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Enhanced antiviral benefit of combination therapy with lamivudine and famciclovir against WHV replication in chronic WHV carrier woodchucks

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Abstract

Cell culture studies in our laboratory and others have previously demonstrated synergistic antiviral activity for combinations of 3TC (lamivudine) and penciclovir against Hepatitis B Virus (HBV) replication and the Duck Hepatitis B Virus (DHBV). Based on these results, a study was designed to determine if an enhanced antiviral effect with combinations of 3TC and famciclovir (FCV, oral prodrug of penciclovir) could be demonstrated in vivo using the Woodchuck Hepatitis Virus (WHV)/woodchuck experimental model of chronic HBV infection. Both antiviral agents have been shown to be effective against WHV replication in WHV chronic carriers in previous studies by our laboratories. The antiviral effects of four different combinations of lamivudine and FCV were found to be greater than those observed for the corresponding monotherapies. All four combination treatments produced antiviral effects that were at least equal to that expected for additive activity based on estimations generated by Bliss Independence calculations. Two of the combination treatments produced antiviral effects that were significantly greater than that expected for additive effects, indicative of synergistic antiviral interactions. These studies demonstrate that combination therapy of chronic WHV infection has enhanced antiviral benefit over corresponding monotherapies and indicate that combination treatment of chronic HBV infection can be superior to therapies using a single antiviral agent.

Keywords: Woodchuck Hepatitis Virus; Hepatitis B Virus; Combination antiviral therapy; Experimental animal model; Lamivudine; Famciclovir

1. Introduction

Several promising nucleoside analogues have been developed for use against chronic Hepatitis

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B virus (HBV) infection, including lamivudine (3TC) which has been recently licensed for use against HBV infection. However, clinical trials have demonstrated that these agents fail to permanently eliminate virus replication in the vast majority of patients, even after prolonged administration lasting up to 2 years. This usually leads to a rapid rebound in virus production once treatment is discontinued. In some cases, viruses resistant to these agents have been observed, further complicating the ability to effectively suppress HBV infection (Pillay et al., 1998). As observed in HIV-infected patients, viral load has been shown to be correlated with the progression of liver disease in HBV chronic carriers treated with lamivudine (Jaeckel and Manns, 1997; Lai et al., 1997). Clearly, more aggressive control of HBV replication than that which can be provided by monotherapy is needed.

Since multiple drug therapy has been shown to be more effective than single drug therapy in combating other viral infections, such as HIV, it is reasonable to expect that combination therapies with two or more drugs may be able to more effectively suppress HBV replication. Cell culture studies in the human HBV-producing hepatoblastoma cell line, 2.2.15, previously reported by our laboratories have demonstrated potential enhanced effectiveness of combination therapies to the treatment of chronic HBV infection (Korba, 1996; Zembower et al., 1998; Perigaud et al., 1999). One of these studies demonstrated that combination treatments with 3TC and penciclovir (PCV) increased the relative potencies of these agents 10-fold above those observed for the corresponding monotherapies (Korba, 1996). A similar enhancement of antiviral effectiveness has also been observed for combinations of 3TC and PCV against Duck Hepatitis B Virus (DHBV) replication in cultures of primary duck hepatocytes (Colledge et al., 1997). Lamivudine (3TC) and famciclovir (FCV, oral prodrug of penciclovir) have been shown to be highly effective inhibitors of hepadnaviral replication in cell culture, experimental animal models, and in human clinical trials (Korba and Boyd, 1996; Bartholomeusz et al., 1997; Jaeckel and Manns, 1997; Lai et al., 1997; Lin et al., 1998; Korba et al., 2000).

The woodchuck hepatitis virus (WHV) and its natural host, the Eastern Woodchuck *(M. monax)*, constitute a useful model of HBV-induced disease, including HCC (Tennant et al., 1988; Gerin et al., 1991). Multiple published studies have utilized the WHV/woodchuck system to investigate potential antiviral therapies for chronic HBV infection (Fourel et al., 1990; Korba et al., 1991; Cullen et al., 1997; Gangemi et al., 1997; Tencza and Newbold, 1997; Block et al., 1998; Genovesi et al., 1998; Hurwitz et al., 1998; Mason et al., 1998; Tennant et al., 1998a,b; Korba et al., 2000). In several of these studies, the response of WHV replication to the antiviral therapies and the toxic effects resulting from the treatment regimens paralleled observations in human clinical trials.

This report discusses the results of the treatment of chronically-infected woodchucks with combinations of 3TC and FCV. These studies demonstrate that combination therapy confers an enhanced suppression of WHV replication in vivo over the corresponding monotherapies, indicating that multiple drug therapies may be of enhanced benefit to the control of chronic HBV infection in man.

2. Material and methods

2.1. Woodchucks

The woodchucks used in these studies were born to WHV-negative females in a breeding colony maintained at Cornell University. Animals were inoculated at 3 days of age with 5 million woodchuck infectious doses of a standardized WHV inoculum pool (WHVp1). This inoculum produces a 65–70% chronic WHsAg carrier rate under these conditions (Tennant et al., 1988). The diet consisted of laboratory animal chow formulated for rabbits (Agway Red Rabbit Food, Syracuse, NY), specially pelleted in blocks for woodchucks. Diet and water were provided ad libitum. Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, revised 1996) and were reviewed and approved by

the Cornell University Institutional Animal Care and Use Committee.

2.2. Experimental design

Nine groups of four WHV carriers each (14–20 months old) were used. One group served as placebo control. Each antiviral agent was used at two doses for monotherapy (one group of animals for each dose). FCV was administered at either 15 or 50 mg/kg and 3TC was administered at 1.0 or 5.0 mg/kg. The remaining four groups of animals each received one combination of these drug doses: 1.0 mg/kg 3TC plus 15 mg/kg FCV, 1.0 mg/kg 3TC plus 50 mg/kg FCV, 5.0 mg/kg 3TC plus 15 mg/kg FCV, and 5.0 mg/kg 3TC plus 50 mg/kg FCV. Treatment was administered once daily for 12 weeks and the animals were followed for an additional 12 weeks post-treatment. FCV and 3TC were given orally in a liquid diet as previously described (Tennant et al., 1998a). Famciclovir was purchased as Famvir™ tablets and 3TC was purchased as Epivir™ tablets though the pharmacy of the Veterinary Hospital of Cornell University. Tablets were ground into powder prior to addition to the liquid diet.

The doses used for this study were based on previous studies in woodchucks conducted by our laboratories (Hurwitz et al., 1998; Korba et al., 2000). The lower doses of drugs were selected to produce minimal to no antiviral effect as monotherapies, and the higher doses were selected to produce moderate, but significant reductions in WHV replication as monotherapies. The monotherapies were selected to induce only modest antiviral effects in order to allow for the discrimination of potential additive or synergistic antiviral effects by the combination treatments.

Serum samples were taken for analysis 1 week prior to the initiation of treatment, on the first day of treatment ('day 0'), at weeks 1, 2, 3, 4, 6, 8, and 12 of treatment, and at 1, 2, 4, 8, and 12 weeks following the end of therapy. Liver biopsies were obtained 1 week pretreatment, at weeks 4 and 12 of treatment, and at 4 and 12 weeks post-treatment.

Serum and liver biopsy specimens were obtained while the animals were under general anesthesia (ketamine/xylazine) as previously described (Tennant et al., 1998a). The general health of the woodchucks was assessed by daily observation at the time they received food and water, at the time of drug (or placebo) administration, and at the times they were anesthetized as previously described (Tennant et al., 1998a). Any abnormalities in behavior, appearance, or food/water intake were recorded. Body weights were monitored weekly when serum and/or liver specimens were taken. Hematologic and routine blood chemistry analyses were performed at the initial pretreatment biopsy, the beginning of treatment, and at 4-week intervals during the experimental periods as previously described (Tennant et al., 1998a).

2.3. WHV markers

WHV viremia in serum samples was assessed by two quantitative methods depending on the concentration of WHV DNA. Serum containing concentrations of WHV virion DNA above 1×10^8 WHV genome equivalents per milliliter of serum (WHVge/ml) were analyzed by dot blot hybridization (four, 10-μl replicates per sample) as previously described (Korba et al., 1986; Tennant et al., 1998a). Samples containing WHV DNA below the dot blot sensitivity cut-off were analyzed using quantitative PCR-based methods (Tennant et al., 1998a; Morrey et al., 1999). Serum samples were analyzed in duplicate. WHV DNA was prepared for amplification by extraction using GeneReleaser™ matrix (Bioventures, Murfreesboro, TN). For the extraction step, 5 μl of serum and 15 μl of GeneReleaser™ were added to 0.2-ml PCR reaction tubes and incubated in a thermocycler with a heated lid (Touchdown™, Hybaid, Middlesex, UK) using the following program: 37°C, 30 s/8°C, 30 s/65°C, 90 s/97°C, 180 s/8°C, 60 s/65°C, 180 s/97°C, 60 s/65°C, 60 s/80°C, 30 min. Following the lysis incubation procedure, the remaining components of the PCR reaction mixture were added (final reaction volume of 40 μl), and the reaction tubes were subjected to the following amplification program: 94°C, 2 min/94°C, 1 min/ 55°C, 1 min/72°C, 1 min/(repeat steps 2–4, 34 times)/hold at 4°C. The final concentration of the reaction components were: 10 mM Tris–HCl

(pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 25 mM dNTPs, 12.5 mM primers, 2.5 U Taq polymerase (Boehringer Mannheim). The oligonucleotide primers used for amplification were TGCTCTC-CGCACCATACAGC (5′ primer, bases 2162–2181) and CCGCGTCGCAGACGCTCTCA-ATCT (3′ primer, bases 2546–2569). These primers were chosen based on the empirical testing of several different primer sets scattered around the WHV genome that were identified by the MacVector™ (Oxford Molecular Group, Oxford, UK) computer program for reproducibility and quantitative yield of WHV DNA in dilution series of woodchuck serum samples containing previously determined amounts of WHVge/ml (Tennant et al., 1988, 1998a).

Following PCR amplification, 60 μl distilled, sterile, filtered water was added to each tube and the samples were centrifuged ($15\,000 \times g$) for 1 min. The contents were transferred to 1.1-ml plastic tubes and 300 μl of 1:1 37% formaldehyde/$20 \times$ SSC was added (Korba et al., 1986). Following 45 min of incubation at 60°C, the samples were applied to nitrocellulose membranes and rinsed with $20 \times$ SSC using a 96-well dot blot manifold (GIBCO-BRL, Gaithersburg, MD), and hybridized to a ^{32}P-labelled, 3.2 kb cloned WHV DNA fragment as previously described (Korba et al., 1986; Tennant et al., 1998a).

Assessments of the level of WHV genome equivalents for the PCR analyses were determined by direct comparisons to parallel PCR amplification of a dilution series (1000–1 000 000 WHVge/ml) of the standardized serum pool (WHV7p1) used to infect these animals which contains a known WHV genome content (Tennant et al., 1988, 1998a). The dilution standards were included, in duplicate, with each run of the thermocycler and were blotted, in duplicate, on each hybridization membrane containing the test samples amplified in the same thermocycler run. Two negative controls were also included, in duplicate, in the standards: uninfected woodchuck serum used for the dilutions and the water/buffers used for the PCR components. The sensitivity cut-off of this procedure is approximately 500 WHVge/ml.

Levels of WHsAg and the presence of anti-WHs and anti-WHc antibodies in serum samples were assessed as previously described using WHV-specific RIA and EIA assays (Wong et al., 1982; Cote et al., 1984; Ponzetto et al., 1984; Cote et al., 1993).

Levels of intrahepatic WHV nucleic acids were quantitatively determined by Southern or Northern blot hybridization as previously described (Korba et al., 1986; Tennant et al., 1998a).

2.4. Evaluation of drug interactions

Statistical comparisons of the levels of WHV nucleic acids were performed using Students two-tailed *t*-test with corrections for small sample sizes. Evaluation of combination treatments for evidence of synergistic, additive, or antagonistic drug interactions was performed using the methods first described by Bliss (Bliss, 1939; Prichard and Shipman, 1996).

3. Results

Treatment of chronic WHV carrier woodchucks with 1 mg/kg 3TC induced a sevenfold decline in WHV viremia after 12 weeks of treatment (Figs. 1 and 3, Table 1). No change in the level of hepatic WHV DNA replication intermediates (WHV RI) was apparent following 12 weeks of therapy with 1 mg/kg 3TC (Figs. 2 and 3, Table 1). Hepadnaviral DNA replication intermediates in liver tissue are comprised of a heterogeneous population of single-stranded and partially double-stranded viral DNA molecules that migrate as a distinctive smear with an apparent molecular size of 0.2–3.0 kb in Southern blot hybridization analyses (Summers and Mason, 1982; Ganem, 1997). Twelve weeks of treatment with 5 mg/kg 3TC induced a 75-fold decline in viremia and a 3-fold decline in WHV RI (Figs. 1–3, Table 1). Viremia returned to pretreatment levels within 1–2 weeks following the end of treatment with either dose of 3TC (Figs. 1 and 3). The levels of WHV RI in the animals treated with 5 mg/kg 3TC had returned to pretreatment levels by 4 weeks post-treatment (Figs. 2 and 3).

Twelve weeks of treatment with 15 mg/kg FCV had no significant effect on the levels of viremia or WHV RI (Figs. 1–3, Table 1). Treatment with 50 mg/kg FCV induced nearly a 10-fold decline in viremia but only a twofold reduction in WHV RI after 12 weeks of therapy (Figs. 1–3, Table 1). Following treatment with 50 mg/kg FCV, viremia rebounded more slowly than that observed in the 3TC-treated animals (Figs. 2 and 3). Viremia generally remained depressed for 1–2 weeks following the termination of FCV therapy and then returned to pretreatment levels by 4–6 weeks post-treatment. The levels of WHV RI in the animals treated with 50 mg/kg FCV had returned to pretreatment levels by 4 weeks post-treatment (Figs. 2 and 3).

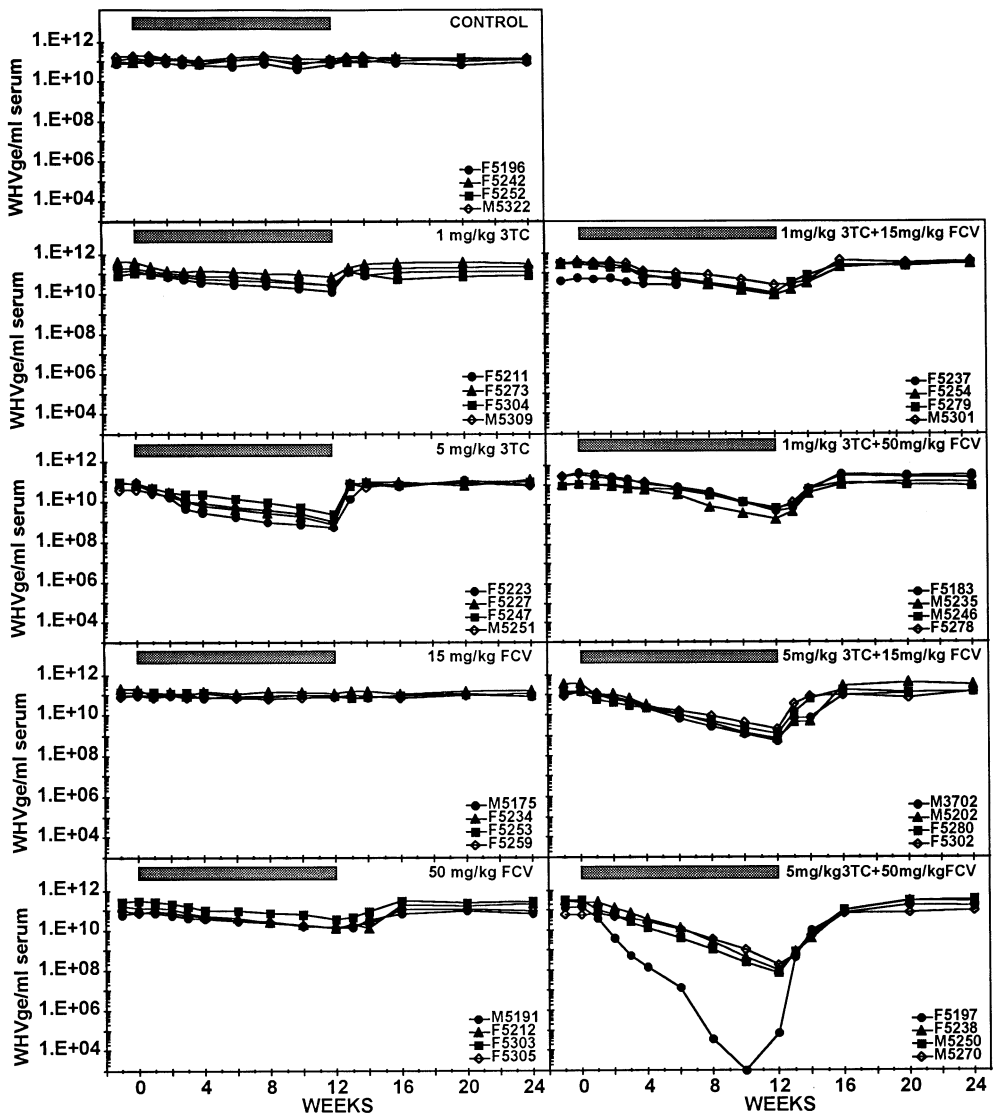

Fig. 1. Effect of antiviral treatments on WHV viremia in chronic WHV carrier woodchucks. Values for individual animals in each treatment group are displayed. See text and Section 2 for experimental details. Horizontal bars denote treatment period. 'WHVge', WHV genomic equivalents (virion or WHV DNA-containing virus particles).

Table 1
Summary of antiviral activities: 3TC and FCV monotherapies[a]

Group	Time point	WHV viremia (log10 WHVge/ml serum)		WHV R.I. (pg/μg total cell RNA)		WHV RNA (pg/μg cell DNA)	
		Mean ± S.D.	(Range)	Mean ± S.D.	(Range)	Mean ± S.D.	(Range)
Placebo	Pretreatment (week 1)	11.1 ± 0.1	(10.9–11.2)	1458 ± 408	(1200–1800)	62 ± 13	(48–79)
	Week 4 of treatment (week 4)	11.0 ± 0.1	(10.8–11.1)	1503 ± 471	(950–2100)	57 ± 9	(50–69)
	End of treatment (week 12)	11.0 ± 0.1	(10.9–11.1)	1367 ± 171	(1200–1600)	58 ± 3	(54–61)
	4 weeks post-treat. (week 16)	11.1 ± 0.1	(10.9–11.2)	1405 ± 351	(1100–1800)	62 ± 7	(55–68)
	12 weeks post-treat. (week 24)	11.1 ± 0.1	(11.0–11.2)	1485 ± 265	(1300–1800)	57 ± 11	(50–70)
1 mg/kg 3TC	Pretreatment (week 1)	11.2 ± 0.3	(10.9–11.6)	1716 ± 206	(1700–2000)	57 ± 10	(44–67)
	Week 4 of treatment (week 4)	10.9 ± 0.2	(10.6–11.2)	1598 ± 480	(1200–2200)	57 ± 10	(49–70)
	End of treatment (week 12)	10.5 ± 0.3 #,*	(10.1–10.5)	1186 ± 216	(1000–1500)	58 ± 10	(45–68)
	4 weeks post-treat. (week 16)	11.2 ± 0.3	(10.7–11.3)	1510 ± 412	(1100–2100)	60 ± 9	(51–70)
	12 weeks post-treat. (week 24)	11.2 ± 0.2	(10.9–11.5)	1414 ± 206	(1200–1700)	62 ± 9	(51–70)
5 mg/kg 3TC	Pretreatment (week 1)	10.9 ± 0.2	(10.6–11.0)	1597 ± 602	(940–2400)	60 ± 10	(49–70)
	Week 4 of treatment (week 4)	9.9 ± 0.3 #,**	(9.5–10.4)	1363 ± 400	(1200–2000)	66 ± 11	(55–80)
	End of treatment (week 12)	9.0 ± 0.3 ##,***	(8.7–9.4)	535 ± 83 ##,**	(440–640)	69 ± 9	(58–70)
	4 weeks post-treat. (week 16)	10.9 ± 0.1	(10.8–11.0)	1444 ± 350	(1100–1900)	64 ± 10	(51–72)
	12 weeks post-treat. (week 24)	10.9 ± 0.1	(10.8–11.1)	1468 ± 479	(1000–2100)	67 ± 7	(61–76)
15 mg/kg FCV	Pretreatment (week 1)	11.1 ± 0.2	(10.9–11.3)	1521 ± 332	(1100–1900)	65 ± 5	(60–70)
	Week 4 of treatment (week 4)	11.0 ± 0.1	(10.8–11.1)	1486 ± 504	(990–2200)	61 ± 11	(50–75)
	End of treatment (week 12)	11.0 ± 0.1	(10.9–11.1)	1520 ± 527	(890–2000)	55 ± 13	(41–70)
	4 weeks post-treat. (week 16)	11.0 ± 0.1	(10.8–11.0)	1613 ± 222	(1300–1800)	61 ± 5	(58–68)
	12 weeks post-treat. (week 24)	11.0 ± 0.1	(10.9–11.2)	1463 ± 222	(1300–1800)	56 ± 7	(47–64)
50 mg/kg FCV	Pretreatment (week 1)	11.1 ± 0.2	(10.8–11.2)	1404 ± 355	(920–2000)	57 ± 15	(43–78)
	Week 4 of treatment (week 4)	10.8 ± 0.2	(10.6–11.0)	1274 ± 519	(950–2100)	58 ± 11	(46–70)
	End of treatment (week 12)	10.2 ± 0.2 ##,***	(10.1–10.6)	704 ± 84 #,*	(610–810)	60 ± 8	(59–69)
	4 weeks post-treat. (week 16)	11.2 ± 0.3	(10.8–11.4)	1497 ± 507	(1200–2300)	58 ± 13	(46–70)
	12 weeks post-treat. (week 24)	11.2 ± 0.3	(10.8–11.5)	1162 ± 356	(930–1700)	56 ± 11	(47–51)

[a] Geometric mean values (± S.D.) and the overall range of values for WHV nucleic acids are presented. WHVge, WHV genome equivalents. Levels of intrahepatic intracellular WHV DNA replication intermediates (WHV R.I.) and total WHV RNA were normalized for total cellular DNA or RNA using the relative levels of β-actin DNA or RNA (β-actin hybridization probe purchased from Oncor, Gaithersburg, MD).

Statistically different from pretreatment ($P<0.01$).

Statistically different from pretreatment ($P<0.001$).

* Statistically different from control ($P<0.05$).

** Statistically different from control ($P<0.01$).

*** Statistically different from control ($P<0.001$).

Combination therapy with 1 mg/kg 3TC and 15 mg/kg FCV induced a 27-fold decline in viremia, but no significant change in the level of WHV RI was observed (Figs. 1–3, Table 2). Treatment with 1 mg/kg 3TC and 50 mg/kg FCV was more effective, inducing a 60-fold decline in viremia but only a 2–3-fold decline in WHV RI (Figs. 1–3, Table 2). Treatment with 5 mg/kg 3TC and 15 mg/kg FCV induced a 200-fold decline in viremia and a threefold decline in WHV RI (Figs. 1–3, Table 2). A combination of 5 mg/kg 3TC and 50 mg/kg FCV was the most effective, reducing viremia more than 10 000-fold and WHV RI 10-fold (Figs. 1–3, Table 2). In one animal (F5197), the combination of the two higher doses of 3TC and FCV reduced viremia nearly one million-fold. Following the end of therapy, viremia rebounded at approximately the same rate as observed for the FCV monotherapies, requiring approximately 2–4 weeks to rise to pretreatment levels (Figs. 1–3). The levels of WHV RI in the animals treated with the combination therapies had returned to pretreatment levels by 4 weeks post-treatment (Figs. 2 and 3).

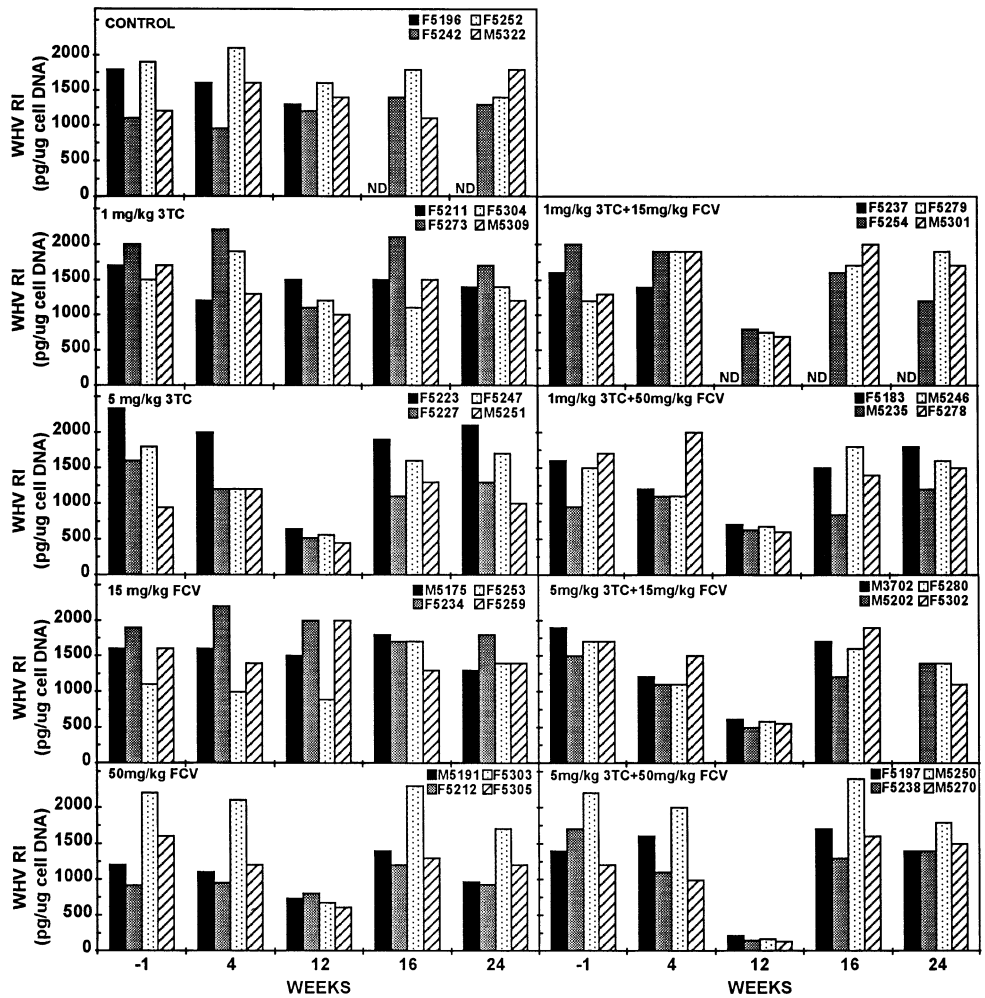

Fig. 2. Effect of antiviral treatments on hepatic WHV replication in chronic WHV carrier woodchucks. Values for individual animals in each treatment group are displayed. See text and Section 2 for experimental details. 'WHV RI', hepatic WHV DNA replication intermediates. Levels of hepatic cellular DNA were quantified by hybridization to a commercial β-actin gene probe (Oncor, Gaithersburg, MD) using Southern or dot blot hybridization techniques as described in Section 2.

Table 2
Summary of antiviral activities: 3TC and FCV combination therapies[a]

Group	Time point	WHV viremia (log_{10} WHVge/ml serum)		WHV R.I. (pg/μg total cell RNA)		WHV RNA (pg/μg cell DNA)	
		Mean ± S.D.	(Range)	Mean ± S.D.	(Range)	Mean ± S.D.	(Range)
1 mg/kg 3TC + 15 mg/kg FCV	Pretreatment (week 1)	11.3 ± 0.4	(10.6–11.6)	1495 ± 359	(1200–2000)	58 ± 11	(44–69)
	Week 4 of treatment (week 4)	10.8 ± 0.2	(10.5–11.1)	1760 ± 250	(1400–1900)	60 ± 11	(48–73)
	End of treatment (week12)	10.1 ± 0.2[c,g,m]	(9.9–10.4)	745 ± 55[c,e,j,l]	(690–800)	57 ± 7	(49–63)
	4 weeks post-treat. (week 16)	11.5 ± 0.1	(11.3–11.7)	1759 ± 208	(1600–2000)	57 ± 3	(55–60)
	12 weeks post-treat. (week 24)	11.5 ± 0.1	(11.5–11.6)	1571 ± 361	(1200–1900)	55 ± 8	(50–64)
1 mg/kg 3TC + 50 mg/kg FCV	Pretreatment (week 1)	11.2 ± 0.2	(11.1–11.4)	1403 ± 335	(950–1700)	58 ± 13	(49–77)
	Week 4 of treatment (week 4)	11.0 ± 0.2	(10.8–11.2)	1305 ± 436	(1100–2000)	55 ± 14	(41–72)
	End of treatment (week 12)	9.6 ± 0.2[d,g,h,m]	(9.2–9.8)	651 ± 46[c,e,i,k]	(600–700)	60 ± 8	(50–64)
	4 weeks post-treat. (week 16)	11.3 ± 0.2	(11.0–11.6)	1335 ± 401	(840–1800)	56 ± 6	(59–63)
	12 weeks post-treat. (week 24)	11.2 ± 0.2	(10.9–11.5)	1509 ± 250	(1200–1800)	56 ± 5	(50–63)
5 mg/kg 3TC + 15 mg/kg FCV	Pretreatment (week 1)	11.2 ± 0.2	(10.9–11.6)	1694 ± 163	(1500–1900)	68 ± 14	(50–80)
	Week 4 of treatment (week4)	10.4 ± 0.1	(10.3–10.5)	1215 ± 189	(1100–1500)	66 ± 9	(59–77)
	End of treatment (week 12)	9.0 ± 0.2[d,g,m]	(8.7–9.3)	561 ± 46[c,g,m]	(500–610)	62 ± 11	(46–69)
	4 weeks post-treat. (week 16)	11.2 ± 0.2	(11.0–11.4)	1578 ± 294	(1200–1900)	60 ± 10	(48–72)
	12 weeks post-treat. (week 24)	11.3 ± 0.2	(11.1–11.5)	1292 ± 173	(1100–1400)	53 ± 4	(50–57)
5 mg/kg 3TC + 50 mg/kg FCV	Pretreatment (week 1)	11.2 ± 0.3	(10.8–11.5)	1583 ± 435	(1200–2200)	58 ± 14	(45–77)
	Week 4 of treatment (week 4)	9.8 ± 0.8[b,e,k]	(8.1–10.6)	1366 ± 468	(990–2000)	58 ± 2	(55–60)
	End of treatment (week 12)	7.2 ± 1.4[c,f,h,l]	(4.8–8.2)	162 ± 34[d,g,j,m]	(150–210)	58 ± 7	(50–68)
	4 weeks post-treat. (week 16)	10.9 ± 0.1	(10.8–11.0)	1707 ± 465	(1300–2400)	49 ± 8	(42–61)
	12 weeks post-treat. (week 24)	11.3 ± 0.2	(11.0–11.6)	1517 ± 189	(1500–1800)	53 ± 6	(47–58)

[a] Geometric mean values (± S.D.) and the overall range of values for WHV nucleic acids are presented. WHVge, WHV genome equivalents. Levels of intrahepatic intracellular WHV DNA replication intermediates (WHV R.I.) and total WHV RNA were normalized for total cellular DNA or RNA using the relative levels of β-actin DNA or RNA (β-actin hybridization probe purchased from Oncor, Gaithersburg, MD). nd, not determined.

[b] Statistically different from pretreatment ($P<0.05$).
[c] Statistically different from pretreatment ($P<0.01$).
[d] Statistically different from pretreatment ($P<0.001$).
[e] Statistically different from control ($P<0.05$).
[f] Statistically different from control ($P<0.01$).
[g] Statistically different from control ($P<0.001$).
[h] Statistically different from 3TC monotherapy ($P<0.5$).
[i] Statistically different from 3TC monotherapy ($P<0.01$).
[j] Statistically different from 3TC monotherapy ($P<0.001$).
[k] Statistically different from FCV monotherapy ($P<0.05$).
[l] Statistically different from FCV monotherapy ($P<0.01$).
[m] Statistically different from FCV monotherapy ($P<0.001$).

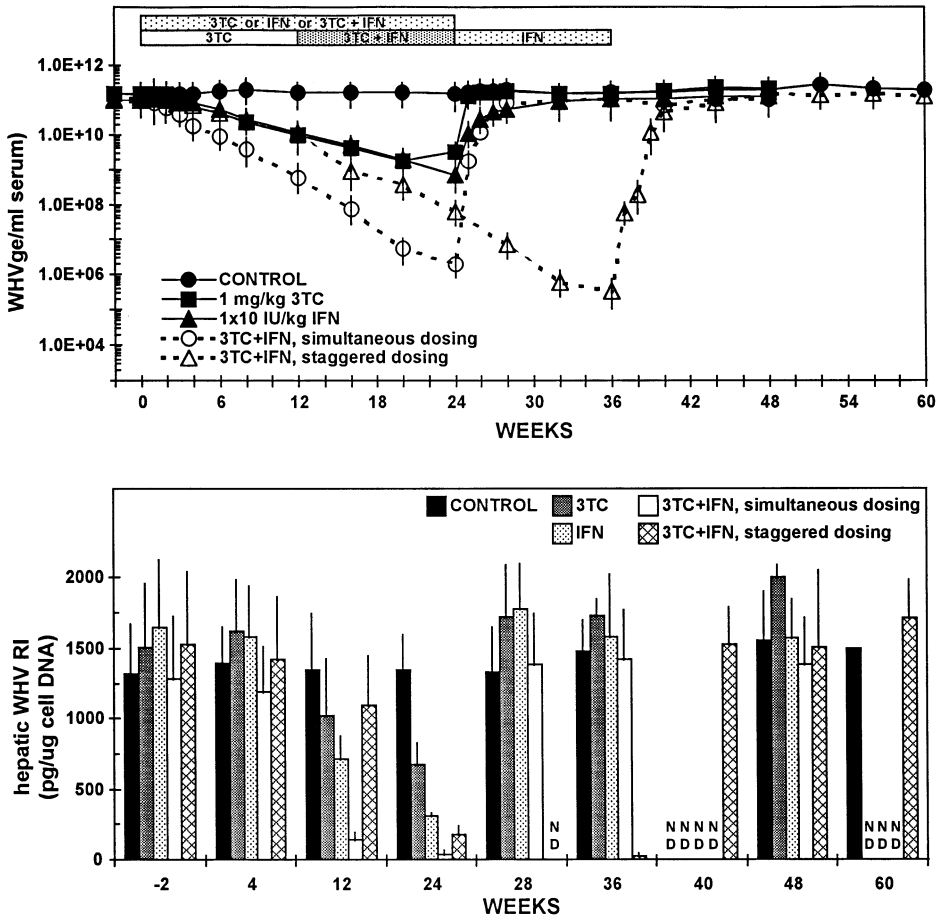

Fig. 3. Effect of antiviral treatments on WHV replication in chronic carrier woodchucks. Geometric mean values for each experimental group are displayed. See text and Section 2 for experimental details. Horizontal bars denote the treatment periods. Vertical lines denote standard deviations. The top panels compare the effects of the four monotherapies relative to the placebo-treated control group. The remaining panels individually compare the effect of each dose of 3TC monotherapy to the two corresponding combination treatments with 3TC plus FCV. 'WHVge', WHV genomic equivalents (virion or WHV DNA-containing virus particles). 'WHV RI', hepatic WHV DNA replication intermediates.

None of the treatments regimens induced significant changes in the levels of WHsAg or antibodies to WHsAg (anti-WHs) or WHcAg (anti-WHc) in the serum of the treated animals (data not shown), or in the levels of hepatic WHV RNA (Table 1). No significant changes in the levels of viremia, WHV RI, hepatic WHV RNA (Figs. 1–3, Table 1), WHsAg, anti-WHs, or anti-WHc (data not shown) were observed in the control (placebo-treated) group of animals during the course of the study.

No obvious treatment-related clinical (e.g. body weight loss), hematologic, or serologic indications of toxicity, including drug-related hepatic toxicity (ALT, AST, SDH) were observed in any of the treated animals. One of the control animals (M3702) died at week 20 due to complications related to the development of hepatocellular carcinoma (HCC). As observed for HBV chronic carriers (Beasley and Hwang, 1991), the development of HCC is a natural consequence of chronic WHV infection in woodchucks (Gerin et al., 1991; Tennant et al., 1991). HCC develops in 100% of

WHV carrier animals within 4 years of infection with a median development interval of approximately 29 months post-infection (Gerin et al., 1991; Tennant et al., 1991). One of the animals treated with a combination of 1 mg/kg 3TC and 15 mg/kg FCV (F5237) died of hepatic hemorrhage 24 h following the biopsy at the fourth week of treatment. Both deaths were believed to be unrelated to treatment.

The combination of 1 mg/kg 3TC and 50 mg/kg FCV was significantly more effective at reducing viremia ($P < 0.01$) than treatment with 1 mg/kg 3TC and 15 mg/kg FCV. The use of 5 mg/kg 3TC and either 15 or 50 mg/kg FCV was more effective at reducing viremia ($P < 0.001$) and WHV RI ($P < 0.01$ and $P < 0.001$, respectively) than the combination of 1 mg/kg 3TC and 15 mg/kg FCV. The combination of 5 mg/kg 3TC and either 15 or 50 mg/kg FCV was more effective at reducing viremia ($P < 0.01$ and $P < 0.001$, respectively) and WHV RI ($P < 0.05$ and $P < 0.001$, respectively) than the combination of 1 mg/kg 3TC and 50 mg/kg FCV. Treatment with 5 mg/kg 3TC and 50 mg/kg FCV was more effective at reducing viremia ($P < 0.05$) and WHV RI ($P < 0.001$) than the combination of 5 mg/kg 3TC and 15 mg/kg FCV.

A major issue in the use of combination therapy is not simply that a particular drug mixture is more effective than corresponding monotherapies, but a question of whether the mixture is more effective (i.e. synergistic) or less effective (i.e. antagonistic) than what could be expected for the combined effects of the individual monotherapies. Currently, there is no consensus on how to compare the kinetics of viral load for a given treatment period to discriminate these differences. While it is possible to determine if the viral load curve in one group of animals is statistically different from another, it is not easy to determine if the curve resulting from treatment with a combination of drug 'A' plus 'B' is different from what could be 'expected' from the independent (i.e. 'additive') effects of drug 'A' plus drug 'B'. There are, however, several generally accepted algorithms to determine such interactions at a single time point (see Prichard and Shipman, 1996, for a review).

We compared the antiviral effects of the combination treatments and the monotherapies in this study at a single point, the end of the treatment period, using one of these algorithms, Bliss Independence Estimation (Bliss, 1939). This evaluation states that for two independently acting drugs, the combined antiviral effect (Z) is equivalent to the effect of drug A (X) plus the effect of drug B (Y) on the portion of the target (i.e. viral) population not affected by drug A: $Z = X + Y(1 - X)$. Experimental results that are smaller than that predicted by this method indicate antagonism and values greater than that predicted indicate synergism.

Using this method, it was possible to calculate an average 'expected' reduction of viremia and WHV RI for each of the combination treatments based on the antiviral effects of the monotherapies in each of individual animals (Table 3). The average 'expected' value was derived from a set of 16 individual 'expected' values that were generated by using all possible combinations of the monotherapy data from each of the four 3TC-treated and four PCV-treated animals in the Bliss estimation algorithm. All four combination treatments induced reductions in viremia and WHV RI that were at least as great as those predicted by Bliss independence estimation, indicating that the antiviral activities of 3TC and FCV were generally independent and not antagonistic (Table 3). Furthermore, treatments with either 1 mg/kg 3TC and 15 mg/kg FCV, or 5 mg/kg 3TC and 50 mg/kg FCV, induced reductions in viremia and WHV RI that were significantly greater than those predicted, indicating probable synergistic antiviral effects for these two drug combinations (Table 3).

4. Discussion

In this report, using the WHV/woodchuck experimental model of chronic HBV infection, we compared the relative antiviral activity of several combinations of 3TC and FCV to matched monotherapies in a placebo-controlled study. In these experiments, combination therapy induced more pronounced suppressions of WHV replica-

tion than that observed for the corresponding monotherapies. Furthermore, the combination treatments suppressed WHV replication at least a much as that predicted by Bliss Independence estimations, indicating that the antiviral activities of 3TC and FCV are independent in vivo. It is interesting to note that the kinetics of the rebound of viremia following the end of combination therapy more closely paralleled that observed for FCV monotherapy rather than 3TC in this and previous woodchuck studies (Hurwitz et al., 1998; Korba et al., 2000), providing additional evidence for independent antiviral activities.

Two of the combination regimens induced suppression of WHV replication significantly more than that predicted, indicating potential synergistic antiviral interactions. Previously, we demonstrated that 3TC and penciclovir acted in a synergistic manner to suppress HBV replication in human cell culture (Korba, 1996). A synergistic interaction for the suppression of DHBV replication in primary duck hepatocyte cultures has also been reported (Colledge et al., 1997). In our studies, the most effective treatment was that using the combination of 5 mg/kg 3TC and 50 mg/kg FCV, the doses that most closely approached the metabolic equivalents used in human clinical trials.

None of the treatments examined induced long-term reductions in WHV replication. The amount of tissue in the needle biopsy specimens was not sufficient to directly measure the levels of cova-

Table 3
Evaluation of drug interactions by Bliss Independence Estimation[a]

	Relative decrease in levels of WHV DNA at end of treatment period versus pretreatment period							
	Serum WHV Virion DNA				Hepatic WHV R.I.			
	'Expected'		Observed		'Expected'		Observed	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Control	–	–	1.1 ± 0.3	1.0–1.6	–	–	1.1 ± 0.2	0.9–1.4
1 mg/kg 3TC (12 weeks)	–	–	7.1 ± 1.8	4.0–13	–	–	1.4 ± 0.3	1.1–1.7
5 mg/kg 3TC	–	–	75 ± 27	40–159	–	–	3.0 ± 0.3	2.1–3.8
15 mg/kg FCV	–	–	1.4 ± 0.4	1.0–2.0	–	–	1.0 ± 0.2	0.8–1.2
50 mg/kg FCV	–	–	8.4 ± 2.3	6.3–13	–	–	2.0 ± 0.8	1.1–3.2
1 mg/kg 3TC + 15 mg/kg FCV	10 ± 6.0	4–25	27 ± 5.0*	16–40	1.4 ± 0.4	1.0–2.2	2.0 ± 0.4	1.6–2.5
1 mg/kg 3TC + 50 mg/kg FCV	50 ± 17	32–158	60 ± 13	16–79	2.5 ± 1.4	1.3–4.6	2.2 ± 0.5	1.5–2.8
5 mg/kg 3TC + 15 mg/kg FCV	106 ± 43	40–316	204 ± 75	100–631	3.0 ± 0.8	1.7–5.9	3.0 ± 0.1	2.9–3.1
5 mg/kg 3TC + 50 mg/kg FCV	631 ± 270	251–2000	11 000 ± 5800**	700–1 000 000	6.0 ± 1.4	3.5–12	10 ± 1.2*	6.7–12

[a] Values for 'expected' antiviral effect were calculated by the method of Bliss (1939) using the antiviral effects of each individual animal treated with the corresponding monotherapies. WHV R.I., WHV DNA replication intermediates.

* Statistically different from 'expected' ($P<0.05$).

** Statistically different from 'expected' ($P<0.01$).

lently closed circular WHV DNA (cccDNA) (Ganem, 1997). The rebounds in viremia and WHV replication following the termination of therapy, as well as the lack of an effect on hepatic WHV RNA or WHsAg levels in serum, are presumably due to a failure of the treatment protocols used to significantly reduce the levels of this critical viral template.

In this study there was a general correlation between reductions in intrahepatic WHV replication and viremia. However, the relative rate and degree of change in these two markers of WHV replication were substantially different. This is a common observation in both in vitro and in vivo antiviral studies with HBV, WHV, and DHBV (Fourel et al., 1990; Korba and Boyd, 1996; Colledge et al., 1997; Cullen et al., 1997; Gangemi et al., 1997; Lai et al., 1997; Genovesi et al., 1998; Lin et al., 1998; Mason et al., 1998; Tennant et al., 1998a; Zembower et al., 1998; Perigaud et al., 1999; Korba et al., 2000). The maintenance and the clearance of hepadnavirus particles obviously involves complex interactions between host and virus. We speculate that the differences in the rates and degree of changes in serum WHV DNA and WHV RI are a reflection of the relative ability of host processes to turnover viral particles in these different compartments.

It is not known why the rebound of viremia was slower following the end of the treatments with FCV relative to the other agents. Similar patterns of viremia have been observed in FCV treatments in human clinical trials for chronic HBV infection, FCV therapy of DHBV infections in Peking ducks, and in PCV monotherapy or PCV/3TC combination therapy of cultures of DHBV-infected primary duck hepatocytes (Tsiquaye et al., 1996; Bartholomeusz et al., 1997; Colledge et al., 1997; Lin et al., 1998). Pharmacokinetic and pharmacologic studies have indicated that the intracellular half-life of penciclovir triphosphate (PCV-TP, the active metabolite for this agent) is considerably longer than that observed for the other agents (Tsiquaye et al., 1996; Ono et al., 1998), thereby providing the potential for sufficient PCV-TP to suppress WHV replication to remain in hepatocytes following the withdrawal of therapy.

The cumulative antiviral effect of the 5 mg/kg dose of 3TC in this study was less than that previous reported by our laboratories (Korba et al., 2000). The antiviral effect of 5 mg/kg 3TC in this study was, however, within the range of antiviral responses previously reported by our laboratories for this dose of 3TC (Hurwitz et al., 1998). We have observed a considerable degree of variability in the antiviral effectiveness of this dose of 3TC in our woodchuck studies as compared to other doses of this nucleoside (Hurwitz et al., 1998). This result reinforces the concept of the necessity for performing appropriate monotherapies in parallel to combination treatments within each individual experiment.

These studies provide evidence that combination therapy of chronic HBV infection with 3TC and FCV will confer an enhanced antiviral benefit over monotherapy with either agent. The continued use of the WHV/woodchuck model system should be of considerable assistance in the development of new, more effective strategies for the control of chronic HBV infection.

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