHsAg during the 48 weeks of treatment. Three
woodchucks each in the groups treated with TDF (M3303, F6526, F7030), TDF + 3TC
(F3143, M7010, F7026), and TDF + FTC (F6524, F6531, M7040) had pronounced and
sometimes sustained reductions in serum WHsAg during treatment. In most drug-treated
woodchucks having reductions in serum WHsAg, the levels returned to the pretreatment
level immediately after drug withdrawal, but reductions were sustained in one
woodchuck each in the groups receiving TDF (F6526), TDF + 3TC (M7010), and TDF +
FTC (M7040 until the time of death during week 56). Two woodchucks from the
placebo-treated control group (F3073, M6539) had reductions in serum WHsAg, but the
decline was sustained only in the one control woodchuck in which WHV infection
spontaneously resolved (F3073). Overall, although remarkable and sometimes sustained
declines in serum WHsAg of individual woodchucks were observed, the mean serum
WHsAg levels in the drug-treated groups were not significantly different from those in
the placebo control group throughout the study ($P > 0.05$; Table 1). Comparison of serum
WHV DNA concentrations with serum WHsAg levels showed a direct relationship between the magnitudes of the reductions in serum viral DNA and serum antigenemia in individual woodchucks (Figures 1, 3).

**Serum anti-WHc and anti-WHs antibodies.** No meaningful significant differences in serum anti-WHc antibody levels were evident between the pretreatment determinations and those during or after treatment in any of the treatment groups, or between the placebo and drug-treated groups at any time point (data not shown). None of the drug-treated woodchucks developed anti-WHs antibody except for the one woodchuck from the group receiving ADV + FTC (F6536) that had low-level anti-WHs antibody throughout the study. In fact, a remarkable anti-WHs antibody response was detected only in the one woodchuck from the placebo-treated control group (F3073) in which WHV infection spontaneously resolved (data not shown). The observations on anti-WHs are important because they indicate the antiviral effects observed in drug-treated groups were not related to the spontaneous resolution of WHV infection.

**Hepatic WHV DNA replicative intermediates.** Remarkable reductions were observed in the hepatic concentrations of WHV DNA replicative intermediates (RI) of individual woodchucks across all groups (Figure 4). Hepatic WHV DNA RI concentrations returned to pretreatment levels in most woodchucks immediately or shortly after the end of treatment at week 48, but the reductions were sustained in one woodchuck from each of the groups receiving 3TC (F7033), TDF (F6526), TDF + 3TC (M7010), and TDF + FTC (F6524). Overall, mean hepatic WHV DNA RI levels were significantly reduced compared with the mean pretreatment level ($P < 0.05$) in several liver samples obtained through the end of treatment (weeks 12, 36, 48) and follow-up.
(week 60) in the groups receiving 3TC (week 48), FTC (weeks 36, 48), ADV (weeks 12, 36, 48), TDF (weeks 36, 48), ADV + 3TC (weeks 12, 36, 48, 60), TDF + 3TC (weeks 12, 36, 48), and TDF + FTC (weeks 12, 36, 48) (Table 1). Furthermore, hepatic WHV DNA RI were significantly lower than those in the placebo control group ($P < 0.05$) in the groups receiving ADV (weeks 12, 36), ADV + 3TC (weeks 12, 48), TDF + 3TC (weeks 36, 48), and TDF + FTC (week 36) (Table 1). After 12 weeks of treatment, combination therapy with ADV and 3TC produced the greatest antiviral effect on hepatic WHV DNA RI, with a 6.0-fold reduction from the pretreatment level. This was followed in rank order by monotherapy with ADV, combination therapy with TDF and 3TC, combination therapy with TDF and FTC, monotherapy with TDF or FTC, combination therapy with ADV and FTC, and monotherapy with 3TC. After 48 weeks of treatment, the combination of ADV and 3TC had the most sustained effect, with a 25.2-fold reduction in hepatic WHV DNA RI from pretreatment level. This was followed in rank order by combination therapy with TDF and 3TC, combination therapy with TDF and FTC, monotherapy with ADV, monotherapy with 3TC or FTC, and monotherapy with TDF. At the end of treatment, the single survivor of combination therapy with ADV and FTC had a 4.5-fold reduction in hepatic WHV DNA RI from pretreatment level.

**Hepatic WHV RNA.** Pronounced reductions also were observed in the hepatic WHV RNA of individual woodchucks from the various treatment groups during the 48 weeks of treatment (Figure 5). Hepatic WHV RNA returned to pretreatment levels in most woodchucks immediately after the end of treatment at week 48, but reductions were sustained in one woodchuck each in the groups receiving TDF (F6526), TDF + 3TC (M7010), and TDF + FTC (M7040). Overall, although remarkable declines in hepatic
WHV RNA of individual woodchucks were observed, the mean WHV RNA levels in the drug-treated groups were not significantly different from those in the placebo control group throughout the study ($P > 0.05$; Table 1).

**Hepatic WHcAg and WHsAg.** Transient reductions in the expression of WHcAg and of cytoplasmic WHsAg in hepatocytes were observed in individual woodchucks across all groups (Figures 6, 7). Individual variation was evident, with occasional differences between group mean values (Table 1). The percentages of hepatocytes staining positive for WHcAg were significantly reduced compared with that at pretreatment ($P < 0.05$) in the groups receiving ADV (weeks 12, 48), TDF (week 36), ADV + 3TC (weeks 12, 48), and TDF + FTC (weeks 12, 48). In the group treated with ADV + 3TC, the percentage of hepatocytes positive for WHcAg was significantly lower than that in the placebo control at week 12 ($P < 0.05$). Declines in the percentages of hepatocytes staining positive for WHsAg of individual woodchucks were also observed, but the declines in the drug-treated groups were not significantly different from the changes in the placebo control group throughout the study ($P > 0.05$).

**Histopathology.** Portal and lobular hepatitis were either absent or mild in all woodchucks before the start of treatment (Figures 8, 9). Transient reductions in portal and lobular hepatitis were observed in individual woodchucks from the various treatment groups but individual variation was evident, with occasional differences between group mean values (Table 1). At week 12 of treatment, portal hepatitis was reduced transiently in the groups receiving TDF and TDF + 3TC. A transient reduction in portal hepatitis also was observed in the group treated with TDF + FTC at week 36 of treatment. By the end of treatment at week 48, portal hepatitis was reduced in the group treated with TDF,
and this reduction was sustained until the end of the study at week 60. Reductions from pretreatment and differences between the drug-treated and placebo control groups in portal hepatitis, however, were not significant ($P < 0.05$). Transient reductions in lobular hepatitis were observed for the groups receiving 3TC and TDF at week 12 of treatment. At week 36 of treatment, lobular hepatitis was transiently reduced in the single surviving woodchuck from the group treated with ADF + FTC. By the end of treatment at week 48, lobular hepatitis was transiently reduced in the group treated with TDF + 3TC. By the end of the study at week 60, lobular hepatitis was again reduced in the group treated with TDF and in the single surviving woodchuck from the group treated with ADF + FTC. The reduction in lobular hepatitis observed in the group receiving TDF + 3TC was significantly greater than that in the placebo control at week 48 ($P < 0.05$). Overall, portal and lobular hepatitis remained mild in woodchucks across all experimental groups at the end of the study.

**Toxicity.** No clinical signs of toxicity were observed in woodchucks treated with 3TC, FTC, ADV, or TDF, alone or in combination. Changes in body weights of woodchucks in the drug-treated groups were similar to those of placebo-treated control woodchucks. The hematological and biochemical profiles of woodchucks in the drug-treated groups also were similar to those of placebo-treated controls during treatment and after drug withdrawal for most parameters (data not shown). One or more woodchucks in each experimental group had increases in serum SDH, GGT, ALT, AST, and ALP activity during the period of drug treatment (data not shown). Increases in SDH activity between weeks 4 and 24 of treatment in individual woodchucks almost always were associated with proportional elevations in ALT activity, suggesting increased hepatitic activity.
Increases in SDH activity between weeks 28 and 48 of treatment and after drug withdrawal almost always were associated with proportional increases in GGT, ALT, AST, and/or ALP activities, which suggested the development of HCC.

One woodchuck each from the groups receiving ADV + FTC (F3122), ADV (F6554), and TDF + FTC (M7040) died unexpectedly during week 24, 47, or 59 of the study, respectively, but the deaths were not attributed to drug treatment. One woodchuck treated with TDF + 3TC (F3143) was discovered partially paralyzed during week nine of treatment and was euthanized. Seven other woodchucks were euthanized or found dead during the period of drug treatment because of seizures that were recognized clinically as a pre-terminal sign of HCC. All seven of these woodchucks had developed pronounced elevations in serum GGT activity, and HCC was evident by ultrasound examination. Three of these seven woodchucks from the group receiving ADV + FTC (M3306, F7007, M6501) were euthanized or found dead during week 22, 29, or 41 of treatment, respectively; two others that had received TDF (F7030, M3303) were euthanized during week 41 and 44 of treatment, respectively; one each from the groups receiving TDF + FTC (F7004) and ADV +3TC (M7039) were euthanized at weeks 32 and 36 of treatment, respectively.

DISCUSSION

In this placebo-controlled study, the antiviral activity of oral dosing with 3TC, FTC, ADV, and TDF, alone or in combination, was assessed against WHV in chronically infected woodchucks. In the woodchuck model of chronic HBV infection, suppression of WHV replication in serum and liver in vivo was significantly greater in woodchucks
treated with combination therapy of ADV and 3TC or TDF and FTC than in placebo-
treated control animals, and no evidence of toxicity was observed during the 48-week
period of daily oral administration.

During the initial 12 weeks of treatment, the combination of ADV + 3TC produced
the greatest antiviral response, reducing serum WHV viremia from pretreatment level by
4.7 log_{10}. This was followed in rank order by TDF + FTC (4.2 log_{10}), TDF + 3TC (3.7
log_{10}), TDF alone (3.6 log_{10}), ADV alone (3.4 log_{10}), ADV + FTC (3.1 log_{10}), 3TC alone
(1.9 log_{10}), and FTC alone (1.7 log_{10}). After 48 weeks of treatment, the combination of
ADV + 3TC or of TDF + FTC produced the most sustained antiviral response, reducing
serum viremia from the pretreatment level by 6.2 and 6.1 log_{10}, respectively. This was
followed by TDF + 3TC (5.6 log_{10}), ADV alone (4.8 log_{10}), ADV + FTC (one survivor,
4.4 log_{10}), TDF alone (2.9 log_{10}), 3TC alone (2.7 log_{10}), and FTC alone (2.0 log_{10}).

In general, the antiviral responses to the drugs and drug combinations in the present
study varied across individual woodchucks, with the exception that minimal variation
was noted for monotherapy with ADV. Varying degrees of recrudescence of viral
replication were observed in all drug-treated groups from 12 to 24 weeks, with a
progressive reduction in serum viremia during the remainder of drug treatment.
Recrudescence of viral replication after drug withdrawal was observed in most
woodchucks. Woodchucks with pronounced and sustained reductions in serum viremia
had the most pronounced declines in serum WHs antigenemia, hepatic WHV DNA RI
and WHV RNA, and expression of viral antigens in liver. Treatment of woodchucks for
48 weeks did not elicit detectable antibodies against WHsAg in any of the drug-treated
groups.
The generally robust antiviral responses observed during the initial 12 weeks in the groups of woodchucks treated with ADV or TDF alone, or in combination with 3TC and FTC, were followed by recrudescence of WHV replication, which characteristically peaked after 24 weeks. Beginning at 30 weeks, serum viremia in these groups decreased progressively until the end of the 48-week drug treatment period. The reason for the transient viral recrudescence is not known and has not been observed previously in long-term antiviral studies in woodchucks treated with 3TC, entecavir, or L-FMAU (clevudine) (8, 24, 36, 41, 55, 71). Partial sequencing of the WHV polymerase gene at week 24 of treatment indicated that recrudescence of viral replication was not related to the development of drug-resistant mutations within the B domain (data not shown). This finding is consistent with the observation that recrudescence of viral replication was transient, and that pronounced reductions in serum viremia then were observed during the remainder of drug treatment.

Laboratory woodchucks, like their counterparts that live in the native habitat, have a rigorous endogenous circannual metabolic and reproductive rhythm. Food intake and metabolic rate are significantly reduced in the laboratory woodchuck from August until December when increased metabolism and reproductive activity begin. Following completion of the breeding season in March and April, food intake and metabolic rate increase significantly, and this is associated with a significant increase in body weight, primarily in the form of body fat. Food intake then decreases significantly beginning in July and August and the annual cycle is repeated (9-11). Drug treatment in this study was initiated in January, and the height of recrudescence of serum WHV DNA was observed after 24 weeks, during the month of June, when food intake and body weight gain both
would be maximal. The role of circannual metabolic and hormonal changes in
determining the observed differences in antiviral response is not known. It can only be
speculated that the conversion of antiviral drugs to their active metabolites might have
been influenced by season or age of the experimental woodchucks. Blood levels of drug
also may have varied during treatment either because of changes in intestinal absorption
or in the rate of drug clearance. However, seasonal changes in antiviral response
independent of drug-resistant mutations have not been reported previously in long-term
antiviral studies in woodchucks (8, 24, 36, 41, 55, 71).

The present results on antiviral effects are consistent with those from long-term
antiviral studies of 3TC and ADV in woodchucks, and of 3TC, FTC, ADV, or TDF on
duck HBV (DHBV) and HBV in transiently or stably transfected cells and primary duck
hepatocytes (14, 15, 17, 24, 26, 36, 52, 55, 67-69, 71).

The magnitude of reduction in serum WHV viremia after 12 or 48 weeks of treatment
with 3TC, FTC, ADV, or TDF, and the time to viral recrudescence following drug
withdrawal observed in this study, were similar to those previously reported for some
antiviral drugs after long-term administration to chronic WHV carrier woodchucks.

Treatment with 3TC for 24 weeks reduced serum WHV DNA by 1.5 \( \log_{10} \), and WHV
DNA returned to pretreatment levels within 1 or 2 weeks (26). Treatment with interferon
alpha for 24 weeks reduced serum WHV viremia by 2.2 \( \log_{10} \). WHV DNA returned to
pretreatment levels in 1 to 2 weeks in the majority of woodchucks but was extended by 8
to 12 weeks in one woodchuck (26). Treatment with ADV for 12 weeks reduced serum
WHV viremia by 2.5 \( \log_{10} \), and WHV DNA returned to pretreatment levels within 6
weeks following drug withdrawal (14).
Treatment of chronic WHV carrier woodchucks with other antiviral drugs, however, induced greater antiviral effects on serum WHV viremia after long-term treatment than were observed in this study. Daily treatment with entecavir for 8 weeks, and then weekly treatment for 14 or 36 months reduced serum WHV DNA by 5 to 8 log_{10} (8). Treatment with L-FMAU (clevudine) for 32 weeks reduced serum WHV viremia by more than 8 log_{10} in most woodchucks (28, 41). Recrudescence of viral replication was not observed until 8 weeks after drug withdrawal at the earliest in a few woodchucks, and the antiviral effect was sustained much longer in the majority of drug-treated woodchucks (i.e., through the end of the study).

A significant percentage of patients infected with HIV are co-infected with HBV and are at risk of developing HBV-associated liver diseases. Many patients co-infected with HIV and HBV have received 3TC as part of their combination therapy for HIV. Antiviral activity against HBV in such patients is characteristically transient because of the development of 3TC resistance (6, 18, 21, 60). In several recent studies, HIV patients with 3TC-resistant HBV infection have been treated with TDF or ADV and a significant inhibition of HBV replication was demonstrated (1, 5, 7, 18, 43, 45, 47, 49-51). A combination of TDF + FTC has been used to treat patients co-infected with HIV and HBV, and robust anti-HBV response was observed with the drug combination which was reversed upon withdrawal of the combination (44).

Combination antiviral therapy currently is the standard of care for patients with HIV infection. Hypothetical advantages of combination therapy for HBV infection include the prevention or delay in development of drug resistant viral mutations and possible synergistic or additive antiviral drug interactions. Possible disadvantages include negative
antiviral drug interactions and enhanced drug toxicity (33). During treatment of chronic 
WHV carrier woodchucks, monotherapy with either 3TC or FTC was modest and inferior 
to monotherapy with either ADV or TDF. At the end of the 48 week period of treatment, 
the combination of 3TC with either ADV or TDF was greater than ADF or TDF 
monotherapy. Similarly, FTC combined with TDF was greater than TDF at the end of 
treatment. However, the ADV + FTC combination was not superior to ADV 
monotherapy. It was not possible to assess the inhibitory effect of drug combinations on 
the development of drug resistant mutants because no such mutants had been detected at 
the end of the 48 week period of drug treatment in the monotherapy groups. No negative 
drug interactions were observed in groups that received combinations of drugs. The 
durability of the antiviral response was not increased by combination therapy because 
recrudescence of viremia after drug treatment ended was similar in monotherapy and 
combinations groups.

There is increasing interest in the clinical use of drug combinations for treatment of 
chronic HBV infection (33, 42). In HBV patients, the effect of ADV combined with 3TC 
on viral load was no greater than that associated with ADV monotherapy initially but 
after two years, the viral load of those receiving the drug combination was lower than that 
of the ADV monotherapy group and rates of 3TC resistance were significantly lower in 
the combination group (53). TDF monotherapy has been shown to be safe and highly 
effective in HBV-infected patients in whom ADV therapy had failed (54).

The results of the present study in woodchucks indicate that stronger antiviral effects 
were observed with drug combinations than with monotherapies after 48 weeks of 
treatment. At the doses used, treatment with ADV in combination with 3TC or treatment
with TDF in combination with FTC produced sustained antiviral responses and led to significant reductions in the concentrations of serum WHV DNA, hepatic WHV DNA RI, and hepatic WHV RNA, and the hepatic expression of WHcAg and WHsAg in the woodchuck model of chronic HBV infection. Significant suppression of viral replication also was observed with TDF in combination with 3TC. Forty-eight weeks of therapy with 3TC, FTC, ADV, and TDF, alone or in combination, at oral doses of 15 mg/kg per day for each drug were well tolerated in WHV-infected woodchucks and produced no physical, biochemical, or hematological evidence of toxicity. The antiviral activity and the apparently favorable safety profile observed in woodchucks with drug doses comparable or higher than those used in humans support the continued clinical development of combination treatments with ADV or TDF for long-term treatment of chronic HBV infection.

ACKNOWLEDGEMENTS

This work was supported by contract N01-AI-05399 (College of Veterinary Medicine, Cornell University) from the National Institute of Allergy and Infectious Diseases (NIAID).

We gratefully acknowledge the expert assistance of Mary Ascenzi, Betty Baldwin, Lou Ann Graham, Erin Graham, David Dietterich, and Dr. Chris Bellezza (Cornell University).

REFERENCES

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FIGURES

Figure 1. Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on serum WHV DNA in chronic WHV carrier woodchucks. Treatment groups: A. Placebo. B. 3TC. C. FTC. D. ADV. E. ADV + 3TC. F. ADV + FTC. G. TDF. H. TDF + 3TC. I. TDF + FTC. Horizontal bars denote the 48-week treatment period. Log₁₀ changes in serum WHV DNA from baseline at week 0 prior to drug administration for individual woodchucks in each treatment group are displayed. WHVge, WHV genomic equivalents (virion or WHV DNA-containing virus particles).

Figure 2. Comparison of antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on serum WHV DNA in chronic WHV carrier woodchucks. A. Monotherapy with ADV or 3TC and combination therapy with ADV and 3TC. The placebo control group is displayed for comparison B. Monotherapy with TDF or 3TC and combination therapy with TDF and 3TC. C. Monotherapy with ADV or FTC and combination therapy with ADV and FTC. D. Monotherapy with TDF or FTC and combination therapy with TDF and FTC. Horizontal bars denote the 48-week treatment period. Mean log₁₀ changes in serum WHV DNA from baseline at week 0 prior to drug administration for each treatment group are displayed. Vertical lines denote standard deviations. Each group contained five woodchucks at the start of treatment. Differences in geometric mean serum WHV DNA concentrations from pretreatment values were
significant for the following treatment groups and time points ($P < 0.05$): 3TC, days 1-5 and weeks 1-8; FTC, days 2-5 and weeks 1-12 and 28-52; ADV, days 1-5 and weeks 1-44; TDF, days 2-5 and weeks 1-6 and 28-36; ADV + 3TC, days 1-5 and weeks 1-36; ADV + FTC, days 2-5 and weeks 1-24; TDF + 3TC, days 2-5 and weeks 1-8; and TDF + FTC, days 1-5 and weeks 1-32 of the study. Differences in geometric mean serum WHV DNA concentrations from those in the placebo control group were significant for the following treatment groups and time points ($P < 0.05$): 3TC, days 2-5 and weeks 1-2; FTC, days 2-5 and weeks 1-2; ADV, days 2-5 and weeks 1-3; TDF, days 2-5 and weeks 1-3; ADV + 3TC, days 1-5 and weeks 1-3; ADV + FTC, days 2-5 and weeks 1-3; TDF + 3TC, 2-5 days and weeks 1-3; and TDF + FTC, days 2-5 and weeks 1-3 of treatment.

WHVge, WHV genomic equivalents (virion or WHV DNA-containing virus particles).

**Figure 3.** Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on serum WHsAg in chronic WHV carrier woodchucks. Treatment groups: 
A. Placebo.  
B. 3TC.  
C. FTC.  
D. ADV.  
E. ADV + 3TC.  
F. ADV + FTC.  
G. TDF.  
H. TDF + 3TC.  
I. TDF + FTC. Horizontal bars denote the 48-week treatment period. Serum WHsAg concentrations for individual woodchucks in each treatment group are displayed.

**Figure 4.** Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on hepatic WHV replication in chronic WHV carrier woodchucks. Treatment groups: 
A. Placebo. Hepatic tissue was not available from M7006 at week 60.
B. 3TC. Hepatic tissue was not available from F3121 at week 48. C. FTC. D. ADV. Hepatic tissue was not available from F6554 at weeks 48 and 60 and from F7028 at week 48. E. ADV + 3TC. Hepatic tissue was not available from M7039 at weeks 48 and 60. F. ADV + FTC. Hepatic tissue was not available from F3122, M3306, M6501, and F7007 at weeks 36, 48, and 60. G. TDF. Hepatic tissue was not available from M3003 and F7030 at weeks 48 and 60. H. TDF + 3TC. Hepatic tissue was not available from F3143 at weeks 12, 36, 48, and 60 and from M3082 at week 60. I. TDF + FTC. Hepatic tissue was not available from F7004 at weeks 36, 48 and 60 and from M7040 at week 60. WHV DNA RI, WHV DNA replicative intermediates. Levels of hepatic cellular DNA were quantified by hybridization to a woodchuck-specific β-actin gene probe by Southern blot hybridization technique.

Figure 5. Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on hepatic WHV RNA in chronic WHV carrier woodchucks. Treatment groups: A. Placebo. B. 3TC. C. FTC. D. ADV. E. ADV + 3TC. F. ADV + FTC. G. TDF. H. TDF + 3TC. I. TDF + FTC. For availability of hepatic tissue of individual woodchucks from the experimental groups see legend to Figure 4. Levels of hepatic cellular RNA were quantified by hybridization to a woodchuck-specific 18S gene probe by northern blot hybridization technique.

Figure 6. Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in
Combination, on hepatic expression of WHcAg in chronic WHV carrier woodchucks.

Treatment groups: **A.** Placebo. **B.** 3TC. **C.** FTC. **D.** ADV. **E.** ADV + 3TC. **F.** ADV + FTC. **G.** TDF. **H.** TDF + 3TC. **I.** TDF + FTC. For availability of hepatic tissue of individual woodchucks from the experimental groups see legend to Figure 4.

**Figure 7.** Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adeovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on hepatic expression of cytoplasmic WHsAg in chronic WHV carrier woodchucks. Treatment groups: **A.** Placebo. **B.** 3TC. **C.** FTC. **D.** ADV. **E.** ADV + 3TC. **F.** ADV + FTC. **G.** TDF. **H.** TDF + 3TC. **I.** TDF + FTC. For availability of hepatic tissue of individual woodchucks from the experimental groups see legend to Figure 4.

According to the number of hepatocytes, staining was scored on a scale of 0 to 4 (1 = staining of up to 1% of hepatocytes, 2 = staining of up to 2% of hepatocytes, 3 = staining of up to 5% of hepatocytes, and 4 = staining of 10% or more hepatocytes).

**Figure 8.** Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adeovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on portal hepatitis in chronic WHV carrier woodchucks. Treatment groups: **A.** Placebo. **B.** 3TC. **C.** FTC. **D.** ADV. **E.** ADV + 3TC. **F.** ADV + FTC. **G.** TDF. **H.** TDF + 3TC. **I.** TDF + FTC. For availability of hepatic tissue of individual woodchucks from the experimental groups see legend to Figure 4. According to their severity, the specific lesions were graded on a scale of 0 to 4 (representing absent lesion to the most severe lesion, respectively).
Figure 9. Antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on lobular hepatitis in chronic WHV carrier woodchucks. Treatment groups: A. Placebo. B. 3TC. C. FTC. D. ADV. E. ADV + 3TC. F. ADV + FTC. G. TDF. H. TDF + 3TC. I. TDF + FTC. For availability of hepatic tissue of individual woodchucks from the experimental groups see legend to Figure 4. According to their severity, the specific lesions were graded on a scale of 0 to 4 (representing absent lesion to the most severe lesion, respectively).
Change in serum WHV DNA (log_{10} WHV ge/mL serum)

Time (Weeks)

Figure 1
Figure 2

Change in mean serum WHV DNA (log_{10} WHV ge/ml serum)

Figure 2
Figure 3
Figure 4
Figure 5

Hepatic WHV RNA (pg/µg total cell RNA) vs. Time (Weeks)

A. Placebo Control
B. TDF
C. FTC
D. ADV
E. ADV + 3TC
F. ADV + FTC
G. TDF
H. TDF + 3TC
I. TDF + FTC
Figure 6
Figure 7
Figure 8
Figure 9
Table 1. Comparison of antiviral effect of oral lamivudine (3TC), emtricitabine (FTC), adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate (TDF) administration, alone and in combination, on serum WHsAg, hepatic WHV DNA replicative intermediates and WHV RNA, hepatic expression of WHcAg and cytoplasmic WHsAg, and portal and lobular hepatitis in chronic WHV carrier woodchucks at selected time points during the study.

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<th>Treatment</th>
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<th>Number of animals</th>
<th>Geometric mean serum WHsAg (log₁₀ ng/ml)</th>
<th>Mean hepatic WHV DNA RI (pg/µg total cell DNA)</th>
<th>Mean hepatic WHV RNA (pg/µg total cell RNA)</th>
<th>Mean WHcAg-positive hepatocytes (%)</th>
<th>Mean WHsAg-positive hepatocytes (score)</th>
<th>Mean portal hepatitis (score)</th>
<th>Mean lobular hepatitis (score)</th>
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<td>36 wk treatment (wk 36) 5</td>
<td>5.2 ± 0.2</td>
<td>1133 ± 443</td>
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<td>59 ± 9</td>
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a, Each group contained five woodchucks at the start of treatment. Values are means ± standard deviations.
b, Difference from pretreatment value was statistically significant (P < 0.05).
c, Difference from placebo control group was statistically significant (P < 0.05).

Hepatic staining for cytoplasmic WHsAg was scored on a scale of 0 to 4 (1 = staining of up to 1% of hepatocytes, 2 = staining of up to 2% of hepatocytes, 3= staining of up to 5% of hepatocytes, and 4= staining of 10% or more hepatocytes). According to their severity,
portal and lobular hepatitis were graded on a scale of 0 to 4 (representing absent lesion to the most severe lesion, respectively). WHV DNA RI, WHV DNA replicative intermediates.