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Evaluation of Single and Combination Therapies with Tenofovir Disoproxil Fumarate and Emtricitabine In Vitro and in a Robust Mouse Model Supporting High Levels of Hepatitis B Virus Replication

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Next-generation therapies for chronic hepatitis B virus (HBV) infection will involve combinations of established and/or experimental drugs. The current study investigated the in vitro and in vivo efficacy of tenofovir disoproxil fumarate (TDF) and/or emtricitabine [(−)-FTC] alone and in combination therapy for HBV infection utilizing the HepAD38 system (human hepatoblastoma cells transfected with HBV). Cellular pharmacology studies demonstrated increased levels of (−)-FTC triphosphate with coinfection of increasing concentrations of TDF, while (−)-FTC had no effect on intracellular tenofovir (TFV) diphosphate levels. Quantification of extracellular HBV by real-time PCR from hepatocytes demonstrated the anti-HBV activity with TDF, (−)-FTC, and their combination. Combination of (−)-FTC with TDF or TFV (ratio, 1:1) had a weighted average combination index of 0.7 for both combination sets, indicating synergistic antiviral effects. No cytotoxic effects were observed with any regimens. Using an in vivo murine model which develops robust HBV viremia in nude mice subcutaneously injected with HepAD38 cells, TDF (33 to 300 mg/kg of body weight/day) suppressed virus replication for up to 10 days posttreatment. At 300 mg/kg/day, (−)-FTC strongly suppressed virus titers to 14 days posttreatment. Combination therapy (33 mg/kg/day each drug) sustained suppression of virus titer/ml serum (<1 log10 unit from pretreatment levels) at 14 days posttreatment, while single-drug treatments yielded virus titers 1.5 to 2 log units above the initial virus titers. There was no difference in mean alanine aminotransferase values or mean wet tumor weights for any of the groups, suggesting a lack of drug toxicity. TDF−(−)-FTC combination therapy provides more effective HBV suppression than therapy with each drug alone.

Chronic hepatitis B has been a major target for the development of therapeutics for more than 20 years. Given that the pathogenesis of chronic hepatitis B virus (HBV) infection is immune mediated, early approaches attempted to boost immune responses against virus-infected hepatocytes by using alpha interferon and, later, pegylated interferon, but the success of these approaches has been limited (30, 38).

Another approach targeted the HBV-encoded DNA polymerase reverse transcriptase (RT). For example, lamivudine (3TC) (8, 16) resulted in the partial or total clearance of virus from blood and in the improvement of liver histology in most infected persons (22, 44). However, prolonged treatment was also associated with the appearance of drug-resistant mutants in up to 20% of subjects per year (4, 15, 45). Other drugs, such as adefovir (ADV) (19) and entecavir (ETV) (23, 33, 40, 46), were effective against 3TC-resistant mutants (47). Clevudine [CLV; 1-(2-fluoro-5-methyl-beta,1-arabinofuranosyl) uracil] has shown potent in vitro activity against HBV in HepG2.2.15 cells (11) and in clinical trials (2, 6, 11), but studies in the United States and Europe were discontinued due to clevudine-associated myopathy (12). The newer nucleoside analogs have lower rates of drug resistance, although rebound viremia often occurred after therapy was withdrawn. Tenofovir disoproxil fumarate (TDF; Viread), a prodrug of tenofovir (TFV), is also effective against HBV in cell lines supporting viral replication and in clinical trials (7, 14). However, most single nucleoside analog therapies do not suppress virus replication after the end of treatment and may result in the appearance of drug-resistant mutants, which limits their utility in chronic virus infection. Combination therapy of nucleoside analogs with emtricitabine [(−)-FTC; also called FTC or Emtriva] showed synergistic antiviral activity in the same culture system (26) and in clinical trials (13, 18, 21, 36, 42).

Clinical experience suggests that stronger and more prolonged suppression of virus replication is associated with a significantly decreased risk for disease progression and development of hepatocellular carcinoma (HCC) (9), as well as a decreased frequency of drug-resistant mutants (48). This provides a strong rationale for the development of simultaneous combination therapies to replace monotherapies and sequential combination therapies. With an ever expanding list of L-nucleosides [e.g., 3TC, (−)-FTC, CLV, 1-deoxyribosylthymine, and 1-deoxyribosylcytosine], acyclic phosphonates (e.g., ADV, TDF, and GS-3740, an isopropyl-alanylmonoamidate phenyl monoester prodrug of tenofovir), and 2′-deoxyguanosine analogs (e.g., ETV), opportunities now exist to evaluate combination therapies using compounds with complementary chemistries and mechanisms of action. Recently, a multicenter study reported the efficacy and tolerance of the combination tenofovir disoproxil fumarate plus emtricitabine in persons with chronic HBV infection (42).
Part of the problem in developing new drugs against HBV is the lack of suitable in vivo models with sustained virus replication. It is impractical to test antiviral drug combinations in HBV-infected chimpanzees. Related hepadnaviruses exist in ground squirrels, woodchucks, and ducks (31, 32), and these systems have been used to evaluate new drugs against hepadnaviruses (25, 34), but the availability and handling of these models are limited. Alternatively, several groups have made HBV transgenic mice, but these have not been used extensively for preclinical characterization of lead compounds or for testing putative combination therapies (1, 5, 24, 35, 39).

Some years ago, researchers constructed HepAD38 cells in which HBV replication was under the control of the tetracycline (Tet) suppressor in HepG2 (human hepatoblastoma) cells. In the presence of Tet, virus replication was suppressed, while in the absence of Tet, very high titers of HBV were observed (29). Subsequent studies have shown the utility of this cell line for drug discovery and the development of lead compounds active against HBV (41). Previously, we demonstrated that HepAD38 cells transplanted subcutaneously into nude mice resulted in the development of viremia, and treatment of these mice with drugs active against HBV demonstrated significant antiviral activity in vivo, suggesting that this simple small-animal model could be used to assess new therapeutic approaches and combination therapies against wild-type (wt) HBV (17). The present study utilized the HepAD38 system to evaluate TDF and (-)-FTC alone and in combination therapy in vitro and in vivo.

(A portion of this work was presented at the HepDART 2011 Meeting, Koloa, Kauai, Hawaii, 4 to 8 December 2011 [4a].)

MATERIALS AND METHODS

Source of antiviral agents. TDF was extracted from commercially available Viracept obtained from the U.S. Department of Veterans Affairs pharmacy. TFV was obtained from the NIH AIDS Research and Reference Reagent Program (catalog number 10199; https://www.aidsreagent.org/index.cfm). (-)-FTC was synthesized in our laboratory and was greater than 99% pure. 14C- or 3H-radiolabeled deoxycytidine analog triphosphates such as (-)-FTC-TP alone was 4.13 h, following increases to 5.03 h with 1/2 the EC90 of TDF, TFV, and (-)-FTC alone or in combination and incubated at 37°C in a humidified 5% CO2 atmosphere. On day 2, medium was removed and cells were washed with 1× phosphate-buffered saline (PBS). Compounds and controls were prepared in medium without tetracycline and added in duplicate at various concentrations. On day 7, total DNA was extracted using a DNeasy 96 blood and tissue kit (Qiagen, Valencia, CA), and HBV DNA was amplified by real-time PCR using primers specific for the highly conserved sequences complementary to the DNA sequences present in HBsAg, as described previously (37).

Assessment of drug efficacy in mice. All studies were conducted under approval by Temple University’s IACUC. Briefly, 1 × 103 HepAD38 cells were injected subcutaneously into a single site on the backs of nude mice (day 0). At day 21, mice with palpable tumors were bled and the initial levels of virus DNA were determined by real-time PCR. Mice were then randomly divided into different groups containing 5 mice each and kept on tetracycline from days 21 to 31. The tetracycline was then removed from the drinking water and the groups were treated with different doses of TDF, (-)-FTC, combination therapy (TDF plus (-)-FTC), or PBS from days 31 to 36. Drugs were administered intraperitoneally to ensure that each mouse received the same amount of drug at the same time as every other mouse in the same group. Mice were then taken off drug and observed until day 51, at which time they were all sacrificed. Blood samples were obtained on days, 21, 31, 36, 41, 46, and 51. Virus titers were determined at each time point by real-time PCR, and the results were plotted as the log of the virus titer. On day 51, blood samples were evaluated for alanine aminotransferase (ALT) levels using a commercially available kit (ALT/AST 30; Sigma Chemical Co., St. Louis, MO) and tumors were removed and weighed.

Statistical analysis. Comparisons between data sets were evaluated using the Student t test, where significance was recognized when P was <0.05. The computer-simulated combination index (CI) for drug-drug interactions at 50, 75, 90, and 95% inhibition of HBV replication in HepAD38 cells was calculated as described previously (10).

RESULTS

Cellular pharmacology. Intracellular levels of (-)-FTC triphosphate [(-)-FTC-TP] were determined in vitro from extracts of HepG2 cells incubated with radiolabeled nucleoside analog. Coincubation of (-)-FTC with A analog TDF significantly increased (-)-FTC-TP levels in a concentration-dependent manner (Fig. 1A) (P < 0.01). In contrast, coincubation of TDF with (-)-FTC did not significantly alter TFV diphosphate (TFV-DP) levels (Fig. 1B). Although TDF does not compete with 2′-deoxyadenosine or guanosine (Fig. 1B), these observations suggest an additive or synergistic effect of TDF on the increased formation of 2′-deoxycytidine analog triphosphates such as (-)-FTC-TP (Fig. 1A). The effect of TDF upon intracellular levels of (-)-FTC-TP was also observed in decay studies using increasing concentrations of TDF (Fig. 1C). In these studies, the half-life of (-)-FTC-TP alone was 4.13 h, following increases to 5.03 h with 1 μM TDF, to 5.97 h with 10 μM TDF, and to 6.82 h with 30 μM TDF. In addition, these results demonstrate that (-)-FTC (Fig. 1B) did not cause any effect on the intracellular formation of TFV-DP. Given the high antiviral potency and high potential selection of (-)-FTC-resistant virus during treatment, these drug-drug interaction studies suggest that TDF plus (-)-FTC warrants further development as a possible combination therapy.

In vitro toxicity and anti-HBV activities of nucleosides in HepAD38 cells. The 50% effective concentration (EC50) and EC90 of TDF, TFV, and (-)-FTC alone or in combination were determined in HepAD38 cells. Potent inhibition of HBV production was observed with (-)-FTC and TDF (Table 1). In a standard...
equal variance were used to determine a
- deoxyadenosine (dAdo) or guanosine (Guo) and various concentrations
of TDF for 4 h. Gray bars, a significant increase in intracellular con-
centration of dAdo (CI50 = 0.01–0.02, CI75 = 0.13–0.18)

Drug-drug interaction assay. (A) Effect of TDF on intracellular levels of
(-)-FTC-TP. HepG2 cells were treated with 10 μM (-)-FTC alone or in
combination with controls (2'-deoxycytidine or cytidine) and various concen-
trations of TDF for 4 h. Gray bars, a significant increase in intracellular con-
centrations. (B) Effect of (-)-FTC on intracellular levels of TFV-DP. HepG2 cells
were treated with 10 μM TDF alone or in combination with controls
(2'-deoxyadenosine [dAdo] or guanosine [Guo]) and various concentrations
of (-)-FTC for 4 h. (C) HepG2 cells were treated with 10 μM (-)-FTC alone
(-)-FTC 1:1 0.9 0.8 0.7 0.7 0.7
(-)-FTC 1:2 0.9 0.8 0.8 0.8 0.8
(-)-FTC 1:5 10.0 7.3 5.4 4.4 5.8
(-)-FTC 1:10 12.2 9.7 7.7 6.6 8.1
(-)-FTC 2:1 3.3 3.3 3.3 3.4 3.3
(-)-FTC 5:1 15 11 7.8 6.2 8.5

**TABLE 1** In vitro anti-HBV activity and cytotoxicity of the three
compounds used singly and in two-drug combinations in HepAD38
system

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio</th>
<th>EC50 (µM)</th>
<th>EC90 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDF</td>
<td>NA</td>
<td>0.01 (0.002–0.02)</td>
<td>0.13 (0.03–0.25)</td>
</tr>
<tr>
<td>TFV</td>
<td>NA</td>
<td>0.34 (0.11–0.93)</td>
<td>3.72 (1.11–13.7)</td>
</tr>
<tr>
<td>(-)-FTC</td>
<td>NA</td>
<td>0.05 (0.02–0.07)</td>
<td>0.72 (0.18–1.42)</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:1</td>
<td>0.01 (0.01–0.02)</td>
<td>0.13 (0.01–0.18)</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:2</td>
<td>0.01 (0.005–0.02)</td>
<td>0.12 (0.07–0.22)</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:5</td>
<td>0.02 (0.02–0.03)</td>
<td>0.14 (0.13–0.15)</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:10</td>
<td>0.02 (0.01–0.02)</td>
<td>0.13 (0.11–0.15)</td>
</tr>
<tr>
<td>TFV + (-)-FTC</td>
<td>1:1</td>
<td>0.02 (0.02–0.03)</td>
<td>0.15 (0.12–0.18)</td>
</tr>
<tr>
<td>TFV + (-)-FTC</td>
<td>2:1</td>
<td>0.33 (0.21–0.51)</td>
<td>5.64 (2.04–15.6)</td>
</tr>
<tr>
<td>TFV + (-)-FTC</td>
<td>5:1</td>
<td>1.00 (0.75–1.34)</td>
<td>5.07 (3.47–7.40)</td>
</tr>
</tbody>
</table>

*All values represent the average of two experiments, and samples were performed in
duplicate. EC50 and EC90, effective concentrations required to reduce HBV levels by
50% and 90%, respectively, on day 5. The values in parentheses indicate 95% confidence
intervals generated by using the CalcuSyn program. The cytotoxic concentrations that inhibited cell growth by 50% (CC50) in parent HepG2 cells were
>10 μM for all compounds or compound combinations tested. Note that for the
combination-drug studies, values were determined from standard curves for the first
compound (either TDF or TFV). NA, not applicable.

5-day assay with HepG2 cells, toxicity was not detected for any of
the anti-HBV compounds (Table 1). This was evident even among several drug combinations tested, suggesting that these drugs pro-
duced strong antiviral efficacy in the absence of toxicity.

Evaluation of drug interactions between (-)-FTC and TDF
or TFV in HepG2 and HepAD38 cells. HepAD38 cells were
exposed to antiviral drugs for 5 days, and antiviral drug combination
analyses were performed by the methodology described above.
Data analyses were performed using the CalcuSyn program.
Cytotoxicity was determined by MTT assay. The combination of
(-)-FTC with TDF or TFV at ratio of 1:1 had a weighted average
combination index of 0.7 for both combination sets (Table 2),
indicating synergistic antiviral effects (since CIs of <1, 1, and >1
indicate synergism, an additive effect, and antagonism, respec-
tively). In contrast, antagonism was observed when the ratio con-
sisted of an increased amount of (-)-FTC relative to the amount
of TDF or TFV (Table 2). No cytotoxic effects were observed with
any of the combinations when tested at the highest concen-
trations.

**TABLE 2** Computer-simulated combination index (CI) of two-drug
combinations at 50, 75, 90, and 95% inhibition of HBV replication in
HepAD38 cells

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Ratio</th>
<th>CI at inhibition of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:1</td>
<td>0.9</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:2</td>
<td>0.9</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:5</td>
<td>7.3</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>1:10</td>
<td>7.7</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>2:1</td>
<td>3.3</td>
</tr>
<tr>
<td>TDF + (-)-FTC</td>
<td>5:1</td>
<td>15</td>
</tr>
</tbody>
</table>

*CI values were determined for a mutually exclusive interaction using the CalcuSyn
program; a CI of <1, 1, or >1 indicates synergism, an additive effect, and antagonism,
respectively. Clint, weighted-average CI, which was assigned as (CI50 + 2CI75 +
3CI90 + 4CI95)/10, where CI50, CI75, CI90, and CI95 represent CIs for 50, 75, 90,
and 95% inhibition, respectively (10).
cessation of treatment. In Fig. 2B, the highest dose of (-)-FTC (300 mg/kg/day) suppressed virus titers/ml 3 or more log units for up to 2 weeks posttreatment compared to those in PBS-treated mice (P < 0.001 for days 41, 46, and 51). Rebound by day 51 was modest, being less than 1 log_{10} unit above the initial titer (at 21 days postinjection of HepAD38). Lower doses of (-)-FTC (33.3 and 100 mg/kg/day) suppressed the virus titer/ml serum during the period of treatment (days 31 to 36, inclusive), but once treatment was completed, virus titers rebounded about 1.5 log_{10} units over the following 2 weeks. At the lowest doses of (-)-FTC, inhibition of the virus titer/ml serum was modest (at 11.1 mg/kg/day) or absent (at 3.7 mg/kg/day) during the period of treatment, and by 2 weeks posttreatment, virus titers were about 2 log units higher than the initial titers. However, even at these lower doses of (-)-FTC (3.7 to 100 mg/kg/day), the increase in the virus titer/ml serum following cessation of therapy (1.5 to 2 log_{10} units) (Fig. 2B) was smaller than that observed among mice treated with PBS (4.5-log-unit rise in virus titer/ml serum) (P < 0.001 on days 36, 41, 46, and 51). Hence, both TDF and (-)-FTC demonstrated strong antiviral activity in vivo, and this activity was sustained for 10 to 14 days after cessation of therapy.

The antiviral activities of combination therapies are shown in Fig. 2C. At 300 mg/kg/day for each drug, combination therapy with TDF plus (-)-FTC suppressed virus levels to nearly the same extent through day 51 as therapy with (-)-FTC alone, while rebound was observed with TDF alone (Fig. 2A). Compared to PBS-treated mice, combination therapy suppressed the virus titer/ml serum between ~3 log_{10} units on day 41 (P < 0.001) to 4.2 log_{10} units by day 51 (P < 0.001) (Fig. 2C). At 100 mg/kg/day, combination therapy suppressed the virus titer/ml serum throughout the period of observation (Fig. 2C).

In summary, with drug doses of 3.7 mg/kg/day, little difference between monotherapy, combination therapy, and placebo occurred (Fig. 2). When monotherapy and combination therapies are compared over the range of concentrations used, the antiviral effect of 300 mg/kg/day (-)-FTC in Fig. 2B is duplicated by combination therapy at 11.1 mg/kg/day in Fig. 2C, suggesting that the addition of TDF to (-)-FTC results in the use of 27-fold less (-)-FTC to achieve nearly the same antiviral effect. With regard to TDF, even 300 mg/kg/day did not suppress virus levels to the extent or for the duration that combination therapy down to 11.1 mg/kg/day did. These data suggest that combination therapy is more effective at inhibiting HBV during treatment and has more sustained antiviral activity following cessation of treatment than therapy with either drug alone at the same dose.

**In vivo toxicity of combination therapy.** Although clinical trials with (-)-FTC and TDF alone or in combination (as for tenofovir-emtricitabine [Truvada]) demonstrate low toxicity, little is known about these combined modalities in animal models. Hepatotoxicity was determined by measuring serum ALT activities at the time of each blood draw (days 21, 31, 36, 41, 46, and 51) to assess toxicity. The results from day 51 for TDF or (-)-FTC monotherapy compared to those for (-)-FTC plus TDF combination therapy are shown in Fig. 3. The results show no statistical difference in mean ALT values (Fig. 3A). These results were indistinguishable from those obtained when ALT values were obtained at earlier times (data not shown). In addition, there was no statistical difference in the mean ALT values from day 21 to day 51 (data not shown), indicating that (-)-FTC plus TDF combination therapy was not toxic to the liver, at least by this criterion.

In addition, there was no significant difference in mean tumor wet weights when tumors were excised and weighed at day 51 (Fig. 3B), again suggesting little or no toxicity at different doses of monotherapy or combination therapy used.

At the end of the experiment, at day 51, livers and tumors from the mice were removed and stained with hematoxylin-eosin. Normal histology was observed in all mouse livers examined, and little to no necrosis was observed in the growing tumors (data not shown).

**DISCUSSION**

The development of multiple nucleoside and nucleotide analogs by academic institutions and biotechnological and pharmaceutical companies over the past 2 decades has provided new and powerful weapons against chronic hepatitis B. Each of these has been developed and many have successfully been marketed as monotherapies for HBV infections (20). However, continued use of these agents as monotherapy has also been associated with the appearance of drug-resistant mutations, and although the frequency of drug resistance is low with many of the newer agents (3, 6, 20, 21, 27, 28, 45), most have not been around long enough that their long-term use can be assessed. As is the case with treatment of HIV infection, it is likely that effective control/treatment of chronic hepatitis B will require the development of simultaneous combination therapies that can suppress virus replication to ultralow levels over an extended period of time with a minimal incidence of resistance. However, it is unlikely that these combined modalities using nucleoside analogs will eradicate HBV in humans. The novel nude mouse-HepAD38 model was designed as a rapid, in vivo system to assess multidrug toxicity and efficacy in the development of combination therapies (17).

Prior to in vivo assessment of multidrug combinations, various nucleoside inhibitors of HBV were evaluated in vitro using HepAD38 cells. Testing of nucleoside analogs individually showed that (-)-FTC and TDF were potent inhibitors of HBV (Table 1). Further, in vitro characterization showed that these drugs were not toxic to HepG2 cells (Table 1). These observations are consistent
DNA polymerase are distinct and that they may act in a complementary and perhaps additive or synergistic way. Results of cellular pharmacology studies with coincubation of TDF and (−)-FTC supported this hypothesis, as TDF increased the intracellular levels of (−)-FTC-TP in a dose-dependent manner (Fig. 1A).

In order to develop a meaningful combination therapy, an ideal model system must be robust enough to detect single and combination therapies that reduce virus titers by at least 3 to 4 log units. Previous results showed that the nude mouse-HepAD38 model is such a system (17). Accordingly, in order to determine the antiviral efficacy of TDF and (−)-FTC, dose-response curves were generated for each drug individually. As expected, each drug strongly suppressed the virus titer at high doses up to 10 days (for TDF) or 14 days [for (−)-FTC] postadministration (Fig. 2). Even the lowest dose of each drug used (3.7 mg/kg/day) partially suppressed the virus titer compared to that in placebo (PBS)-treated mice. When dose-response curves were generated with TDF plus (−)-FTC combination therapy, the combination was effective in suppressing virus titers at all doses except the lowest one (3.7 mg/kg/day) compared to those in mice treated with each drug alone or PBS (Fig. 2C). The finding that combination therapy with reduced levels of the individual drugs alone demonstrates antiviral efficacy validates the synergistic effects of (−)-FTC plus TDF in combination in vivo. Moreover, the observation that these drugs are apparently not toxic in vitro (Table 1) or in vivo (Fig. 3A and B) suggests that the reduction in virus titer is likely to be associated with the antiviral properties of the compounds used and not likely to be due to hepatotoxicity or the failure of tumors to grow in the nude mice.

Results from our studies further support the HepAD38 system as a valuable model to test parameters that may identify more efficient combination therapies for HBV and further development in human clinical trials. In particular, this work has important implications for the use of the product Truvada, which is a fixed-dose combination of TDF and (−)-FTC widely used for the treatment of HIV and is also used off-label in certain countries like France for the treatment of HBV infection. If the results in human clinical trials demonstrate the same synergy that was observed in the nude mouse-HepAD38 model herein, that synergy may provide future opportunities for the use of smaller amounts of drugs in combination to provide sustained antiviral suppression in a form that will be affordable in developing countries where HBV is endemic and current therapies are often too expensive. The possibility also exists that the use of three drugs will prove to be even better. For example, work is under way to test whether the combination described above, together with ETV, a 2′-deoxyguanosine analog, will provide even greater synergy in long-term suppression of the HBV titer in vivo. It can also be envisioned that unrelated drugs developed for other viral targets could be tested in this nude mouse-HepAD38 system. Furthermore, drug combinations effective against clinically relevant HBV mutants over the long term could also be evaluated in this in vivo system.

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