**The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance**

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After receiving lamivudine for 3 years to treat chronic hepatitis B, 67–75% of patients develop B-domain L528M, C-domain M552I, or M552V mutations in the HBV polymerase that render hepatitis B virus (HBV) drug-resistant. The aim of this study was to evaluate the influence of these mutations on viral replication and resistance to antiviral agents. We investigated the replication fitness and susceptibility of the wild-type and five mutant HBVs (L528M, M552I, M552V, L528M/M552I, and L528M/M552V) to 11 compounds [lamivudine, adefovir, entecavir (BMS-200475) (+)-BCH-189 (±)-FTC (racivir) (–)-FTC (emtricitabine) (–)-FTC, L-DAPD, and (–)-carbovir] by transfecting HBV DNA into hepatoma cells and monitoring viral products by Southern blotting. The replication competency of the single C-domain mutants M552I and M552V was markedly decreased compared with that of wild-type HBV. However, addition of the B-domain mutation L528M restored replication competence. Only adefovir and entecavir were effective against all five HBV mutants, and higher doses of these compounds were necessary to inhibit the double mutants compared with the single mutants. The B-domain mutation (L528M) of HBV polymerase not only restores the replication competence of C-domain mutants, but also increases resistance to nucleoside analogues.


**Introduction**

Despite the availability for almost 20 years of safe and effective vaccines against hepatitis B, chronic infection with hepatitis B virus (HBV) remains among the ten most common causes of death worldwide (1). Recently, lamivudine [(-)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC)] became the first approved oral therapy for the treatment of HBV (2). Clinical data have shown that lamivudine treatment rapidly reduces the levels of HBV DNA, is well tolerated, and improves liver histology (3, 4). However, a defined course of 52 weeks’ treatment provides a sustained response rate of 17–33% (loss of HBeAg). Discontinuation of therapy at 52 weeks is followed by relapse in patients who do not lose HBeAg by that point, and these patients may benefit from long-term therapy (5, 6). However, prolonged use of lamivudine therapy has been associated with increased emergence of lamivudine-resistant HBV with amino-acid substitutions in the B domain (L528M) and in the YMDD motif of the C domain (M552I and M552V) of the viral DNA polymerase (7–14). The emergence rate of lamivudine-resistant HBV ranges from 17–46% at 1 year to as high as 67–75% after 3–4 years of continuous therapy (4, 6, 15).

The YMDD motif, a conserved motif in RNA-dependent DNA polymerase, is involved in nucleotide binding in the catalytic site of the polymerase (16, 17). It was previously demonstrated that owing to replication competence and lamivudine sensitivity, viruses having M552I or M552V sequences may appear during treatment with lamivudine (18, 19). On the other hand, the B domain of DNA polymerase is an element responsible for template positioning (20). Amino acid substitution in the B domain (L528M) of HBV polymerase was described in patients receiving lamivudine, accompanying the M552I or M552V mutation, and in patients receiving famciclovir without any mutation in the YMDD motif (10, 21).

Liaw et al. recently reported that exacerbation occurred in 41% of patients after the emergence of YMDD motif mutations during continued use of lamivudine (22). In addition, lamivudine-resistant HBV is associated with advanced hepatic fibrosis and severe microinflammatory changes in patients with recurrent HBV infection after orthotopic liver transplantation (23). Lamivudine-resistant HBV also can cause severe hepatitis in patients coinfected with HIV and HBV (24). Thus, the need to develop new antivirals...
and new strategies to treat HBV infections is becoming clear. Previously, we demonstrated that adefovir decreased replication of wild-type and lamivudine-resistant HBV (25); however, new antivirals are becoming available, and there is a pressing need for further studies to determine their potential as single agents and for combination chemotherapy.

The aim of this study was to evaluate the replication competence and susceptibility of wild-type HBV and five different mutants (L528M, M552I, M552V, L528M/M552I, and L528M/M552V) to 11 drugs [lamivudine; 9-(2-bis[pivaloyloxymethyl] methoxethyl) adenine (adefovir); 1S-(1R,3α,4β)-2-amino-1,9-dihydro-9H-[4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one (entecavir); (+)-β-2',3'-dideoxy-3'-thiacytidine (BCH-189) (the plus enantiomer of lamivudine); (z)-β-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC) (racivir); (–)-FTC (emtricitabine, clevudine); (–)-FTC (the plus enantiomer of emtricitabine); (–)-β-L-2',3'-dideoxy-5-fluorocytidine (L-D4FC); 2'-Fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU, clevudine); (–)-β-D-2,6-diaminopurine dioxolane (DAPD); and (–)-carbovir].

Methods

Chemicals. Lamivudine was generously donated by GlaxoWellcome (Middlesex, United Kingdom). Adefovir was a gift from Gilead Sciences (Foster City, California, USA). Entecavir (BM 5-200475), a guanosine analogue, was synthesized by the method of Bisacchi et al. (26). The other antiviral agents were synthesized in R.F. Schinazi’s laboratory. All compounds were coded and remained coded until the preliminary results were obtained.

Cells. HuH-7 cells (Human Science Research Resource Bank, Osaka, Japan) (27) were cultured in DMEM (Life Technologies Inc., Rockville, Maryland, USA) supplemented with 10% FBS. The 2.2.15 cells (clonal cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) that secrete HBV virions were kindly provided by G. Acs (Mount Sinai Medical Center, New York, New York, USA) (28). The 2.2.15 cells were maintained in DMEM supplemented with 20% FBS.

HBV plasmids (wild-type, “single” and “double” mutants and dimer). Wild-type HBV DNA, extracted from the serum of a 54-year-old Japanese man with HBV positive (subtype ayw, genotype D) cirrhosis, was amplified and cloned as described previously (18, 25). HBV DNA was extracted from 100 µl serum using a SepaGene kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer’s instructions. Five mutants were prepared. First, three single mutants (L528M, M552I, and M552V) were made by substituting nucleotides in order to change the codon for Leu in the B domain to Met (L528M) using a codon for Leu in the B domain to Met (L528M) using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, California, USA). Similarly, two double mutants (L528M/M552I and L528M/M552V) were constructed by adding L528M substitution to the C-domain single mutants M552I and M552V. To confirm the introduction of mutations, the polymerase genes of the mutants were sequenced using a cycle DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, California, USA) as described previously (29).

Plasmid pSM2 containing a head-to-tail dimer of HBV was kindly provided by S. Günther (Hennrich-Pette-Institut fur Experimentelle Virologie, Hamburg, Germany) (30).

Transfection of HBV DNA into HuH-7 cells. HuH-7 cells at 80–90% confluence (in 60-mm dishes) were transfected with 0.9 µg full-length HBV DNA wild-type, mutants, or pSM2 using Effectene transfection reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Twenty-four hours after transfection, the medium was changed and reincubated with drug-free medium or medium containing 0.0001, 0.001, 0.01, 0.1, 1, or 10 µM of each compound. Medium and cells (rinsed three times with cold PBS) were harvested 3 days later. The efficiency of transfection was monitored by cotransfecting 0.1 µg β-galactosidase expression plasmid, pCMV β (CLONTECH Laboratories Inc., California, USA). Assays for β-galactosidase in extracts of HuH-7 cells were performed as described previously (25).

The medium of 2.2.15 cells at 80–90% confluence (in 60-mm dishes) was changed and reincubated with drug-free medium or medium containing 0.0001, 0.001, 0.01, 0.1, 1, or 10 µM of lamivudine or entecavir. Medium and cells (rinsed three times with cold PBS) were harvested 3 days later.

Experiments were performed at least in duplicate.

Isolation of core-particle-related HBV DNA. Purification of HBV DNA from intracellular core particles was accomplished using the method described by Günther et al. (30) with minor modifications. Briefly, cells were suspended in 500 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% N-P-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 MgCl2, 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% sodium dodecyl sulfate were added and incubated at 50°C for 4 hours. Phenol-chloroform (1:1) extraction was performed, and then 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 gels, transferred to nylon membranes (Hybond N+; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) by Southern blotting, and hybridized with an alkaline-phosphatase-labeled wild-type full-length HBV DNA probe generated with the Gene Images AlkPhos Direct labeling system (Amersham Pharmacia Biotech). Chemiluminescent detection was performed with CDP-Star (Amersham Pharmacia...
Biotech) and analyzed using an LAS1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Drug susceptibility analysis (determination of EC50) by measuring single-stranded HBV DNA. To compare the effect of the 11 antiviral agents on the wild-type HBV and five mutants, HuH-7 cells were transfected with HBV DNA, and the antivirals were added at a concentration of 10 µM. For those antivirals that inhibited the replication of HBV by more than 50% at this concentration, increasing concentrations (0.0001 to 10 µM) of the compound were applied to calculate the effective concentration required to reduce HBV replication by 50% (EC50). To evaluate the susceptibility of HBV to the antiviral agents, Southern blot hybridization of DNA extracts from transfected cells was performed. The single-stranded HBV DNA band, previously shown to represent HBV intermediates (30, 31), was analyzed to assess the efficacy of the RT inhibitors on HBV replication. This single-stranded band was quantified and normalized for transfection efficiency based on β-galactosidase activity. Data are shown as the mean ± SD of at least two experiments.

Results
Replication competency of the five HBV mutants. To evaluate the effect of mutations in the polymerase gene on HBV replication, the replication ability of the wild-type HBV and five mutants (L528M, M552I, M552V, L528M/M552I, and L528M/M552V) was examined in a transient transfection cell culture assay system. Southern blot hybridization of DNA extracts showed the presence of a single-stranded band (representative of HBV replication intermediates) for each construct, indicating that they were replication-competent (Figure 1). These bands were quantified, adjusted for the efficiency of transfection according to the β-galactosidase assay of cotransfected pCMVβ, and determined by taking the relative ratio of the normalized single-stranded band is shown below (Table 1).

Figure 1
Southern blot hybridization analysis of replication of wild-type HBV and five mutants. Lanes correspond to DNA extracted from viral core particles derived from HuH-7 cells that were transfected with DNA of wild-type HBV or one of five mutants. Single-stranded bands (SS) were quantified using an LAS1000 image analyzer and then normalized for transfection efficiency based on β-galactosidase activity. The relative ratio of the normalized single-stranded band is shown below each lane, assuming the single-stranded band of wild-type HBV to be 100% OC, open circular; DS, double-stranded HBV DNA.
Representative Southern blot hybridization analysis of replication of the wild-type treated with entecavir. Lanes correspond to DNA extracted from viral core particles derived from HuH-7 cells that were transfected with wild-type HBV DNA and incubated with increasing concentrations (0.0001, 0.001, 0.01, and 0.1 µM) of entecavir. OC, open circular; DS, double-stranded; SS, single-stranded HBV DNA. (b) Diagram of replication of wild-type HBV treated with entecavir. Single-stranded bands (SS) were quantified using an LAS1000 image analyzer and then normalized for transfection efficiency based on β-galactosidase activity. The single-stranded band of the wild-type without entecavir was calculated as 100. EC₅₀ was determined to be 0.00036 µM.

Table 1
EC₅₀ values of compounds and level of replication of wild-type and five mutant HBV at 10 µM concentration treatment

<table>
<thead>
<tr>
<th>Compound</th>
<th>10 µM</th>
<th>EC₅₀</th>
<th>10 µM</th>
<th>EC₅₀</th>
<th>10 µM</th>
<th>EC₅₀</th>
<th>10 µM</th>
<th>EC₅₀</th>
<th>10 µM</th>
<th>EC₅₀</th>
<th>10 µM</th>
<th>EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>1.3 ± 0.2</td>
<td>0.56</td>
<td>59.2 ± 8.6</td>
<td>&gt;10</td>
<td>176.8 ± 17.9</td>
<td>&gt;80</td>
<td>76.9 ± 15.8</td>
<td>33</td>
<td>111.7 ± 17.3</td>
<td>&gt;10</td>
<td>126.7 ± 9.6</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Adefovir</td>
<td>10.5 ± 6.1</td>
<td>0.58</td>
<td>8.8 ± 10.3</td>
<td>0.45</td>
<td>30.8 ± 20.0</td>
<td>4.5</td>
<td>32.5 ± 2.9</td>
<td>4.9</td>
<td>47.2 ± 6.5</td>
<td>9.5</td>
<td>23.8 ± 13.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Entecavir</td>
<td>3.6 ± 0.4</td>
<td>0.0004</td>
<td>12.7 ± 4.9</td>
<td>0.0005</td>
<td>16.4 ± 3.5</td>
<td>0.06</td>
<td>5.2 ± 1.0</td>
<td>0.003</td>
<td>42.3 ± 5.3</td>
<td>0.25</td>
<td>34.7 ± 18.1</td>
<td>0.28</td>
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<tr>
<td>(+)-BCH-189</td>
<td>103.2 ± 22.3</td>
<td>&gt;10</td>
<td>142.2 ± 28.0</td>
<td>&gt;10</td>
<td>107.5 ± 15.6</td>
<td>&gt;10</td>
<td>131.0 ± 10.9</td>
<td>&gt;10</td>
<td>98.3 ± 8.9</td>
<td>&gt;10</td>
<td>173.8 ± 15.4</td>
<td>&gt;10</td>
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<tr>
<td>(–)-FTC</td>
<td>13.6 ± 12.8</td>
<td>1.85</td>
<td>39.6 ± 6.4</td>
<td>5.1</td>
<td>98.7 ± 20.2</td>
<td>&gt;10</td>
<td>83.5 ± 10.6</td>
<td>&gt;10</td>
<td>125.1 ± 30.8</td>
<td>&gt;10</td>
<td>122.3 ± 4.7</td>
<td>&gt;10</td>
</tr>
<tr>
<td>(–)-FTC</td>
<td>7.1 ± 1.0</td>
<td>0.24</td>
<td>31.5 ± 5.5</td>
<td>2.7</td>
<td>105.9 ± 32.2</td>
<td>&gt;10</td>
<td>102.3 ± 25.1</td>
<td>&gt;10</td>
<td>144.1 ± 19.0</td>
<td>&gt;10</td>
<td>87.6 ± 12.1</td>
<td>&gt;10</td>
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<tr>
<td>D-DAPD</td>
<td>79.7 ± 5.4</td>
<td>&gt;10</td>
<td>111.0 ± 21.3</td>
<td>&gt;10</td>
<td>104.8 ± 4.5</td>
<td>&gt;10</td>
<td>108.3 ± 23</td>
<td>&gt;10</td>
<td>96.6 ± 12.4</td>
<td>&gt;10</td>
<td>101.9 ± 6.9</td>
<td>&gt;10</td>
</tr>
<tr>
<td>D-DAPD</td>
<td>1.5 ± 0.2</td>
<td>0.033</td>
<td>4.8 ± 6.7</td>
<td>0.13</td>
<td>145.5 ± 31.6</td>
<td>&gt;10</td>
<td>5.4 ± 6.5</td>
<td>1.8</td>
<td>128.3 ± 28</td>
<td>&gt;10</td>
<td>128.3 ± 28</td>
<td>&gt;10</td>
</tr>
<tr>
<td>L-FMAU</td>
<td>16.5 ± 10.0</td>
<td>0.053</td>
<td>39.6 ± 17.8</td>
<td>1.2</td>
<td>91.8 ± 13.8</td>
<td>&gt;10</td>
<td>9.6 ± 15.9</td>
<td>0.74</td>
<td>127.3 ± 20</td>
<td>&gt;10</td>
<td>104 ± 24.4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>D-DAPD</td>
<td>75.9 ± 23.6</td>
<td>&gt;10</td>
<td>99.4 ± 8.9</td>
<td>&gt;10</td>
<td>132 ± 29.9</td>
<td>&gt;10</td>
<td>118.7 ± 29.6</td>
<td>&gt;10</td>
<td>134.6 ± 4.1</td>
<td>&gt;10</td>
<td>105.7 ± 9.3</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

*Numbers indicate the mean ± SD in percent of replication of wild-type HBV treated with 10 µM of compounds. Ref. 25. **
polymerase gene. The L528M mutation frequently accompanies M552V and has recently been reported to accompany M552I occasionally (7–10, 12). To summarize three published reports, the clinical frequency of lamivudine-resistant mutants was 18.6% for M552I, 1.4% for M552V, 11.4% for L528M/M552I, and 64.3% for L528M/M552V (10, 11, 13).

Given that mutations in the polymerase gene have been associated with changes in the replication competency of the virus (18, 19), we examined the influence, singly or in combination, of the B-domain mutation (L528M) and the two C-domain mutations (M552I and M552V) on replication ability. Compared with the wild-type HBV, the single C-domain mutants M552I and M552V had markedly decreased replication abilities (18). Of particular interest, the double mutants with both B- and C-domain mutations (L528M/M552I and L528M/M552V) replicated significantly better than did the single C-domain mutants, suggesting that the B-domain mutation L528M rescued the defective replication competence of the C-domain mutants. It was previously indicated that L528M could compensate for the impact of a mutation in the YMDD motif on viral replication in vitro (33). The B-domain L528 in the amino acid 521–537 helix is close enough to interact with M552 of the YMDD loop in a hypothetical molecular model of HBV RT (10). It could be speculated that the L528M mutation reduces the imbalance of conformation caused by the M552V and M552I mutations, thereby improving the replication competence of single C-domain mutants.

Of the 11 compounds tested, entecavir, L-D4FC, L-FMAU, and adefovir inhibited the replication of wild-type HBV effectively, with an EC50 less than 1 µM. These results are comparable with previous reports that demonstrated inhibition of HBV replication by 50% at concentrations of less than 0.1 µM in 2.2.15 cells (34–38).

Because several different groups tested these compounds in different ways, it was difficult to compare their EC50 values, as they varied according to the assay used, cell type used, marker of viral replication selected, composition of the medium, and time in culture (39). We studied the effect of the 11 compounds simultaneously. Therefore, we could determine and compare their relative potencies. Entecavir, L-D4FC, L-FMAU, and (+)-FTC inhibited wild-type HBV replication two to 1,556 times more than lamivudine. Similar results were obtained in 2.2.15 cells and pSM2-transfected HuH7 cells. In these cells, entecavir was 237–2,200 times more potent than lamivudine. In general, the more potent the antiviral agents used to suppress viral replication, the less likely the virus is to develop drug-resistant mutations, because mutations arise as replication errors (40–43). Therefore, in the absence of toxicological considerations of these experimental agents, entecavir, L-D4FC, and L-FMAU are potentially useful “first line” drugs for the treatment of HBV. However, it must be noted that in vitro sensitivities do not always match the sensitivities of virus infection in humans in vivo. Therefore, the ultimate test is their effectiveness in patients with chronic hepatitis B.

While adefovir, entecavir (+)-FTC (–)-FTC, L-D4FC, and L-FMAU are efficacious (EC50 < 10 µM) against some of the five lamivudine-resistant HBV mutants, only adefovir and entecavir are effective against all five mutants. As previously described, adefovir might be a good treatment option in those patients who have failed lamivudine therapy because of drug-resistant HBV (25). In fact, it was recently demonstrated that adefovir resulted in a rapid and sustained reduction in HBV DNA levels associated with improvement in liver function in patients who failed to respond to lamivudine therapy (44). In addition, based on our data, entecavir could be an option for the treatment of lamivudine-resistant mutants. However, it must be noted that the EC50 values for lamivudine-resistant mutants were 2 to 778 times higher than those for the wild-type HBV. Therefore, the dose of entecavir necessary

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (µM)</th>
<th>EC50 (µM)</th>
<th>EC50 (µM)</th>
<th>EC50 (µM)</th>
<th>EC50 (µM)</th>
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<tr>
<td>Wild</td>
<td></td>
<td>Entecavir</td>
<td>Entecavir</td>
<td>Entecavir</td>
<td>Entecavir</td>
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</tr>
<tr>
<td>L528M</td>
<td>(0.00036)</td>
<td>(0.00054)</td>
<td>(0.06)</td>
<td>(0.0031)</td>
<td>(0.25)</td>
<td>(0.28)</td>
</tr>
<tr>
<td>M552I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M552V</td>
<td></td>
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</tr>
<tr>
<td>L528M/M552I</td>
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<td></td>
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<tr>
<td>L528M/M552V</td>
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</table>

*EC50 value of each compound is shown in parentheses.*
to treat lamivudine-resistant mutants would be considerably lower than that used for wild-type HBV.

L-D4FC and L-FMAU were effective against single B- or C-domain mutants L528M and M552V with an EC$_{50}$ of less than 2 µM, but were ineffective against the double mutant L528M/M552V, with an EC$_{50}$ exceeding 10 µM. In addition, the EC$_{50}$ of entecavir for the double mutants was about 500 times higher than that for the single B-domain mutant L528M, and 90 times higher than that for the single C-domain mutants. The addition of the B-domain mutation L528M to C-domain mutants may contribute to increased levels of resistance to entecavir, L-D4FC, and L-FMAU but does not seem to increase the level of resistance to adefovir, suggesting that the resistance pattern for adefovir is unique and different from those of entecavir, L-D4FC, and L-FMAU.

Although further studies are needed to address the cytotoxicity of these drugs in humans, the doses of the compounds used in our study are far below the toxic doses reported by others. Using 2.2.15 cells, no apparent cytotoxicity was noted at concentrations greater than 1,000, 150, and 30 µM for lamivudine, adefovir, and entecavir, respectively (34, 45, 46). For L-D4FC, the concentration required to inhibit 50% of HepG2 growth was estimated to be 20 µM (35). Unfortunately, L-D4FC is cytotoxic in various cells and with prolonged treatment has been shown to increase lactate production in HepG2 (47). L-FMAU (2-) FTC, and (−) FTC did not show any cytotoxicity up to 200 µM in 2.2.15 cells (36–38). In addition, with the exception of entecavir, in vivo studies have shown that these compounds are not associated with overt toxicity at high doses (>100 mg/kg). In duck hepatitis B virus-infected ducklings, treatment with 15–30 mg/kg of adefovir or 40 mg/kg of L-FMAU produced no toxic side effects (46, 48). In the woodchuck model, 0.5–0.1 mg/kg of entecavir, 1–4 mg/kg of L-D4FC, or 20–30 mg/kg of (−) FTC inhibited replication of woodchuck hepatitis virus without associated toxicity (49–52).

As learned from the treatment of HIV, it is likely that combinations of HBV drugs should be used to maximize suppression of replication and consequently decrease the probability of the emergence of a drug-resistant virus (40, 53, 54). This approach would permit the use of lower doses of the antiviral agents and, therefore, reduce the likelihood of side effects. It also seems advantageous to combine adefovir with lamivudine, entecavir, L-D4FC, L-FMAU, or (−) FTC in an effort to better suppress HBV replication and delay the development of resistance. Although entecavir, L-D4FC, and L-FMAU had the lowest EC$_{50}$, a combination of these three drugs may be compromised, as they have a similar cross-resistance profile. Further studies are necessary to determine the potential synergistic interaction of compounds in combination therapy.

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