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Antiviral Research 55 (2002) 141–150

Antiviral Research

www.elsevier.com/locate/antiviral

Mutations in the conserved woodchuck hepatitis virus polymerase FLLA and YMDD regions conferring resistance to lamivudine

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Received 28 August 2001; accepted 25 January 2002

Abstract

During more than 104 weeks of treatment with lamivudine (3TC) in chronic woodchuck hepatitis virus (WHV) carrier woodchucks, viral recrudescence occurred. Analysis of WHV DNA polymerase from woodchuck serum samples by PCR followed by DNA sequencing demonstrated that all samples were wild type at the conserved YMDD motif in domain C. Four of the six 3TC-treated woodchucks showed a mixture of the wild-type Ala (GCT) and the mutant Thr (ACT) at the conserved amino acid residue 566 (FLLA) in domain B of the WHV polymerase region. The appearance of the A566T mutation was temporally associated with viral recrudescence. This change is analogous with the amino acid 181 (FLLA) in HBV where 3TC selects for a change from Ala to Thr in humans. In the woodchuck, the Ala to Thr change in the polymerase gene results in a mutation of the WHV surface protein (amino acid 377) from Trp (TGG) to an opal codon (TGA), which may prematurely terminates the polypeptide. Three WHV molecular infectious clones were constructed to study this mutation in greater detail in vitro: A566T, analogous to A181T in HBV; M589V, analogous to the M204V in HBV; and the double mutant A566T/M589V, analogous to A181T/M204V in HBV. These mutants exhibited drug-sensitivity and replication profiles that paralleled those reported for analogous HBV variants. In transfected Huh7 cells, WHV containing the M589V mutation conferred at least 100-fold increased resistance to 3TC, but replicated approximately 5-fold less efficiently than wild-type virus as judged by both extracellular virus production and intracellular DNA replicative forms. In contrast, A566T mutant was approximately 10-fold more resistant to 3TC, replicated intracellularly as well as wild type, but produced 10-fold lower levels of virions than wild type. These findings are consistent with the observation that the A566T mutation alters the overlapping WHV surface antigen reading frame. WHV carrying mutations in the conserved YMDD motif, while not directly selected during lamivudine therapy in WHV carrier woodchucks, are replication competent in cell

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PII: S0166-3542(02)00019-0

culture indicating the potential for their emergence in treated animals. These results further illustrate the utility of the WHV/woodchuck model to studies of HBV-drug resistance.

Keywords: Viral drug-resistance; Woodchuck hepatitis virus (WHV); Woodchuck (*Marmota monax*); Lamivudine (3TC)

1. Introduction

Approximately 300 million people worldwide are chronically infected with hepatitis B virus (HBV), the prototype member of the hepadnavirus family. Hepadnaviruses are some of the smallest and most compact enveloped viruses with a partially double-stranded DNA genome (Ganem and Varmus, 1987). These viruses cause infections of the liver that can become chronic, leading to cirrhosis and primary hepatocellular carcinoma (Beasley and Hwang, 1984).

Currently there are three animal models routinely used for the study of the biological and pathological properties of HBV. These models include woodchuck hepatitis virus (WHV) and its natural host, the Eastern Woodchuck (*Marmota monax*), ground squirrel hepatitis virus (GSHV) and its host the Beechey ground squirrel, and duck hepatitis B virus (DHBV) and its host the Peking duck (Schinazi et al., 1999). The similarity between WHV and HBV genome, reaching 65% overall nucleotide sequence homology with virtually 100% conservation at the amino acid level in essential functional regions, makes WHV a suitable model to study the replication of this virus and to develop strategies to inhibit HBV (Galibert et al., 1982).

The first choice of antiviral therapy for treatment of chronically infected hepatitis B individuals has been interferon alpha. However, due to significant dose-dependent side effects of interferon alpha, the orally bioavailable nucleoside analog lamivudine (3TC) has emerged as a more effective and practical therapeutic agent (Doong et al., 1991; Chang et al., 1992; Schinazi et al., 1994; Bartholomeuw et al., 1998; Dienstag et al., 1999). In its triphosphate form, 3TC can inhibit HBV DNA polymerase activity and also acts as a viral DNA chain terminator (Severini et al., 1995; Furman and Schinazi, 2000). Major mutations in the polymerase region found in humans treated with 3TC are located in the binding (B) domain (which contains the conserved FLLA motif) and in the catalytic (C) domain (which contains the conserved YMDD motif). A new, genotype-independent nomenclature suggested by the HEP DART International Committee for Viral Hepatitis was adopted to obtain a uniform numbering system for the reverse transcriptase (rt) region of the human HBV polymerase (Stuyver et al., 2001). According to the new classification for genotypes B, C, and F, position rtM204V corresponds to M550V, rtL180M to L526M, rtV173L to V519L, and rtA181T to A527T. For genotype A, position rtA181T corresponds to A529T, and rtL180M to L528M. At present, the Committee did not adopt a similar nomenclature approach for WHV polymerase.

As with human immunodeficiency virus type 1 (HIV-1), 3TC therapy selects for alterations in the YMDD motif of the HBV DNA polymerase in which the methionine rtM204V/I (M550) is changed to Ile (I) or Val (V) (Schinazi et al., 1993; Aye et al., 1997; Ono-Nita et al., 1999, 2001). Various levels of HBV-resistant mutants to 3TC are observed containing point mutations inside and outside the YMDD motif (Fu and Cheng, 1998). In HBV-infected patients, treatment with 3TC or famciclovir (orally available prodrug of penciclovir [PCV]) selects for mutations from Leu to Met or Val at position 180 (526) in the FLLA motif of domain B (Fu and Cheng, 1998). Another mutation in the FLLA motif of the B domain, rtA181T (A529T), is found in HBV patients on prolonged 3TC therapy (Yeh et al., 2000). A mutation in the YMDD motif of the HBV polymerase C domain, rtM204V/I (M550V), often is found in conjunction with a mutation in the FLLA region, rtL180M (L526M), in HBV-infected patients on prolonged 3TC therapy (Neisters et al., 1998). In domain B of HBV DNA polymerase, mutations rtL180M (L526M) and rtV173L (V519L) are associated with a reduced

susceptibility to PCV and 3TC (Bartholomeuw et al., 1997; Allen et al., 1998). In HIV-1, a mutation adjacent to the FLLA region from Pro to Ser at position 157 (PFLLA) conferred low-level viral resistance to 3TC (Smith et al., 1999).

A mutation analogous to rtL180M in HBV has been observed during prolonged 3TC therapy of some chronic WHV carrier woodchucks, but no changes in the YMDD motif were observed (Mason et al., 1998; Zhou et al., 1999). Lamivudine therapy of DHBV-infected Peking ducks failed to select for resistant mutants, but molecular clones carrying mutations analogous to rtM204V/I in HBV were infectious in cell culture and in Peking ducks continuously treated with 3TC (Fischer and Tyrrell, 1996; Seigneres et al., 2001).

In this study, WHV chronically infected woodchucks were given a lifetime administration of 3TC. After approximately 1 year of therapy, some animals exhibited a breakthrough of serum virus levels that were temporally associated with the appearance of specific mutations in the B domain of the WHV polymerase. Molecular clones carrying mutations in both the B and C domains of the WHV polymerase were created, and their replicative and drug sensitivity profiles were analyzed.

2. Materials and methods

2.1. Chemicals

(−)-β-2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) used for the woodchuck treatment trials was synthesized at Emory University as previously described (Chang et al., 1992). Lamivudine and penciclovir (PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine) used for cell-culture studies were purchased from Moravek Biochemicals Inc. (La Brea, CA). Adenine arabinoside 5′-monophosphate (ara-A) was purchased from Sigma, Inc. (Cincinnati, OH).

2.2. 3TC therapy of WHV chronic carriers

The chronic carrier woodchucks in this study were produced by laboratory inoculation with WHV7 as previously described (Tennant et al., 1988). Woodchucks selected for this study were persistently WHV DNA viremic and had no ultrasonographic or biochemical evidence of liver disease immediately prior to the start date. The animals were approximately 8–9 months old at the initiation of treatment. Woodchucks received 5 mg/kg once per day orally for the first 10 months. The dose of drug was increased to 15 mg/kg due to viral recrudescence in some animals. The selection of the dose was based on previously published pharmacokinetic studies in woodchucks treated orally with 3TC (Rajagopalan et al., 1996). Their weights were measured monthly. Hematologic and serum biochemical profiles were performed monthly for the first 6 months of the study and thereafter bimonthly. Serum WHV DNA was measured by blot hybridization or PCR-based methods using a full-length homologous WHV probe as previously described (Korba et al., 2000).

2.3. WHV DNA extraction and PCR analysis for sequencing

WHV DNA was extracted from blind-coded woodchuck serum samples using TRI REAGENT® LS (Molecular Research Center, Inc., Cincinnati, OH). The precipitated DNA pellet was solubilized in deionized water. The forward primer (5′-CCTAGGACTCCTCGCAGGATTACAGGTG-3′) and the reverse primer (5′-GGCAGGCGGCAATGAGTTCCGCCGTGGC-3′) was used to obtain a PCR amplicon of 1.1 kb from nucleotides 1210 to 2320 of the WHV DNA polymerase gene (Cohen et al., 1988). The PCR conditions were as follows: 94 °C for 5 min; 94 °C for 45 s, 61.8 °C for 1 min, and 72 °C for 2 min (35 cycles); and 72 °C for 10 min. The PCR fragment was resolved on a 0.7% agarose gel, and the fragment was purified using the QIAquick gel extraction kit (QIAGEN, Chatsworth, CA). The fragment was sequenced using the forward primer WHVseq2 (5′-ATACCAGAGTGCACTGGC-3′) and the reverse primer WHVseq1RC (5′-GATAGGTGGTGGCAATGCC-3′) covering nucleotides 1319 to 2254, which encompassed the highly conserved domains A–E of the WHV DNA polymerase gene (Cohen et al., 1988). The

sequencing was performed on an ABI PRISM™ 377 sequencer (Perkin Elmer Applied Biosystems, Inc., Foster City, CA) by the Emory University/VA Sequencing Core Facility, Atlanta, GA. The electropherograms were analyzed to determine the relative peak height of the two peaks at nucleotide position 1696. The proportion of each mixed population was estimated from the peak heights of the electropherograms from the sequencing reactions (data not shown). Nucleotide and amino acid alignments were performed using the DNASTAR software programs (DNASTAR, Inc., Madison, WI).

2.4. Clonal analysis

The PCR amplicon from woodchuck 5296 at 80 weeks into the study was cloned into the pCR®2.1 vector (Invitrogen, San Diego, CA). Plasmid preps were performed using the QIAprep Spin Miniprep Kit (QIAGEN, Chatsworth, CA) on positive transformants. To confirm the presence of the 1.1 kb WHV DNA polymerase insert, the plasmids were screened by enzymatic digestion.

2.5. Site-directed mutagenesis

The site-directed WHV mutants were made using the Quick Change™ Site-Directed Mutagenesis Kit by Stratagene. The A566T mutation was created using the primers A566T for (5′-CCGTTTCTCTTGACTCAATTTACTAAGTCC-3′) and A566Trev (5′-GGCACCCTAGTAAATTGAGTCAAGAGGAAACGG-3′) with the template pCMW-82 (Girones et al., 1989). The M589V site-directed mutant was created using the same template pCMW-82 with the primers M589V for (5′-GTGGTTTTTGCTTATGTGGATGATTTGGTTTTGG-3′) and M589Vrev (5′-CCAAAACCAAATCATCCACATAAGCAAAAACCAC-3′). The M589V/A566T double mutant was created using the A566T mutant template with the M589Vfor and M589Vrev primers and using the M589V template with the A566Tfor and A566Trev primers. The PCR reaction consisted of 1 × reaction buffer [10 × reaction buffer equals 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–HCl, 20 mM $MgSO_4$, 1.0% Triton X-100, and 1.0 mg/ml nuclease-free bovine serum albumin], 250 ng of forward primer, 250 ng of reverse primer, 0.50 mM dNTPs, 100 ng template plasmid, and 5.0 U Turbo *Pfu* DNA Polymerase with dH_2O added for a total volume of 50 μl. The PCR conditions were as follows: 95 °C for 30 s, 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 30 min (repeat the last three steps for 16 cycles). One microliter of Dpn I restriction enzyme (10 U/μl) was added to each PCR reaction and incubated for 2 h to digest the parental supercoiled dsDNA.

2.6. Transformation and mutation isolation

Epicurian Coli XL1-Blue supercompetent cells were transformed with one-tenth volume of the digested PCR reaction. Aliquots were spread on LB plates with 20 μl of 100 mM IPTG and 50 μl of 40 mg/ml X-gal, containing 50 μg/ml ampicillin. Plasmid preparations were performed on 20 purified white colonies using the QIAprep Spin Miniprep Kit. One-tenth volume of each DNA sample was digested with Hind III to ensure that the WHV insert and pCMW-82 vector were intact. Positive clones containing fragments of 6 and 3 kb were sequenced on an ABI PRISM™ 377 sequencer. Nucleotide and amino acid alignments were performed using the DNASTAR software programs (DNASTAR, Inc., Madison, WI). DNA preparations from the wild type, pCMW-82 and the isolated mutants, pWHV A566T-4, pWHVM589V-7, and pWHVM589V/A566T-10, were performed using the QIAGEN Midi Plasmid Purification protocol to obtain a stock of the molecular infectious clones for transfection assays.

2.7. Transfections and in vitro drug treatments

Human hepatoma cells (Huh7) were obtained from ATCC and maintained in RPMI1640 containing 4% fetal bovine serum (FBS), L-glutamine and antibiotics. The cultures were transfected using the Lipofectamine-Plus™ transfection reagent (Gibco-BRL, Gaithersburg, MD) following the manufacturer's instructions. Extracellular virion DNA and intracellular DNA replication intermediates (WHV R.I.) were quantitatively analyzed

by blot hybridization as previously described (Korba et al., 2000). Four days following transfection, test compounds were added (0.1–100 μM) once daily for four consecutive days. Medium was replaced daily. WHV DNA levels were measured as described above 24 h following the last addition of test compound. The median effective (EC_{50}) and 90% effective concentrations (EC_{90}) were determined from dose response data (Chou and Talalay, 1984; Korba and Boyd, 1996).

3. Results

3.1. Determination of WHV resistance to 3TC in treated woodchucks

As expected, during the initial 12 weeks of 3TC-treatment a 4-log drop in viral DNA was observed. However, subsequently, significant elevations in WHV serum DNA levels were observed in four of six treated animals on continuous 3TC therapy (Fig. 1). PCR-based sequence analysis of serum samples from Week 80 revealed no changes in the conserved YMDD motif (Table 1). A single nucleotide change was observed at position 1696 in the sequence coding for the conserved FLLA motif in the B domain of the WHV polymerase,

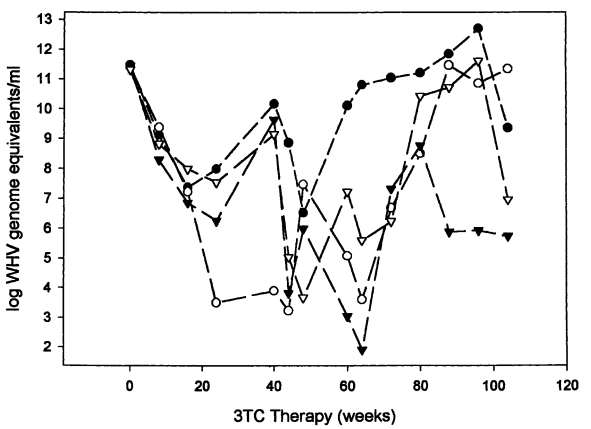

Fig. 1. The WHV viral titer determined at each time of 3TC therapy is indicated in genome equivalents/ml for the four woodchucks. (···●···) = 5193, (···○···) = 5293, (···▼···) = 5296, (···▽···) = 5216.

Table 1
DNA sequencing results from woodchuck serum samples at week 80

Woodchuck	Domain B	Domain C	Treatment
WHV 5193	A (GCT) 65%; T (ACT) 35%	WT[a]	3TC
WHV 5216	A (GCT) 35%; T (ACT) 65%	WT	3TC
WHV 5289	WT[b]	WT	3TC
WHV 5290	WT	WT	3TC
WHV 5293	WT[c]	WT	3TC
WHV 5296	A(GCT) 70%; T(ACT) 30%	WT	3TC
WHV 5178	WT	WT	None
WHV 5195	WT	WT	None
WHV 5201	WT	WT	None
WHV 5210	WT	WT	None
WHV 5245	WT	WT	None
WHV 5294	WT	WT	None

[a] WT is wild type in domain C (YMDD).
[b] WT is wild type in domain B (FLLA), A(GCT).
[c] At Week 104, a mixture of A (GCT) 75% and T (ACT) 25% (FLLA) was noted.

which induced an amino acid change at position 566 from alanine to threonine (A566T) (Table 1), in three of the 3TC-treated animals by Week 80 and one of the 3TC-treated animals by Week 104. Wild-type sequences for this region were also present in each of these four animals (Table 1). No sequence changes from wild type were observed in WHV present in the serum of six age-matched untreated animals (Table 1).

Alignment of the deduced protein sequence of the six 3TC-treated animals with the wild-type WHV sequence (Genbank sequence M18752) (Cohen et al., 1988) and the HBV sequence of the DNA polymerase domain B (Genbank sequence NC001707) is illustrated in Fig. 2A. The mutation from Ala (GCT) to Thr (ACT) in the DNA polymerase resulted in the formation of a stop codon (TGA) from Trp (TGG) at amino acid 377 in the overlapping surface antigen gene (Fig. 2B), potentially creating a truncation in the WHV-surface protein forming a product reduced by 55 amino acids.

To verify that the mutation occurring at nucleotide position 1696 in amino acid A566T was not

a sequencing artifact, a clonal analysis was performed on the WHV from animal 5296 using the Week 80 serum sample. The sequencing results from ten clones revealed that six were wild type (Ala) and four were mutant (Thr) (data not shown) at amino acid 566. These results were comparable to the sequencing results indicating a heterogeneous mixture of 70% Ala and 30% Thr (Table 1).

The appearance of the A566T mutation generally coincided with viral recrudescence in the 3TC-treated animals. Serum samples from the six treated animals were analyzed for mutations at various time points during 3TC therapy immediately prior to the initiation of treatment (Week 0), and Weeks 64, 72, 80, 96 and/or 104. The A566T mutation was not present at the initial (pretreatment) time point in any of the samples. The A566T mutation was detected at Week 64 in 5216 and 5193, at Week 72 in 5296, and at Week 104 in 5293. In 5289 and 5290, very low levels ($\leq 5\%$) of the WHV-serum population contained the A566T mutant by Week 104. The A566T mutation was always present as a mixture of wild type and mutant. In all WHV serum samples, the YMDD motif of domain C was found to be wild type.

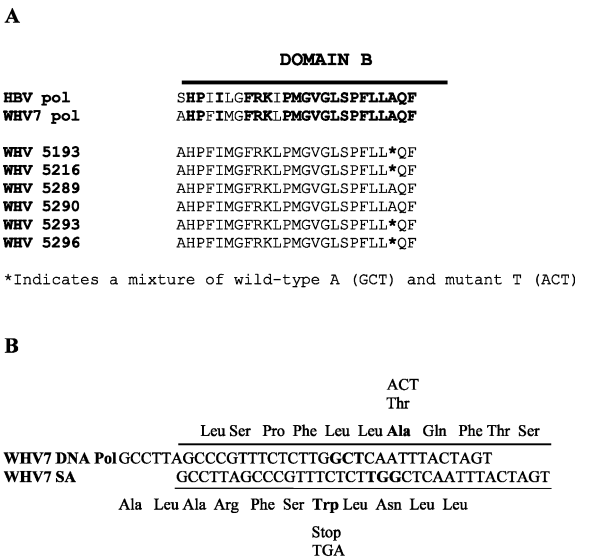

Fig. 2. (A) WHV/HBV sequence alignments in the conserved domain B of the DNA polymerase. The wild-type sequence of the HBV polymerase and the wild-type sequence of the WHV-7 DNA polymerase are indicated (Galibert et al., 1982; Cohen et al., 1988). The deduced protein sequence of the WHV DNA polymerase domain B from the six 3TC treated woodchucks at Week 80 is illustrated. (B) WHV DNA polymerase and surface antigen alignment. The alignment of the WHV DNA polymerase gene and the surface antigen A illustrated that a change at codon 566 in the polymerase will affect the WHV surface antigen by incorporating a stop codon.

Table 2
Replication of 3TC-resistant WHV mutant constructs following DNA transfection into Huh7 cells

Virus	Virion (pg/ml)		WHV R.I. (pg/μg cell DNA)	
	Mean	Range	Mean	Range
Wild-type	37 ± 10	15–70	31 ± 9	20-42
M589V	13 ± 3	9–24	10 ± 3	7-15
A566T	8 ± 1	5–11	25 ± 8	16-40
M589V/A566T	3 ± 1	1–10	22 ± 9	15-38

Geometric mean values of WHV DNA from six experiments are presented $\pm$ S.D. Levels of WHV DNA were obtained 8 days post-transfection. Levels of intracellular DNA were normalized by hybridization to a commercial β-actin probe (Oncor, Inc.). WHV virion DNA levels were obtained from cultures maintained in both 96 and 24-well flat-bottomed culture plates while the levels of WHV DNA replication intermediates (WHV R.I.) levels were obtained only from cultures maintained in 24-well plates.

3.2. Characterization of molecular infectious clones after transfection in Huh7 cells

Utilizing the Quick Change™ site-directed mutagenesis kit, molecular infectious clones for pWHVA566T, pWHVM589V, and pWHVM589V/A566T were made using a previously described infectious WHV construct (Seeger and Maragos, 1989). The M589V mutation, which is analogous to the dominant mutation in the YMDD motif observed in HBV-chronic carriers associated with viral recrudescence during lamivudine therapy, was created to determine if WHV constructs carrying this mutation were viable. Plasmids were transfected into Huh7 cells for analysis of replication competence and drug sensitivity.

All three mutants constructs were found to be replication competent (Table 2). The M589V virus

replicated three-fold less efficiently than the wild-type as determined by both intracellular DNA replicative forms and extracellular virus production (Table 2). The A566T and M589V/A566T viruses replicated intracellularly to the same capacity as the wild type, however, extracellular virus production was decreased 5–12-fold versus wild-type (Table 2).

The mutations created in the B and C domains of the WHV polymerase altered drug sensitivity patterns. The A566T virus was 20-fold more resistant to 3TC than wild-type virus (Table 3). The M589V and M589V/A566T viruses were at least 100-fold more resistant to 3TC than wild-type (Table 3). The A566T and A566T/M589V viruses were at least 10-fold more resistant to PCV than wild-type, while the M589V virus was as sensitive to PCV as wild-type (Table 3). All three mutant viruses were found to be equally sensitive as wild-type to ara-A (Table 3).

4. Discussion

We have shown that in response to prolonged 3TC treatment, WHV chronically-infected woodchucks develop a mutation in amino acid position 566 in the B domain of the WHV DNA polymerase (A566T). In general, the appearance of the A566T mutant in the serum WHV population of 3TC-treated woodchucks coincides with an increase in WHV titers, indicating a role for this mutation in conferring resistance to 3TC. In vitro site-directed mutagenesis experiments confirmed that the A566T mutation confers decreased sensitivity of WHV to 3TC. The G to A nucleotide substitution at nucleotide 1696 causes an Ala to Thr change (A566T), which occurs in the highly conserved FLLA motif. This mutation is analogous to the rtA181T (A529T) mutation observed in HBV-chronic carriers during long-term lamivudine therapy (Yeh et al., 2000). Another mutation in the B polymerase domain, rtL180M (L528M), is commonly observed in HBV patients during lamivudine therapy (Fu and Cheng, 1998). The A566T mutation, as well as a change at amino acid 564 from leucine to methionine (L564M) analogous to rtL180M (L528M) in HBV, has been previously observed in lamivudine-treated WHV carrier woodchucks (Mason et al., 1998; Zhou et al., 1999). In the current study, the L564M mutation was not observed in the 3TC-treated animals.

Unique to this study is the characterization in cell culture of WHV mutants (M589V) that are analogous to rtM204V (M550V) in HBV and the use of a WHV construct that carries mutations in both the B and C polymerase domains. HBV-carrying mutations in both the B and C domains are emerging as the predominant mutants associated with resistance to lamivudine during prolonged therapy (Neisters et al., 1998; Yeh et al., 2000). In this study, both of the WHV B and C domain mutations conferred patterns of relative WHV

Table 3
Susceptibility of 3TC-resistant WHV mutants to nucleoside analogues following DNA transfection into Huh7 cells

Virus	3TC		PCV		Ara-A	
	EC_{50}, (μM)	EC_{90}, (μM)	EC_{50}, (μM)	EC_{90}, (μM)	EC_{50}, (μM)	EC_{90}, (μM)
Wild-type	0.5 ± 0.1	3.6 ± 0.5	3.2 ± 0.6	33 ± 5.5	5.2 ± 1.1	59 ± 6.5
M589V	>300	>300	4.4 ± 0.6	51 ± 5.6	5.6 ± 0.6	53 ± 6.3
A566T	4.7 ± 0.6	62 ± 5.9	>300	>300	8.8 ± 0.9	82 ± 8.9
M589V/A566T	>300	>300	>300	>300	7.9 ± 0.8	78 ± 8.2

Geometric mean values from four experiments are presented ± S.D. EC_{50} and EC_{90} (50 and 90% effective concentrations: concentrations required to reduce WHV DNA by 2-fold or 10-fold) were calculated by linear regression (Korba and Boyd, 1996). In each experiment, a total of 6 cultures were used for each of four drug concentrations (0.1, 1.0, 10 and 100 μM). Cultures were maintained in 96-well flat-bottomed plates following transfection with the Lipofectamine-Plus transfection reagent (Gibco-BRL, Inc.).

replication competence and drug sensitivity to 3TC and penciclovir which are similar to those reported for analogous HBV mutants (Korba and Boyd, 1996; Aye et al., 1997; Bartholomeuw et al., 1997; Allen et al., 1998; Fu and Cheng, 1998; Neisters et al., 1998; Yeh et al., 2000). It is interesting that both the B and C domain mutations did not change the relative sensitivity of WHV to ara-A. Ara-A was the first effective anti-HBV nucleoside analog to inhibit HBV replication in chronically infected individuals, although it is no longer in use due to the induction of peripheral neurotoxicity when taken longer than 3 months (Lok et al., 1984). However, ara-A has also been shown to be a highly effective agent against WHV replication in chronically-infected woodchucks (Korba et al., 2000).

It is significant that the A566T mutation always is observed as a mixture of mutant and wild-type virus. In the woodchuck, the nucleotide substitution in the WHV polymerase gene results in a mutation of the WHV surface protein (WHsAg) gene from amino acid Trp (TGG) to an opal codon (TGA) which potentially terminates the polypeptide at position 377, forming a product reduced by 55 amino acids. A truncation of WHsAg in the overlapping reading frame could explain why the A566T polymerase mutation existed only as a mixture with wild-type and why the mutant virus did not overwhelmingly dominate wild-type WHV in vivo. The cell culture studies indicated that the potential stop codon in the WHV surface antigen gene is either not totally effective (i.e. 'leaky'), or that a low level of wild-type virus is quickly selected for in A566T mutant-infected cells to produce sufficient WHsAg to permit the release of some virus. In HBV, the analogous mutation to the WHV A566T is rtA181T (A529T) (Yeh et al., 2000), found in patients on prolonged 3TC therapy. Interestingly, the nucleotide substitution generates a potential stop codon in the HBV surface antigen gene (HBsAg), and the replication of this mutant is partially 3TC dependent (Yeh et al., 2000).

It is currently not clear why mutations in the YMDD motif of the WHV polymerase were not observed in 3TC-treated woodchucks in this study or in a previous study (Mason et al., 1998). WHV carrying mutations analogous to rtM204V (M550V) in HBV are replication competent, at least in cell culture. Based on metabolic dose conversions, the quantity of 3TC per kg weight administered to the woodchucks in this study was comparable to the quantity typically administered in humans (Hurwitz et al., 1998). However, it is possible that, due to the slightly decreased metabolism of 3TC in woodchucks (Rajagopalan et al., 1996; Schinazi et al., 1997; Hurwitz et al., 1998), the treated animals lacked sufficient levels of 3TC-triphosphate to suppress the replication of the A566T mutant. In HBV patients treated with doses of lamivudine, mutations in the HBV B (FLLA) polymerase domain are not directly associated with viral recrudescence unless accompanied by mutations in the C (YMDD) domain (Bartholomeuw et al., 1997; Fu and Cheng, 1998; Neisters et al., 1998; Yeh et al., 2000). In some studies, single mutations in the HBV B polymerase domain have been observed in 3TC-treated patients far in advance of rises in virus titers that coincide with the appearance of C domain mutations (Fu and Cheng, 1998; Neisters et al., 1998; Yeh et al., 2000). These observations indicate that sufficient levels of 3TC can suppress these mutants in vivo, which is consistent with cell culture observations. In cell culture, HBV and WHV carrying B domain mutations are more resistant to 3TC than wild-type virus, but are far more sensitive than virus carrying mutations in the C domain. It is conceivable that in the woodchuck study population the 3TC levels produced insufficient selective pressure to force the emergence of C domain mutants to overcome drug treatment, as the B domain mutations are sufficient to do so. Since the A566T mutants replicate to the same degree as wild-type, then there would be little additional selective pressure against these variants. Future woodchuck studies to address this issue, perhaps involving higher levels of lamivudine therapy, will need to be conducted.

The results presented in this report confirm the extension of the WHV/woodchuck model to studies of drug-resistant viruses. The WHV/woodchuck model has an established record of predictability with regard to the effectiveness of anti-HBV therapies in humans (see Korba et al.,

2000 for a review). Clinical trials with HBV chronic carriers indicate that viruses resistant to therapy will probably be primarily observed in patients undergoing prolonged periods of continuous therapy (e.g. 1 year or longer). The ability to perform long-term therapy at drug levels comparable to those used in human clinical trials, permitting the emergence and fixation of potential drug-resistant variants into the WHV replication pools of individual chronic carrier animals, is of obvious benefit. The continued use of the WHV/woodchuck and other experimental models of HBV infection and disease will be of assistance in developing effective strategies to treat or circumvent the emergence of HBV-drug resistance.

Acknowledgements

This work was supported by Public Health Service grant RO1-AI-41980 (to R.F. Schinazi), contract N01-AI-35164 (B.C.T.), contract N01-AI-45179 (to J.L. Gerin, B.E. Korba), and the US Department of Veterans Affairs (to R.F. Schinazi). We thank Nitika Kohli for her technical assistance in the site-directed mutagenesis work. Part of this paper was presented at the Eleventh International Conference on Antiviral Research, San Diego, CA, 5–10 April 1998.

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