Background: Long-term management of some chronic hepatitis B patients might require combination therapy using drugs with distinct resistance profiles to sustain viral suppression and to reduce the resistance-associated failure. Tenofovir disoproxil fumarate (TDF), approved for hepatitis B virus (HBV) and HIV-1 treatment, is active against wild-type HBV and HBV containing YMDD mutations, which confer resistance to emtricitabine (FTC), lamivudine (3TC) and telbivudine (LdT) and contribute to entecavir (ETV) resistance. We therefore evaluated the in vitro anti-HBV activity of tenofovir (TFV), the active parent drug of TDF, combined with FTC, 3TC, ETV, LdT and adefovir (AFV).

Methods: The anti-HBV activities of the compounds were tested using the AD38 cell line that expresses wild-type HBV from a tetracycline-controllable promoter. Intracellular HBV DNA levels were quantified using real-time PCR assay and cytotoxicities were assessed with XTT assays. The antiviral data of the drug combinations were evaluated using MacSynergy analyses on the basis of the Bliss independence model as well as isobologram analyses on the basis of the Loewe additivity theory.

Results: All drug combinations tested, FTC+TFV, 3TC+TFV, ETV+TFV, LdT+TFV and AFV+TFV, showed additive anti-viral interactions as analysed by MacSynergy. Isobologram analyses revealed that these combination pairs were additive, with the exception of FTC+TFV, which demonstrated slight synergistic activity. No cytotoxic or antagonistic effects were observed with any of the combinations tested.

Conclusions: The combination of TFV with FTC, 3TC, ETV, LdT or AFV had additive to slightly synergistic anti-HBV effects in vitro. These results support the use of TDF as a component in combination regimens with currently available anti-HBV nucleoside analogues.

Introduction

Chronic hepatitis B (CHB) infection is a significant global public health problem affecting an estimated 350–400 million individuals and leading to 1 million annual deaths worldwide from resultant illnesses, such as cirrhosis and hepatocellular carcinoma [1,2]. Two categories of drugs are used in CHB therapy: the interferons, including standard interferon-α or pegylated interferon-α, and nucleoside/nucleotide hepatitis B virus (HBV) reverse transcriptase (RT) inhibitors. Interferon-α and the chemically modified pegylated interferon-α are cytokines with immunomodulatory and antiviral activities. They are only effective in approximately one-third of indicated patients and are associated with significant side effects [3–5]. Monotherapy with an individual nucleoside/nucleotide analogue is the current standard of care for many patients [6]. There are five approved nucleoside/nucleotide analogues, including lamivudine (3TC), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir disoproxil fumarate (TDF). Several other analogues are in various stages of drug development, including emtricitabine (FTC), clevudine and pradefovir. Because of the persistent nature of CHB infection, which is largely attributable to the stability of HBV covalently closed circular DNA (cccDNA) [7], these therapies rarely produce hepatitis B surface antigen seroconversion and therefore require prolonged administration to control disease in most patients. Long-term therapy, however, can be associated with the emergence of resistant HBV strains, leading to loss of therapeutic benefit and liver disease progression.

Resistance to 3TC results from the selection of HBV RT rtM204V and rtM204I (YMDD) mutations and occurs in approximately 20% of patients per year of treatment [8]. Long-term use of other nucleoside/nucleotide analogues are also associated with resistance development. FTC, an l-nucleoside cytosine analogue approved for the treatment of HIV-1 that is structurally similar to 3TC, also selected the rtM204V and rtM204I
mutations although at a frequency lower than 3TC [9]. LdT is an α-nucleoside analogue of thymidine and has been shown to be more efficacious than 3TC at reducing serum HBV DNA in clinical trials [10]. However, its long-term usage also resulted in virological breakthrough from rtM204V and rtM204I mutations [10]. ETV, a deoxyguanosine analogue, has partially reduced activity against rtM204V and rtM204I mutants [11]. Long-term usage of ETV selects for a number of resistance mutations in HBV RT, including I169T, T184S/G, S202I/G and M250V, which occur in addition to the 3TC rtM204V and rtM204I mutations [12,13]. By contrast, ADV maintains both in vitro and clinical efficacy against 3TC resistance mutations [14,15], but its long-term administration selects for the resistance mutations rtN236T and/or rtA181V/T [16,17].

TDF, an oral prodrug of tenofovir (TFV), showed potent anti-HBV efficacy in vivo [18,19] and was recently approved in the USA and EU for CHB treatment. TDF is also a potent inhibitor of HIV type-1 (HIV-1) and is a recommended component of anti-HIV-1 therapies for HIV-1-infected patients as well as for patients coinfected with HIV-1 and HBV [20]. TFV is structurally similar to adefovir (AFV), and showed similar in vitro activity against wild-type HBV [21–27]. It competitively inhibits HBV RT activity by its incorporation into virus DNA, resulting in chain termination [23]. Like AFV, TFV is also active against 3TC-resistant HBV [11]. HBV resistance to TDF remains to be identified and confirmed. Previously, one report found that two patients coinfected with HIV-1 and HBV who were receiving antiviral treatment including TDF were found to have HBV with the rtA194T mutation in combination with the rtL180M-M204V mutations [28]. In vitro phenotypical analyses showed a reduced susceptibility of virus containing the rtA194T alone or in combination with the rtL180M-M204V mutations to TFV [28]. However, these results were not reproduced by a different group [23]. Therefore, whether rtA194T is associated with resistance to TDF remains to be resolved. The ongoing large scale clinical trials, as well as the increased clinical usage of TDF, will help to answer this question.

Clinical experience in HIV-1 treatment indicates that combination antiretroviral therapy is superior to monotherapy in maintaining viral suppression and delaying the emergence of drug-resistant virus [29]. Combination therapy might offer added benefits in treating CHB because of the probable necessity for long-term treatment with nucleoside/nucleotide analogues. One aspect of such a benefit with combination therapy was reduced resistance development and prolonged suppression of serum HBV DNA when ADV was added on to 3TC treatment as opposed to switching from 3TC to ADV monotherapy [30,31]. Although in these studies combination therapy (ADV+3TC) was initiated after 3TC monotherapy, de novo combination therapy should be at least as beneficial. TDF could be used as an important component of combination regimens for the treatment of CHB, given its superior anti-HBV potency as compared with AFV in vivo [18,19], its lack of cross-resistance with the 1-nucleoside analogues and its effectiveness in the treatment of patients coinfected with HIV-1 and HBV. We therefore investigated the in vitro anti-HBV efficacy of TFV in combination with various nucleoside analogues.

Methods

Cells and compounds

The AD38 cell line, which expresses HBV under the control of an inducible tetracycline promoter, was used [32]. TFV, AFV and FTC were synthesized by Gilead Sciences (Foster City, CA, USA). ETV was extracted from prescription tablets (Bristol–Myers Squibb, Farmington, CT, USA). 3TC and LdT were purchased from a commercial source (Moraveck Biochemicals, Brea, CA, USA).

Evaluation of 50% effective concentrations of individual drugs

AD38 cells were seeded and cultured on 96-well plates as described previously [32]. After incubation for 3 days, cells were washed twice with phosphate-buffered saline (PBS) and fed with drug-containing or plain media without tetracycline. After drug treatment for 3 days, culture supernatants were replaced with fresh drug-containing media and incubated for an additional day. Intracellular HBV DNA was then extracted as previously described [33] and quantified by real-time PCR. Briefly, 5 µl of the above final cell lysate was added to a PCR reaction mixture that contained 0.9 µM of the forward primer, HBVF

\[(5'\text{CCGTCGGTGTCTCAGCTGAGCTG})\] and 0.9 µM of the reverse primer, HBVR

\[(5'\text{ACTGAGATGACCTCATTATGYAAGACCTT-3'})\] which occur in addition to the 3TC rtM204V and rtM204I mutations [12,13]. By contrast, ADV maintains both in vitro and clinical efficacy against 3TC resistance mutations [14,15], but its long-term administration selects for the resistance mutations rtN236T and/or rtA181V/T [16,17].

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and fed with drug-containing or plain media without tetracycline. The drug combinations were prepared in a checker board fashion (concentrations of both drugs increase from the lower left to the upper right corner) as indicated in Figure 1. The highest concentrations of each drug used in combination were 2 µM FTC, 2 µM 3TC, 0.04 µM ETV, 40 µM LdT, 20 µM AFV and 40 µM TFV. After drug treatment for 3 days, culture supernatants were replaced with fresh drug-containing media and incubated for an additional day. After 4 days of drug treatment, intracellular HBV DNA was extracted and quantified using a TaqMan® PCR assay as described above. The average inhibition of HBV DNA levels as a percentage of no drug control was plotted using MacSynergy II software (University of Michigan, Ann Arbor, MI, USA) to give a three-dimensional presentation of the dose–response effect.

Data analysis
The antiviral effects of TFV in combination with FTC, 3TC, ETV, LdT or AFV were assessed using the Bliss independence [34] and Loewe additivity models [35]. The Bliss independence model is defined by the equation Exy=Ex+Ey-(ExEy), where Exy is the additive effect of drugs x and y as predicted by their individual effects (Ex and Ey). The MacSynergy II programme was used to evaluate antiviral data according to the Bliss independence model. MacSynergy II uses a non-parametric three-dimensional approach to quantify areas where observed effects are significantly greater (synergy) or less (antagonism) than those predicted from single-drug control data. Data sets were assessed at the 95% confidence level and interpreted as follows: volumes of synergy or antagonism <25 µM² were considered insignificant, those >25–<50 µM² were considered minor but significant, those >50–<100 µM² were considered moderate and potentially important in vivo and those >100 µM² were considered strong and likely to be important in vivo [36].

The Loewe additivity model is defined by the equation dx/Dx+dy/Dy=1, where Dx and Dy are the doses of individual drugs required to exert the same effect as doses dx and dy used in combination. If the experimental product of this equation (termed the Loewe combination index) is equal to 1, the data are considered additive; indices of <1 or >1 indicate synergy or antagonism, respectively. Isobologram analyses were used to evaluate antiviral activity according to the Loewe additivity model. Dose–response curves were generated for each drug alone and in combination and used to determine EC₅₀ values for each drug alone or in the presence of the fixed concentration of the second drug. The x-coordinate is the fractional inhibitory concentration (FIC) and was calculated by dividing the EC₅₀ of drug A with a fixed overlay of drug B by the EC₅₀ of drug A alone. The y-coordinate is the fixed concentration of drug B divided by the EC₅₀ of drug B alone. These points were plotted on a graph to generate the isobologram. On this same graph, a line representing additivity was included (coordinates [1,0] to [0,1]). Data points that were above the additivity line represent antagonism between the compounds, whereas data points below the additivity line represent synergy between the compounds. Statistical evaluation of the data was conducted on the basis of deviation from additivity (D-value) and tested by a one-tailed Student's t-test [37]. D-values between -0.2 and -0.1 with a statistically significant P-value (P<0.05) were representative of a slight synergistic effect, whereas a D-value of -0.5 with P<0.05 could be interpreted as strong synergy [37]. The same interpretation could be applied to positive D-values as indicative of antagonism.

Cytotoxicity assays
To assess cytostatic or cytotoxic effects of the drug combinations, AD38 cells were seeded into 96-well plates at a density of 3x10⁴ cells/well and exposed to compounds for 4 days with a treatment schedule identical to that described above for the antiviral assays. Each drug was tested alone and in combination with TFV at the highest doses used for antiviral combination assays. Specifically, TFV, FTC, 3TC, ETV, LdT and AFV single drugs were tested at 40 µM, 2 µM, 2 µM, 0.04 µM, 40 µM and 20 µM each, respectively, whereas combinations of drugs were assayed at 2 µM FTC+40 µM TFV, 2 µM 3TC+40 µM TFV, 0.04 µM ETV+40 µM TFV, 40 µM LdT+20 µM TFV...
Table 1. Anti-HBV activity of individual compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean EC&lt;sub&gt;50&lt;/sub&gt; µM (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV</td>
<td>5.46 (1.77)</td>
</tr>
<tr>
<td>FTC</td>
<td>0.22 (0.13)</td>
</tr>
<tr>
<td>3TC</td>
<td>0.25 (0.23)</td>
</tr>
<tr>
<td>ETV</td>
<td>0.01 (0.001)</td>
</tr>
<tr>
<td>LdT</td>
<td>9.49 (1.57)</td>
</tr>
<tr>
<td>AFV</td>
<td>5.24 (3.62)</td>
</tr>
</tbody>
</table>

AFV, adefovir; EC<sub>50</sub>, 50% effective concentration; ETV, entecavir; FTC, emtricitabine; HBV, hepatitis B virus; LdT, telbivudine; TFV, tenofovir; 3TC, lamivudine.

and 20 µM AFV+20 µM TFV. Plain culture media and a serial dilution of DMSO were used as negative and positive controls, respectively. Following the drug treatment, cell viability was assessed by acid 3-(1-phenylimino-carbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) cleavage assay and optical density measured at 450 nm absorbance was read as previously described (4-methoxy-6-nitro) benzene sulfonic acid hydrate (450 nm absorbance). Following the XTT cleavage assay and optical density measured at 450 nm absorbance was read as previously described.

Excluding the data from the row and column corresponding to the metabolic activities of live cells.

Results

Anti-HBV activity of test compounds in the AD38 cell line

AD38 cells express high levels of wild-type HBV (genotype D and subtype ayw) from an integrated HBV genome under the transcriptional control of a tetracycline-controllable promoter (tetracycline-off and HBV expressed after removal of tetracycline) [32]. To test anti-HBV activity of various nucleoside/nucleotide analogues, AD38 cells were incubated with the test compounds for 4 days following release of tetracycline suppression and intracellular HBV DNA were extracted and quantified using a TaqMan® real-time PCR assay. The HBV DNA levels from the positive control wells (without tetracycline or drug) were consistently >200-fold that from negative controls (with tetracycline), a signal/noise ratio allowing differentiation of a range of effects on HBV DNA levels (data not shown). The average EC<sub>50</sub> values for each individual drug obtained with the AD38 cell line are summarized in Table 1 and the results are within the range of previously reported data generated with other HBV expression systems using real-time PCR assays as a detection method [24–27].

Anti-HBV activity of drug combinations

To test the anti-HBV activity of two-drug combinations using AD38 cells, drug A or B was added in a checker board fashion as depicted in Figure 1. Each combination assay experiment was carried out with 5 replicate 96-well plates because of the inherent variability of in vitro HBV expression. As a control experiment for the assay system to evaluate drug interactions, the combination of FTC+FTC was assessed. Using MacSynergy analyses on the basis of the Bliss independence model, the results indicated that this combination was additive with a synergy volume of 0 and an antagonism volume of 16.48 µM<sup>2</sup>% at the 95% confidence interval (CI; Figures 2A and 2B). A careful inspection indicated that inhibition of HBV DNA at the highest test drug concentrations reached a plateau, resulting in the observed small and statistically insignificant antagonism volume. Excluding the data from the row and column corresponding to the highest drug concentrations resulted in both synergy and antagonism volumes of 0. Analyses of the same data using the isobologram method [37] on the basis of the Loewe additivity model resulted in a D-value of 0.063, which was not significantly different from 0 (Student's t-test P=0.38; Figure 2C).

Each TFV drug combination pair was independently tested ≥3x, with the exception of the TFV+LdT and TFV+AFV combinations. TFV+LdT was tested twice. The TFV+AFV combination was tested 5x, as data were noticeably more variable than with other combination pairs. One example of each of the anti-HBV three-dimensional dose–response plots of TFV+FTC, TFV+3TC, TFV+ETV, TFV+LdT and TFV+AFV combinations is shown in Figure 3. Each drug alone (x- or y-axis, where the other drug is absent) showed a dose–response and an EC<sub>50</sub> similar to the values indicated in Table 1. Also, a general trend of dose–response surface for each combination pair is visually indicated, suggesting consistency of data.

Data analyses using MacSynergy demonstrated that each of the TFV+FTC, TFV+3TC, TFV+ETV, TFV+LdT and TFV+AFV combinations had mean synergy and antagonism volumes at the 95% CI that were within the range of -25–25 µM<sup>2</sup>% (Table 2). Volumes between -25–25 µM<sup>2</sup>% at the 95% CI are considered statistically insignificant, indicating additive interactions [34,36]. Therefore by using MacSynergy analyses, all the tested drug combinations were considered additive in anti-HBV activity with no evidence of antagonism. A sample three-dimensional synergy plot at the 95% CI from MacSynergy analyses for each of the TFV combination pairs is shown in Figure 4.

These results were also analysed using the isobologram method [34,37]. One example isobologram for each combination is shown in Figure 5. D-values calculated from combinations of TFV+3TC, TFV+ETV, TFV+LdT and TFV+AFV varied between -0.17–0.24, but each time that the values were outside of the -0.1–0.1 range the P-values were not statistically significant (P>0.05). These small or statistically insignificant D-values were indicative of additive interactions between each of the tested combination pairs (Table 3). Data from TFV+AFV were more variable,
Anti-HBV activity of combinations of tenofovir with other nucleoside/nucleotide analogues

and results from only 1 of the 5 experiments could be analysed with the isobologram method. It is unclear why this combination pair had more variability in anti-HBV activity. Interestingly, TFV+FTC combinations consistently resulted in negative D-values ranging from -0.25 to -0.12 (D-values between -0.2 and -0.1 indicate weak synergy [37]), although statistical significance was not consistently shown (P-values ranged from 0.0007 to 0.22). Therefore, isobologram analyses revealed that all the tested combinations were additive with the exception of the TFV+FTC combination, which showed a weak synergistic effect.

Effects of drug combinations on cytotoxicity and cytostasis
To exclude the possibility that the observed anti-HBV activities of the tested drug combinations were a result of cytotoxicity and/or cytostatic effects of the drug treatments, experiments were conducted at the highest drug concentrations of each drug alone and in combination with TFV (as tested for anti-HBV activity) to evaluate toxicity. The XTT-based cytotoxicity assays were conducted ≥3× for each drug or drug combination. No significant differences (Student’s t-test, P>0.05) were observed between the untreated control and any of the drug-treated cultures. Treatment with 5% DMSO resulted in a >50% reduction in the assay signals (Figure 6).

Discussion
Several new drugs have been developed in recent years for the treatment of CHB. However, the available anti-HBV agents only prevent virus replication and have no direct or permanent effect on eliminating the existing cccDNA, the viral form that does not undergo semi-conservative Replication.

Figure 2. FTC+FTC combination

(A) Three-dimensional inhibition plot. (B) Synergy plot at the 95% confidence interval from MacSynergy analysis. (C) Isobologram analysis plot showing means ±se. The line between coordinates (0,1) and (1,0) indicates additivity. FIC, fractional inhibitory concentration. FTC, emtricitabine.
Figure 3. Three-dimensional dose–response plots of drug combinations

One example of the three-dimensional graph for each tested drug combination is shown. (A) tenofovir (TFV)+emtricitabine (FTC). (B) TFV+lamivudine (3TC). (C) TFV+entecavir (ETV). (D) TFV+telbivudine (LdT). (E) TFV+adefovir (AFV).
Table 2. MacSynergy analyses of anti-HBV drug combination results

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Mean synergy(^a) volume, µM(^{3%}) (±s d)</th>
<th>Mean antagonism(^a) volume, µM(^{3%}) (±s d)</th>
<th>Net effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV+FTC</td>
<td>5.88 (6.67)</td>
<td>-1.88 (3.26)</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+3TC</td>
<td>7.16 (12.41)</td>
<td>-11.35 (4.64)</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+ETV</td>
<td>0.4 (0.69)</td>
<td>-9.53 (4.95)</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+LdT</td>
<td>3.5 (4.95)</td>
<td>-8.81 (11.22)</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+AFV</td>
<td>2.33 (5.20)</td>
<td>-13.57 (6.46)</td>
<td>Additive</td>
</tr>
</tbody>
</table>

\(^a\)Mean [±s d] synergy and antagonism volumes at the 95% confidence interval. AFV, adefovir; ETV, entecavir; FTC, emtricitabine; HBV, hepatitis B virus; LdT, telbivudine; TFV, tenofovir; 3TC, lamivudine.

Figure 4. MacSynergy analyses of antiviral combination data

Effects of each drug combination pair are represented as synergy plots at the 95% confidence interval by MacSynergy analyses (MacSynergy II; University of Michigan, Ann Arbor, MI, USA). A representative graph for each drug combination is shown. (A) tenofovir (TFV)+emtricitabine (FTC). (B) TFV+lamivudine (3TC). (C) TFV+entecavir (ETV). (D) TFV+telbivudine (LdT). (E) TFV+adefovir (AFV).
DNA replication but plays a central role in maintaining an infected state [7]. Because cccDNA has a very long half-life [38,39] and is probably distributed into daughter cells during cell division [39,40], it will likely take a very long period of treatment, even with a very effective drug, to significantly clear the virus from infected hepatocytes.

In addition, no clinical study has demonstrated that any currently approved drugs completely inhibited HBV DNA replication as a monotherapy, which could allow for the rise of drug resistance mutations from the residual virus replication in some patients. Aside from the issue of whether drug resistance mutations pre-existed

**Figure 5.** Isobolograms of anti-HBV activities of the drug combinations

A representative graph for each drug combination is shown. Data are means ± se. (A) tenofovir (TFV)+emtricitabine (FTC). (B) TFV+lamivudine (3TC). (C) TFV+entecavir (ETV). (D) TFV+telbivudine (LdT). (E) TFV+adefovir (AFV). FIC, fractional inhibitory concentration; HBV, hepatitis B virus.
or evolved because of the low level of ongoing virus replication, long-term antiviral treatments (3TC, ADV, LdT and ETV) against a single target (RT) are associated with the emergence of drug resistance mutations, or predicted to be so with the ensuing loss of therapeutic benefits [41]. Combination therapy using drugs with at least additive interactions and without cross-resistance might provide the added efficacy necessary to reduce the risk of antiviral drug resistance.

Data on nucleoside/nucleotide drug combinations against HBV, either from preclinical or clinical studies, are still limited. One of the earlier preclinical studies by Korba [42] showed that combinations of 3TC and penciclovir had synergistic activity against HBV in HepG2 2.2.15 cells. Using the woodchuck hepatitis virus model, the combination of 3TC and famciclovir was shown to have additive to synergistic antiviral effects in chronically infected woodchucks [43]. In duck hepatitis B virus (DHBV)-infected duck primary hepatocytes, AFV, 3TC and penciclovir showed additive or synergistic antiviral effects when used in combination [44]. Also using the DHBV model, Seigneres et al. [45] reported enzymatic, cell culture and in vivo results demonstrating that combinations of FTC, amdoxovir and clevudine were more efficacious than any of the drugs alone in antiviral activity. More recently, Delaney et al. [46] showed that combinations of AFV and 3TC, FTC, ETV, LdT or TFV had additive to synergistic effects in anti-HBV activity in a stably transfected cell line that constitutively expresses a wild-type genotype A HBV. Clinically, de novo 3TC+ADV combination therapy was compared with 3TC monotherapy in treatment-naive hepatitis B e antigen (HBeAg)-positive patients [47]. Initially, both treatments resulted in an equally effective antiviral response by week 16, with a 4–5 log10 copies/ml serum HBV DNA reduction. However, by week 52, the ADV+3TC group maintained viral suppression with a median 5.22 log10 copies/ml reduction in HBV DNA as compared with a 3.41 log10 copies/ml reduction in the 3TC monotherapy group, largely owing to the development of 3TC resistance in the 3TC monotherapy group. In another study, 30 treatment-naive HBeAg-positive patients were treated with ADV+FTC combination therapy or ADV monotherapy [48]. At week 48, the combination group showed

### Table 3. Isobologram analyses of anti-HBV drug combination results

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>D-value range</th>
<th>P-value range</th>
<th>Net effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV+FTC</td>
<td>-0.25–0.12</td>
<td>0.0007–0.22</td>
<td>Synergistic</td>
</tr>
<tr>
<td>TFV+3TC</td>
<td>-0.17–0.12</td>
<td>0.02–0.44</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+ETV</td>
<td>0.036–0.24</td>
<td>0.1–0.36</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+LdT</td>
<td>0.066–0.047</td>
<td>0.25–0.32</td>
<td>Additive</td>
</tr>
<tr>
<td>TFV+AFV</td>
<td>-0.12</td>
<td>0.14</td>
<td>Additive</td>
</tr>
</tbody>
</table>

*a* For this combination n=1. AFV, adefovir; ETV, entecavir; FTC, emtricitabine; HBV, hepatitis B virus; LdT, telbivudine; TFV, tenofovir; 3TC, lamivudine.

### Figure 6. Cytotoxicity assay

The absorbance readings were averages of three replicate wells. AFV, adefovir; ETV, entecavir; LdT, telbivudine; TFV, tenofovir; 3TC, lamivudine.
a median HBV DNA reduction of 3.95 log10 copies/ml as compared with a 2.44 log10 copies/ml reduction in the ADV monotherapy group.

In this study, we used TFV as a common component of in vitro combinations with 3TC, FTC, ETV, LdT or AFV, and assessed their anti-HBV activities in the AD38 cell line. Two types of analyses were performed to evaluate the degree of synergy between these combinations. MacSynergy and isobologram analyses, on the basis of the Bliss independence and the Loewe additivity models, respectively, demonstrated that with the exception of TFV+FTC, these combinations were additive with respect to inhibition of HBV DNA replication. The combination of TFV+FTC had a slight synergistic effect according to isobologram analyses. None of the drug combinations had any significant antagonistic effects. Also, none of the drugs alone or in combination with TFV showed any cytotoxic effects at the highest tested combination doses. One combination pair, TFV+AFV, was previously tested in a different cell line [46]. Data analyses using the MacSynergy method in both studies indicated that the two drugs were additive with regards to anti-HBV activity, albeit with some degree of variability in this study. Data analyses using the isobologram method could only be carried out on one set of data in this study, which yielded a slightly negative D-value (-0.12). However, this did not achieve statistical significance and visual inspection of the isobologram curve (Figure 3E) also suggested additive anti-HBV activity for this combination.

It is not unexpected that analyses of the same data using MacSynergy and isobologram methods, which are each based on a different theory, would lead to slightly different results [34,46,49], as was shown for the TFV+FTC combination in this study. The basis for such a disagreement is mostly attributable to the shape of the individual dose–response curves of each drug. Both models are in agreement when two drugs have identical exponential dose–response curves [50]. It is unclear what mechanism contributed to the slight synergistic interaction between TFV and FTC observed in the isobologram analyses of this study. One possible explanation could be deduced from published drug metabolism studies in CEM cells [36]. In those studies, when 10 µM each of TFV and FTC were included in culture media, the intracellular concentrations of TFV diphosphate and FTC triphosphate each were significantly increased compared with when individual drugs were added alone [36]. This observation correlated with an additive to synergistic anti-HIV-1 effect of the TFV+FTC combination in peripheral blood mononuclear cells, and strong synergy in MT-2 cells [36]. Whether higher levels of the active metabolites of TFV and FTC are formed in the AD38 cell line used in this study, is unknown.

Because TFV remained active against mutations known to be associated with resistance to 3TC, FTC, LdT or ETV [11,26,27], the finding in this study that TFV is at least additive with each of these nucleoside analogues supports a clinical evaluation of therapy using any of these combinations. Among the combinations, TDF+FTC (Truvada®) is widely used for the treatment of HIV-1 AIDS. Because TDF and FTC each showed potent antiviral activity in treating CHB [9,18,19], it was assumed that their combination would be more potent against HBV, particularly because the two drugs do not share cross-resistance. Therefore, TDF+FTC combination therapy is already used in disease management in patients with prolonged viraemia following TDF monotherapy [18,19], and has been used successfully in treating patients with ADV-resistant HBV mutations [51]. Double-blind randomized clinical trials to evaluate the efficacy of TDF+FTC are currently ongoing. As with TDF+FTC, TDF+3TC combination therapy might also offer added activity while reducing the risk of resistance [52–53]. Other combinations, such as TDF+LdT and TDF+ETV could offer added benefits for long-term patient treatment because of the additive interaction as well as lack of cross-resistance [11,26,27]. However, unlike the combinations of TDF+FTC or TDF+3TC, there are little data on the safety profile of TDF+LdT or TDF+ETV; therefore, the overall clinical benefit of these combinations is unknown. By contrast, TDF+ADV combination therapy, although additive in antiviral activity as demonstrated in this study, should not be used in the treatment of CHB. TFV and AFV are structurally similar analogues, with similar activities against wild-type and various drug resistance mutations [11,33]. In particular, previous in vitro studies have demonstrated low-level cross-resistance to TFV of the rtA181V and rtN236T ADV-associated mutations [33]. Although the clinical significance of this observed in vitro cross-resistance has not been demonstrated, the use of combination therapy consisting of TDF and ADV would not be recommended at this time despite the lack of antagonism demonstrated in vitro.

In summary, the combinations of TFV plus FTC, 3TC, ETV, LdT or AFV each showed additive to slightly synergistic anti-HBV activity in vitro. There was no evidence of in vitro cytotoxicity with any of the drug combinations at the tested drug concentrations. These results provide a rationale for and support the use of TDF as an important component in combination therapy for the treatment of CHB.

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References

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