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Article

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Susceptibility of lamivudine-resistant hepatitis B virus to other reverse transcriptase inhibitors

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The emergence of resistant hepatitis B virus (HBV), with mutations in the YMDD motif of the polymerase gene after treatment with lamivudine, is becoming an important clinical problem. In this study, susceptibility of wild-type and lamivudine-resistant HBV *M552I*, *M552V*, and *L528M/M552V* mutants to other reverse transcriptase inhibitors was investigated by transient transfection of full-length HBV DNA into human hepatoma cells. HBV DNA replication was monitored by Southern blot hybridization, which showed the presence of a single-stranded band (representative of the HBV replicative intermediates) in the drug-free, wild-type HBV-transfected cells. This band was diminished in the samples of wild-type HBV DNA treated with either lamivudine, adefovir, or lobucavir. The band intensities from the lamivudine-resistant mutants were not decreased by treatment with lamivudine, but were decreased by the treatments with adefovir or lobucavir. In contrast, penciclovir and nevirapine did not diminish the intensity of the single-stranded band of wild-type HBV or the lamivudine-resistant mutants. These results demonstrate that lamivudine-resistant HBV is susceptible to adefovir and lobucavir. Lamivudine-resistant HBV should be treated with adefovir or lobucavir, and combination therapy with lamivudine and adefovir/lobucavir may prevent the emergence of lamivudine-resistant HBV.

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Introduction

Hepatitis B continues to be a major health problem, accounting for 1.2 million deaths per year worldwide, according to the 1997 World Health Organization (WHO) report (1). Despite the existence of an effective vaccine, there is no completely effective antiviral treatment for patients chronically infected with hepatitis B virus (HBV). The overall response rate to IFN therapy, as measured by hepatitis B e antigen (Hb$_e$Ag) loss, is less than 40% (2). Because replication of HBV DNA proceeds through reverse transcription (3), the use of reverse transcriptase inhibitors is potentially an attractive option.

Lamivudine (3TC) is a promising reverse transcriptase inhibitor. It is well tolerated, produces a rapid and profound decrease in serum HBV DNA levels in patients with chronic hepatitis B (4), and induces histologic improvement (5). However, in recent clinical trials, 14–39% of immunocompetent patients developed drug-resistant virus during long-term treatment of hepatitis B (6–8). These lamivudine-resistant viruses contain mutations *M552I* or *M552V* in the Tyr, Met, Asp, Asp motif (YMDD motif) of the polymerase (6–11) considered to be a part of the catalytic center of the reverse transcriptase domain (12).

As a variety of new antiviral agents have been developed, it may be important to evaluate their action on these resistant mutants. Among these drugs, adefovir (PMEA) is a broad-spectrum nucleotide analogue that acts against HIV, herpesviruses, and hepadnaviruses (13–16). Adefovir dipivoxil (the oral prodrug of PMEA) is in clinical trials for use in the treatment of HIV and HBV infection (15, 16). It has been shown to be safe, well tolerated, and to reduce serum HBV DNA levels by a median of 4.1 log$_{10}$ and induce seroconversion in 20% of the 15 patients who were treated for 12 weeks with the drug at 30 mg/day (17).

Lobucavir is a nucleoside analogue with properties similar to ganciclovir that is active against a broad range of herpesvirus and HBV in vitro (18–21). Currently, it is undergoing phase I and phase II clinical trials in the United States to determine both its safety and antiviral activity in adults with chronic hepatitis B (22).

Penciclovir is a potent inhibitor of herpesvirus, varicella-zoster virus, and Epstein-Barr virus (23–25); its antiviral effect on HBV has been demonstrated in vitro and in vivo (24–29). Recently, famciclovir (the oral form of penciclovir) was used as prophylaxis for HBV recurrence and to treat patients with hepatitis B infection after liver transplants (30–33).

Reverse transcriptase can also be inhibited by agents that are not nucleoside or nucleotide analogues. Nevirapine (non-nucleoside reverse transcriptase inhibitor) binds to a hydrophobic pocket in the enzyme-DNA complex near the polymerization active site of HIV reverse transcriptase and blocks the chemical reaction without interfering with nucleotide binding or nucleotide-induced conformational change (34). Nevirapine has been approved for use in combination with nucleoside analogue anti-HIV drugs in HIV-infected patients whose current treatments have failed (35).

Lamivudine is a promising treatment; however, because a high number of lamivudine-resistant HBV mutants have emerged after long-term administration of the drug, the study of the efficacy of other reverse transcriptase inhibitors against lamivudine-resistant HBV has become imperative. Therefore, we decided to examine the effect of other reverse transcriptase inhibitors on the replication of the lamivudine-resistant HBV, using transient transfection of a full-length HBV DNA in human hepatoma cells.

Methods

Chemicals. Lamivudine was generously donated by Glaxo Wellcome (Greenford, United Kingdom); adefovir was a gift from Gilead Sciences (Foster City, California, USA); lobucavir was a gift from Bristol-Myers Squibb (Wallingford, Connecticut, USA); nevirapine was a gift from Boehringer Ingelheim (Ridgefield, Connecticut, USA); and penciclovir was a gift from SmithKline Beecham (Worthing, United Kingdom).

Plasmids. HBV DNA was amplified and cloned as described previously (36). Three types of lamivudine-resistant mutants were created by substituting nucleotides to change the codon for Met in the YMDD motif to Ile (*M552I* mutant) or Val (*M552V* mutant) and codon 528 for Leu in the B-domain motif to Met (*L528M/M552V* mutant) (37), using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA). The polymerase gene of the mutants was sequenced using a cycle DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, California, USA), as described previously (38), to confirm the introduction of mutations.

Transfection of full-length HBV DNA into HuH-7 cells. Full-length HBV DNA wild-type and lamivudine-resistant mutants were prepared for transfection as previously described (39).

Figure 1
Southern blotting analysis of HBV DNA extracted from HuH-7 cells transfected with wild-type, *M552I, M552V,* and *L528M/M552V* mutants of HBV treated with antivirals at a concentration of 8.7 μM. Lane a: control; lane b: lamivudine; lane c: nevirapine; lane d: adefovir; lane e: lobucavir; and lane f: penciclovir. OC, open circular; DS, double-stranded; SS, single-stranded.

HuH-7 cells (Human Science Research Resource Bank, Osaka, Japan) (40) were cultured in RPMI-1640 supplemented with 0.5% FBS and 0.2% lactalbumin. Approximately 10^6 cells were plated onto a 60-mm-diameter dish, and 24 hours later were transfected with 2 μg of full-length HBV DNA using Lipofectamine Plus reagent (GIBCO BRL, Gaithersburg, Maryland, USA). Twenty-four hours after transfection, the medium was changed and reincubated with drug-free medium or medium containing several different concentrations of lamivudine, adefovir, lobucavir, nevirapine, or penciclovir, as described previously (41). Medium and cells (rinsed 3 times with ice-cold PBS) were harvested 3 days later. The efficiency of transfection was monitored by cotransfecting a β-galactosidase expression plasmid, pCMVβ (CLONTECH Laboratories Inc., Palo Alto, California, USA). Assays for β-galactosidase in extracts of HuH-7 cells were performed as described (42). Experiments were performed in triplicate.

Isolation of HBV DNA from transfected cells. Purification of HBV DNA from intracellular core particles was accomplished using the method described by Günther et al. (39), with a minor modification. Briefly, cells were suspended in 500 μL of lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, and 1% NP-40), transferred to an Eppendorf tube, vortexed, and allowed to stand on ice for 15 minutes. Nuclei were pelleted by centrifugation at 4°C, 15,000 *g*, for 1 minute. The supernatant was transferred to a new tube, adjusted to 10 mM $MgCl_2$, and digested with 100 μg/mL of DNase I for 30 minutes at 37°C. To stop the reaction, EDTA was added to a final concentration of 25 mM. Then, 0.5 mg/mL proteinase K and 1% SDS were added and incubated at 0°C for 4 hours. Phenol-chloroform (1:1) extraction was performed, and the nucleic acids were ethanol precipitated along with a glycogen carrier.

Southern blot hybridization of HBV DNA. HBV DNA was resolved in 1.5% agarose gel, transferred to a nylon membrane (Hybond N+; Amersham International, Little Chalfont, United Kingdom) by Southern blotting, and hybridized with a ^{32}P-labeled wild-type, full-length HBV DNA probe generated with Ready-To-Go DNA labeling kit (–dCTP) (Pharmacia Biotech AB, Uppsala, Sweden). Autoradiography was performed and analyzed by using a BAS 2000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Effect of antiviral drugs on the replication of wild-type or mutant HBV. To compare the effect of the antiviral drugs on wild-type and lamivudine-resistant mutants of HBV, we transfected HuH-7 cells with HBV DNA and added the antiviral drugs at a concentration of 8.7 μM. We chose this concentration because it was previously demonstrated to be the IC_{90} for lamivudine of the wild-type HBV (41) and would allow direct comparison with the other drugs. We also applied several concentrations of the antiviral drugs to calculate their IC_{50}, to standardize the assay, and to view the of an inhibition curve. To evaluate the susceptibility of HBV to lamivudine, lobucavir, adefovir, penciclovir, and nevirapine, Southern blot hybridization on DNA extracts from transfected cells was performed. We selected these drugs because the first 4 are already in ongoing clinical trials for hepatitis B (8, 16, 22, 33), and chose nevirapine because it is a reverse transcriptase inhibitor. The single-stranded HBV DNA band, previously shown to represent HBV intermediates (39, 43), was analyzed to assess the efficacy of the reverse transcriptase inhibitors on HBV replication. This band was quantified and normalized for transfection efficiency based on β-galactosidase activity.
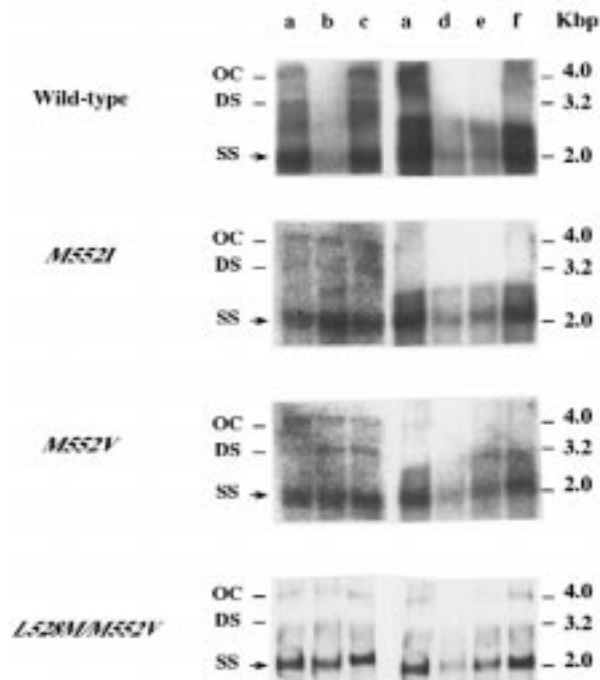
Results

Wild-type HBV is susceptible to lamivudine, lobucavir, and adefovir. Southern blot hybridization on DNA extracts showed the presence of a single-stranded band (representative of the HBV replicative intermediates) in the

Diagram comparing the mean ± SD susceptibility of wild-type, *M552I*, *M552V*, and *L528M/M552V* mutants of HBV transfected into HuH-7 cells to lamivudine, lobucavir, adefovir, penciclovir, and nevirapine at a concentration of 8.7 µM. Single-stranded bands were quantified using a BAS 2000 image analyzer and normalized for transfection efficiency based on β-galactosidase activity. The single-stranded band of control was calculated as 100.
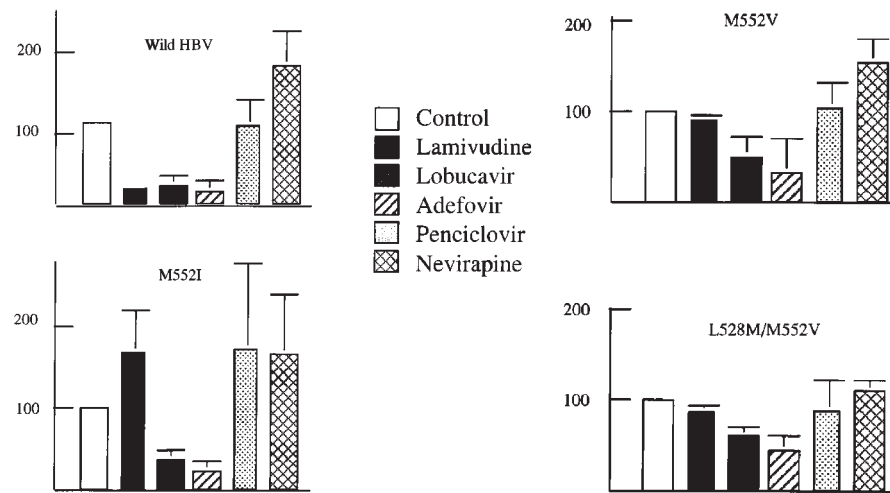
drug-free samples (Figure 1, lane a). This band was diminished by 83.3 ± 2.2%, 87.2 ± 14.5%, and 79.7 ± 12.6% (Figure 2) in the samples transfected with wild-type HBV DNA that was treated with lamivudine (Figure 1, lane b), adefovir (Figure 1, lane d), or lobucavir (Figure 1, lane e), respectively. Inhibition curves for each drug are provided in Figure 3, and calculated antiviral IC_{50} values are presented in Table 1.

Lamivudine-resistant HBV is susceptible to lobucavir and adefovir. To evaluate the susceptibility of lamivudine-resistant mutants of HBV to adefovir, lobucavir, penciclovir, and nevirapine, Southern blot hybridizations on DNA extracts from transfected cells were performed (Figure 1). Southern blot hybridization on DNA extracts showed the presence of a single-stranded band (representative of the HBV replicative intermediates) in the drug-free samples (Figure 1, lane a). When treated with lamivudine, the *M552I*, *M552V*, and *L528M/M552V* mutants' band intensities were not decreased, thus confirming their lamivudine-resistant phenotype. However, following treatment of the mutants with adefovir and lobucavir, the band intensities were decreased by 79.3 ± 12.6% and 64.1 ± 11.5% for the *M552I* mutant; by 69.2 ± 37.5% and 51.7 ± 21.7% for the *M552V* mutant; and by 57.0 ± 9.9% and 41.0 ± 14.8% for the *L528M/M552V* mutant, respectively (Figure 2). In contrast, penciclovir and nevirapine did not diminish the band intensities of the lamivudine-resistant mutants.

We determined the inhibition of HBV DNA synthesis using several concentrations of the drugs. Inhibition curves for each drug showed that the properties of inhibition were sigmoidal (Figure 3). The antiviral drugs' IC_{50} values are presented in Table 1. It is interesting to note that whereas the wild-type HBV has an IC_{50} for adefovir and lobucavir lower than 1 µM, the lamivudine-resistant variants showed a higher IC_{50}, ranging from 2.2 to 7 µM.

Lack of susceptibility of HBV to penciclovir and nevirapine. The single-stranded band in the samples of wild-type and lamivudine-resistant HBV treated with penciclovir was not decreased as expected (Figure 1, lane f). To evaluate whether a higher dose of penciclovir would inhibit wild-type HBV replication in this system, HuH-7 cells transiently transfected with wild-type HBV DNA were treated with increasing doses of penciclovir (0–80 µM) and lamivudine (0–10 µM). While lamivudine clearly inhibited the replication of wild-type HBV, the replication of wild-type HBV was not diminished by even higher doses of penciclovir (Figures 4 and 5). The sequence of wild-type HBV DNA was checked for mutations within the B domain of the polymerase gene and associated with reduced penciclovir sensitivity as described previously (32). The sequence of the HBV DNA used in this study did not contain the described mutations (data not shown). Nevirapine also did not diminish the replication of wild-type HBV or the lamivudine-resistant mutants (Figure 1, lane c; and Figure 5). Because penciclovir and nevirapine did not inhibit replication of wild-type HBV, we did not estimate the dose-dependency curve for the lamivudine-resistant HBV.

Discussion

Hepatitis B continues to be a life-threatening disease worldwide (1), and IFN-α — the only approved treatment — has dose-limiting side effects and limited benefit (2). Thus, more effective and safer treatments for hepatitis B are urgently required. Currently, lamivudine is a promising treatment for chronic hepatitis B and is in widespread use. However, resistance to lamivudine has been observed in both immunocompetent patients and in patients after liver transplants, and is associated with a mutation Met to Ile or Val in

Table 1

Susceptibility of HBV constructs to reverse transcriptase inhibitors

	IC50 (µM)[A]			
Antiviral	Wild-type	*M552I*	*M552V*	*L528M/M552V*
Lamivudine	<1	>80	33	>80
Adefovir	<1	4.5	4.9	2.2
Lobucavir	<1	5.0	3.6	7.0
Penciclovir	>80	ND	ND	ND
Nevirapine	>80	ND	ND	ND

[A]IC50 values represent the mean of 3 experiments. ND, not done.

the YMDD motif of the HBV polymerase gene (7–10). In addition, a mutation from Leu to Met has been described in the B domain of the polymerase gene associated with the *M552V* mutant (37). Because reverse transcriptase has a fundamental role in the replication of retroviruses and HBV, it became an attractive target in chemotherapy for these viruses. Reverse transcriptase inhibitors can be broadly classified into 2 groups: nucleoside (or nucleotide) analogues (lamivudine, adefovir, lobucavir, and penciclovir) that are active in the triphosphate form; and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine) that interact directly with reverse transcriptase. Therefore, we decided to study the effect of 3 antiviral agents (adefovir, lobucavir, and penciclovir) currently used in clinical trials and to study the effect of nevirapine on lamivudine-resistant HBV, using transient transfection of a full-length HBV DNA in human hepatoma cells.
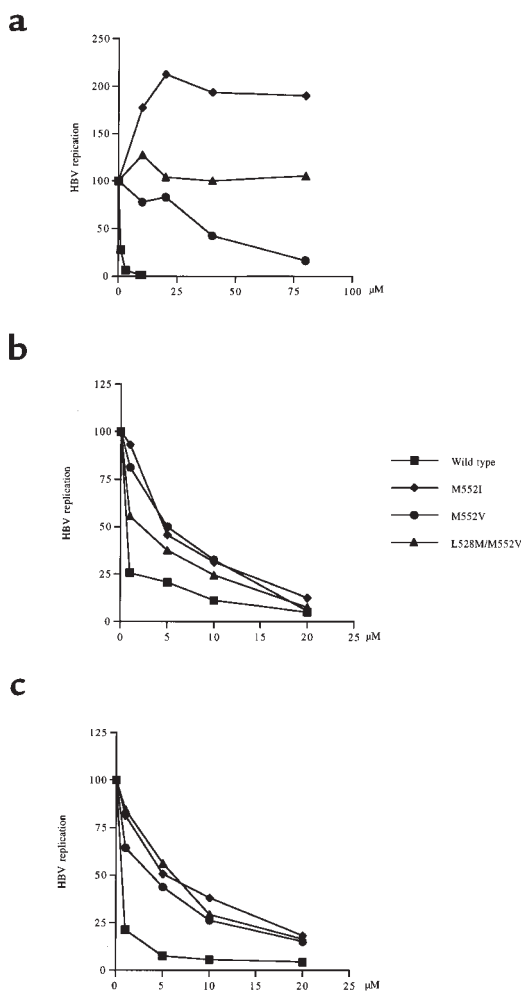

a

b

c

Figure 3
Drug inhibition curves of wild-type and lamivudine-resistant HBV transfected into HuH-7 cells treated with indicated concentrations of lamivudine (**a**), adefovir (**b**), and lobucavir (**c**). Values represent the mean of 3 experiments.

The HepG2.2.15 cell line has been used to evaluate the effect of antiviral drugs against HBV (26–28, 44). However, this system does not permit analysis of HBV mutants that are now being reported, because these cells are stably transfected with wild-type HBV. Using the system of transient transfection of a full-length HBV DNA into HuH-7 cells, we were able to evaluate other potential therapeutic agents that might improve the treatment of chronic hepatitis B.

To test the replication ability of the wild-type and lamivudine-resistant mutant HBV, Southern blot hybridization on DNA extracts was performed because it is an established method to monitor the antiviral activity of drugs for HBV (26–28, 44–47). As was reported previously (39, 43), open circular, double-stranded, and single-stranded HBV DNA bands were seen at 4.0 kbp, 3.2 kbp, and 2.0 kbp, respectively. The single-stranded HBV DNA band, previously shown to represent HBV replicative intermediates (3), was analyzed to study the function of inhibitors of reverse transcriptase by inhibiting the conversion of the pregenomic RNA to single-stranded DNA. The effects of the antiviral drugs were determined by the addition of a drug 24 hours after transfection in order to avoid interference of these drugs in the transfection step. This system may be a valid way to evaluate the effects of antiviral agents on HBV replication, because the formation of single-stranded DNA occurred 72 hours after transfection (data not shown). Because the levels of HB_sAg did not change in the samples treated in this system, HB_sAg expression could not be used to evaluate antiviral efficacy. This lack of change could be explained by the drugs' mechanisms of action, which inhibit only the reverse transcription step and cannot inhibit transcription and translation of transfected HBV DNA.

Clinical studies have shown that adefovir is safe, well tolerated (15,16), and reduces serum HBV DNA levels by more than $4 \log_{10}$ (99.99%) (17). The results presented here confirm the in vitro anti-hepadnaviral activity of adefovir. Following administration of adefovir, the single-stranded band of wild-type HBV DNA was significantly diminished (Figure 1). This result is in agreement with the results of Yokota et al. (45) and Heijtink et al. (46), indicating an inhibitory effect of adefovir in human and duck HBV in vitro infection.

Our study demonstrates that lobucavir could decrease replication of wild-type HBV in vitro, and this result is in accordance with other studies that show that lobucavir has in vitro and in vivo activity against HBV (20, 21, 47). Currently, clinical trials are underway to determine its safety and its antiviral activity against HBV and cytomegalovirus (20, 22).

In contrast to the significant inhibitory effect of lamivudine on wild-type HBV, the resistant mutants *M552I*, *M552V*, and *L528M/M552V* did not show significant decrease in replication, even with higher doses of lamivudine (Figure 3a). In the present study, we demonstrate that adefovir and lobucavir markedly reduce viral DNA production by the lamivudine-resistant mutants. However, even though adefovir and lobucavir are effective against lamivudine-resistant HBV, the dose used to inhibit replication of the wild-type virus is not enough

Figure 4

Southern blotting hybridization analysis of HBV transfected into HuH-7 cells treated with lamivudine and penciclovir. Lanes correspond to DNA extracted from viral core particles derived from HuH-7 cells. The arrow indicates the single-stranded band, representative of HBV replication intermediates. OC, open circular; DS, double-stranded; SS, single-stranded.
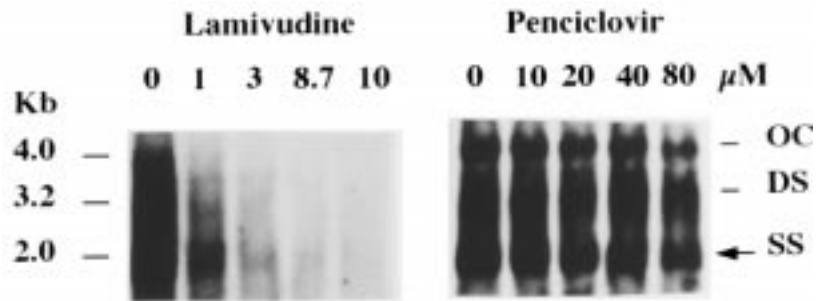
to completely inhibit the lamivudine-resistant mutants. This result suggests not only that mutations associated with lamivudine resistance resulted in a small decrease in susceptibility to adefovir and lobucavir, but also indicates that higher doses of the drugs may be required to achieve complete suppression of viral replication. Also, it is interesting to note that this decrease in susceptibility to lamivudine and lobucavir is progressive among the mutants, where the *M552V* mutant is more susceptible than the *L528M/M552V* mutant. Allen et al. (37) had shown previously that in the double mutant (*L528M/M552V*), each mutation may contribute partially to the loss of sensitivity to lamivudine in vitro. Our study suggests that the same loss of sensitivity can occur for lobucavir but not for adefovir.

Although mutations in HIV reverse transcriptase associated with decreased susceptibility to adefovir were described in vitro (48), these mutations have been rarely observed in vivo and were not associated with rebound viremia (48); to date there has been no description of mutations in the HBV reverse transcriptase leading to adefovir resistance. Interestingly, these mutations in the HIV reverse transcriptase are not located in any of the 5 conserved motifs of RNA-dependent polymerases of HIV.

Unexpectedly, penciclovir did not show any antiviral effects against HBV wild-type or lamivudine-resistant mutants in our study, despite previous evidence of a capacity to inhibit replication of HBV in vitro and in vivo (23–29). The sequence was confirmed to be wild-type HBV in the B domain of polymerase because mutations that confer resistance to penciclovir have been described previously in this region (32). Stably transfected cells, HepG2.2.15, were also used to check the susceptibility to penciclovir (data not shown). This experiment confirmed the results obtained using HuH-7 cells, showing that up to 80 μM of penciclovir did not diminish the production of replicative intermediates in either cell line, and is in agreement with another study that found an EC_{50} of 100 μM for penciclovir (21). A previous report showed that penciclovir could diminish the production of the covalently closed circular (CCC) DNA form of duck HBV, an event that occurs prior to transcription and reverse transcription (27). These conflicting results may suggest the possibility that penciclovir could inhibit HBV replication by blocking a step (other than reverse transcription) that is not detected in our assay, which monitors only the formation of the single-stranded DNA intermediate band.

Nevirapine is the first NNRTI to be approved for the treatment for HIV (35). Because it inhibits replication by blocking reverse transcription (34), we attempted to evaluate whether it could inhibit the replication of HBV. Like other NNRTIs, nevirapine is a potent inhibitor of HIV reverse transcriptase, but the spectrum of activity is extremely narrow because it is not effective against other retroviruses. Our results may confirm that nevirapine might be specific for HIV.

Given the high incidence of lamivudine-resistant HBV, monotherapy with lamivudine for HBV is unlikely to be sufficient to eradicate it (49, 50). A more efficient strategy to prevent the emergence of a resistant virus should be a goal in the treatment of chronic hepatitis B (51). The combination of lamivudine with adefovir or lobucavir could be expected to be one approach to preventing the emergence of lamivudine resistance, because lamivudine-resistant mutants are susceptible to both adefovir and lobucavir.
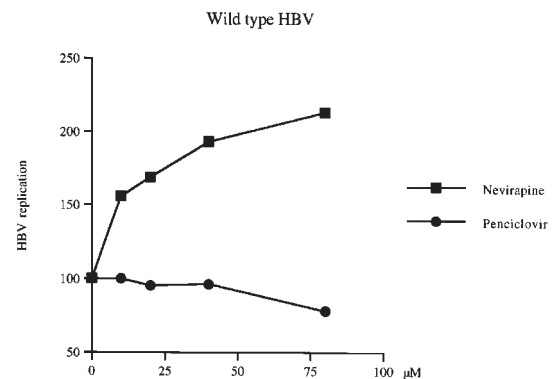
Acknowledgments

Figure 5

Lack of susceptibility of HBV to penciclovir and nevirapine. Drug inhibition curves of wild-type HBV transfected into HuH-7 cells treated with indicated concentrations of penciclovir and nevirapine. Values represent the mean of 3 experiments.

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