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Susceptibility to Antivirals of an HBV Strain With Mutations Conferring Resistance to Both Lamivudine and Adefovir

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Abstract

Mutations within the Hepatitis B virus (HBV) polymerase gene conferring drug-resistance are selected during prolonged lamivudine (3TC) or adefovir dipivoxil (ADV) treatment. As there is no other approved drug against HBV, treatments with 3TC or ADV are used either sequentially or in addition depending on treatment response or failure. Considering the use of *de novo* or add-on 3TC+ADV bitherapy, we investigated the possibility of emergence of an HBV strain harboring polymerase mutations conferring resistance to both 3TC (rtL180M+M204V) and ADV (rtN236T). We constructed the L180M+M204V+N236T mutant and determined its replication capacity and its susceptibility to different nucleos(t)ide analogs in transiently transfected hepatoma cell lines. The triple mutant replicates its genome *in vitro*, but less efficiently than either the wild-type (wt) HBV or L180M+M204V and N236T mutants. Phenotypic assays indicated that the L180M+M204V+N236T mutant is resistant to pyrimidine analogs (3TC, -FTC, β-L-FD4C, L-FMAU). Compared to wt HBV, this mutant displays a 6 fold decreased susceptibility to ADV and entecavir and a 4 fold decreased susceptibility to tenofovir. Interferon α inhibited equally the replication of wt and L180M+M204V+N236T HBV. In conclusion, the combination of rtL180M+M204V and rtN236T mutations impairs HBV replication and confers resistance to both 3TC and ADV *in vitro*. This suggests that the emergence of the triple mutant may be delayed and associated with viral resistance in patients treated with 3TC+ADV. However, other nucleos(t)ide analogs in development showed an antiviral activity against this multiresistant strain *in vitro*. This provides a rationale for the clinical evaluation of *de novo* combination therapies.

Introduction

The main goals of the treatment of chronic hepatitis B virus (HBV) infection are the sustained suppression of HBV replication, and a remission of liver disease. An immunomodulator, interferon α (IFN-α), and two synthetic nucleos(t)ide analogs, lamivudine (3TC) and adefovir dipivoxil (ADV) are currently approved for the treatment of chronic hepatitis B (CHB). With respect to IFN-α, the overall response rate to this drug is less than 40 %, and IFN-α therapy is associated with a number of adverse effects (1). 3TC, a nucleoside analog of L-deoxycitydine, and ADV, a phosphonate nucleotide analog of adenosine monophosphate, target the HBV reverse transcriptase (RT) activity, thus inhibiting viral replication. They are both powerful antivirals and monotherapy with 3TC or ADV results in significant improvement in virologic, biochemical, and histologic status in most of the patients (2, 3, 4, 5).

However, long-term 3TC or ADV monotherapy leads to the emergence of drug-resistant HBV strains. 3TC-resistance increases at a rate of approximately 20 % of patients per year to reach about 70 % of the treated patients after four years (6,7). Mutations conferring resistance to 3TC have been mapped in the conserved YMDD motif within the C domain of the viral RT (M204I/V) (8, 9, 10). They are frequently associated with compensatory mutations in the conserved B domain (V173L, L180M) that restore partially the replicative capacity of YMDD mutant strains *in vitro* (11, 12, 13). Mutations associated with 3TC-resistance induce a reduction in the susceptibility to 3TC (9) and may be associated with a progression of liver disease in a significant number of patients (6).

ADV, the latest antiviral approved for the treatment of CHB, is used successfully for the treatment of patients with 3TC-resistant CHB either in monotherapy (14), or in combination with 3TC (14, 15). This agrees with *in vitro* studies that showed that 3TC-resistant mutations do not confer resistance to ADV (16, 17, 18). ADV-resistance is less frequent and more belated compared to 3TC. Two recent clinical studies reported cases of ADV-resistance associated with the selection of the mutation N236T within the D domain of HBV RT after two years of ADV monotherapy (19, 20). This mutation induces a reduction in the susceptibility to ADV by 4.4 (20) to 23 (19) fold compared to wt HBV *in vitro*, but retains susceptibility to 3TC.

Several other synthetic nucleos(t)ide analogs are currently in clinical development. Emtricitabine (-FTC), a close analog of 3TC, has been recently approved for the treatment of human immunodefiency virus (HIV) infection and is currently in Phase III clinical trials for the treatment of HBV infection (21, 22), whereas clevudine (L-FMAU), a thymidine analog, has entered Phase II clinical trials (23). Elvucitabine (β-LFD4C), another L-deoxycytidine analog, is also a potent antiviral against HBV and hepadnaviruses in animal models (24, 25, 26). However, 3TC-resistance mutations confer cross-resistance to these three pyrimidine analogs at the exception of the M204V mutant that retains susceptibility to L-FMAU and β-L-FD4C (12, 27). Entecavir (ETV), a deoxyguanosine analog, inhibits specifically the replication of wt and 3TC-resistant HBV *in vitro* and *in vivo* (12, 28, 29). ETV is currently in Phase III clinical trials (30). Tenofovir (PMPA), a nucleotide analog closely related to ADV, has already obtained approval for the treatment of HIV infection (22), and inhibits efficiently the replication of 3TC-resistant M204V HBV mutant *in vitro* (31). Moreover, promising results were

reported with respect to the efficacy of a 3TC+PMPA combination to reduce HBV DNA levels in HIV/HBV co-infected patients carrying wt or 3TC-resistant HBV (32, 33, 34).

To date, there are two options to treat patients who developped a 3TC-resistance (14, 15). 3TC can be switched to ADV with the risk of developing ADV-resistance on the long-term, or ADV can be added to the ongoing 3TC monotherapy to delay further resistance as there is no cross-resistance between these drugs (19, 20). However, a 3TC+ADV bitherapy could select for HBV mutants resistant to both drugs. In this study, we determined the phenotypic characteristics of an HBV laboratory strain carrying mutations conferring resistance to both 3TC (L180M+M204V) and ADV (N236T). The combination of L180M+M204V+N236T mutations was assembled in an HBV replication-competent plasmid. Its effects on the replication of the HBV genome and the susceptibility to nucleos(t)ide analogs (3TC, -FTC, L-FMAU, β-L-FD4C, ETV, ADV, PMPA) and to IFN-α were assessed in transiently transfected hepatoma cell lines.

Materials and Methods.

Antiviral drugs: 3TC, -FTC, L-FMAU, ADV and PMPA were provided by Gilead Sciences (Foster City, USA). ETV was obtained from Moravek (Brea, USA), and IFN α 2b from Schering Plough (Kenilworth, USA). β-L-FD4C was a generous gift from Dr. Y.C. Cheng (Yale University, USA).

Construction of N236T and L180M+M204V+N236T laboratory strains. Plasmids pCMV-HBV containing wt or L180M+M204V HBV genome (genotype D, subtype ayw) (35) were engineered with the QuickChange kit (Stratagene, La Jolla, USA) to install the N236T mutation. All constructs were sequenced to confirm this modification. The different EcoRI-NcoI restriction fragments encompassing the B, C and D domains were transferred into the plasmid pTriEXModHBV containing 1.1 genome unit of the same wt HBV strain (D, ayw). This expression vector enables the production of pregenomic RNA under the control of the chicken beta actin promoter and therefore triggers HBV DNA synthesis (36). Three pTriEXModHBV plasmids with L180M+M204V, N236T or L180M+M204V+N236T mutations were thus obtained.

Viral DNA synthesis capacity of HBV mutants. Transient transfection of plasmids containing wt or mutated HBV genome into Huh7 and HepG2 cell lines were performed using Fugene6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer protocol. Transfected cells were grown for one week, and culture medium was changed every four days. Intracellular encapsidated HBV DNA was purified and submitted to Southern Blot analysis as previously described (35, 36, 37). Viral DNA synthesis levels were determined after phosphorimager analysis of Southern Blot. Transfection efficiency was monitored by quantifying the amount of HBsAg secreted into

the cell culture supernatant at the end of the experiment with the Monolisa HBsAg Ultra kit (Bio-Rad, Hercules, USA).

Drug susceptibility assay. Huh7 cells were transfected as described above with wt or mutant HBV constructs. Treatment with drugs or combination of drugs started 60 hours post-transfection and was renewed every other day for 5 days. To screen which drugs in development display an antiviral activity against the triple mutant replication, transfected cells were treated with a single drug concentration (-FTC: 15 µM; L-FMAU: 100 µM; β-L-FD4C: 1.25 µM; PMPA: 100 µM; ETV: 5 µM; IFN-α: 10^3 IU/ml). To determine inhibitory concentrations (IC50), transfected cells were treated with 0, 0.5, 15 and 100 µM of 3TC, PMEA, or 3TC+PMEA, 0, 1.25, 2.5, 5 and 10 µM of ETV or 0, 12.5, 25, 50, 100 and 200 µM of PMPA. For hydroxyurea (Sigma, Saint Louis, USA) susceptibility assay, HEK 293 cells were transfected as described previously and treated with 0 or 0.1 mM of hydroxyurea for 2 days (11). At the end of treatments with drugs, intracellular encapsidated HBV DNA were extracted, submitted to Southern Blot analysis which was quantified after a phosphorimager analysis. For each drug, the IC50 was determined by a 50 % decrease in the amount of intracellular viral DNA detected in treated cells at the end of the treatment compared to untreated cells (35). Analysis of cellular toxicity was performed as previously described (35).

Results

The association of 3TC- and ADV-resistant mutations impairs HBV genome replication.

First, we asked whether an HBV polymerase mutant gene carrying mutations conferring resistance to both 3TC and ADV was replication competent. Huh7 and HepG2 cells were transiently transfected with plasmids containing wt, L180M+M204V, N236T or L180M+M204V+N236T HBV genome. One week post-transfection, DNA from intracellular core particles were extracted and submitted to Southern Blot analysis. Relative replication capacities of the three HBV mutants were determined after quantification by phosphorimager analysis of Southern Blots and standardized according to the amount of HBsAg secreted in the culture medium (data not shown). The level of replication of the wt HBV was taken as standard of replication (Fig. 1B). The relative replication capacities of the L180M+M204V mutant in Huh7 and HepG2 cells, 83.7 ± 11.3 % and 22.9 ± 2.8 % respectively, were similar to those previously measured in these cell lines (12, 38). The replication efficiency of the N236T mutant was not significantly different in Huh7 (104 ± 10 %) and HepG2 cells (87.3 ± 12 %). With respect to the L180M+M204V+N236T mutant, the relative level of viral DNA synthesis was 27.8 ± 2.1 % in Huh7 cells and 6.8 ± 1.8 % in HepG2 cells. Thus, the triple mutant, like the L180M+M204V mutant, replicates its genome about 4 fold less efficiently in HepG2 cells than in Huh7 cells. Differences in the cellular concentration of the deoxynucleotides (dNTPs) pool may explain this discrepancy in the viral DNA synthesis levels. Indeed, Melegari *et al.* (11) previously observed that YMDD-mutants, but not wt HBV, displayed

a reduced capacity of replication under conditions of intracellular dNTPs depletion produced by hydroxyurea in HEK 293 cells. To address this point, the effect of 0.1 mM of hydroxyurea on the replication of wt, L180M+M204V, N236T or L180M+M204V+N236T HBV genomes was determined in transiently transfected HEK 293 cells. The viral DNA synthesis of wt HBV and N236T mutant were both inhibited by less than 30 % by hydroxyurea administration (Fig. 2). However, the replication of L180M+M204V and L180M+M204V+N236T mutants were both inhibited by more than 70 %, suggesting that the mutant polymerases harboring the L180M+M204V mutations in the YMDD motif are more sensitive to intracellular dNTPs depletion than HBV strains with a wt YMDD motif.

Susceptibility of the L180M+M204V+N236T mutant to 3TC and ADV.

As the replication of the triple mutant is very low in HepG2 cells, we assessed the susceptibility of this mutant to antiviral drugs in Huh7 cells. We first determined the triple mutant susceptibility to 3TC and ADV, individually or in combination, and compared it to wt, N236T, and L180M+M204V HBV counterparts. As previously observed, ADV and 3TC were efficient against the replication of the L180M+M204V (ADV IC50 = 15.5 µM ± 1.8) and N236T (3TC IC50 = 2.65 µM ± 0.52) mutants respectively (Table 1). The L180M+M204V mutant was resistant to 3TC (fold resistance > 40) and the N236T mutant was about 3 fold less susceptible to ADV than wt HBV (IC50 = 50.3 µM ± 11 and 15.8 µM ± 1.9 respectively). The L180M+M204V+N236T mutant was as resistant to 3TC as the L180M+M204V mutant (fold resistance > 40) and it presented a 6.3 fold decreased susceptibility to ADV (IC50 = 100 µM ± 20) compared

to wt HBV. Thus, the combination of drug resistance mutations did not restore susceptibilty to one or the other drug. As a consequence, the triple mutant was resistant to the treatment combining 3TC and ADV showing an IC50 (100 µM ± 20) similar to the IC50 for ADV that is more efficient on the triple mutant replication than 3TC. Similarly, the response of the N236T and L180M+M204V mutants to the 3TC+ADV combination reflected their susceptibility to these two drugs (Table 1). With respect to wt HBV, the IC50 for the bitherapy (1.7 µM ± 0.36) and 3TC (2.48 µM ± 0.67) were not significantly different, 3TC being more efficient at lower concentrations than ADV on wt HBV.

Susceptibility of the L180M+M204V+N236T mutant to different nucleos(t)ide analogs in development.

Since the L180M+M204V+N236T mutant was resistant to both 3TC and ADV, we next determined its susceptibility to several nucleos(t)ide analogs in development (-FTC, β-L-FD4C, L-FMAU, ETV and PMPA). Huh7 cells transfected with plasmids containing wt, N236T, L180M+M204V or L180M+M204V+N236T genomes were treated for five days with each compound at a high single concentration. All the nucleos(t)ide analogs reduced the viral DNA synthesis level of wt HBV and N236T mutant by more than 70 % and 50 % respectively, which confirmed the antiviral potency of these drugs (Table 2). The levels of viral genome replication of the L180M+M204V and L180M+M204V+N236T mutants were decreased by less than 20 % in presence of 15 µM of -FTC, 1.25 µM of β-L-FD4C or 100 µM of L-FMAU. However, the replication of the triple mutant was reduced by 48.9 % with 5 µM of ETV and by 70.8 % with 100 µM

of PMPA. The IC50s of these two drugs were thus determined by treating transiently transfected Huh7 with increasing drug concentrations ranging from 0 to 10 µM for ETV (data not shown) and from 0 to 200 µM for PMPA (Figure 3). With ETV, the IC50s for the wt and N236T HBV strains were 0.8 µM ± 0.1 and 0.7 µM, respectively (Table 3). The L180M+M204V and L180M+M204+N236T mutants both displayed an approximatively 6 fold resistance to ETV (IC50s = 5 µM ± 0.25 and 5 µM ± 0.7 respectively) compared to wt HBV. With PMPA, a 3.4 fold resistance compared to wt HBV was observed for the L180M+M204V mutant (IC50 = 35.2 µM ± 5.1), a 4.5 fold resistance for the N236T mutant (IC50 = 46 µM ± 6) and 4.4 fold resistance for the L180M+M204V+N236T mutant (45.5 µM ± 6.1). The efficiency of IFN-α 2b against the replication of the triple mutant was also tested. It reduced equally the levels of viral DNA synthesis of the wt HBV and the three mutants by approximately 50 % (Table 2).

Discussion.

The emergence of HBV strains resistant to antiviral treatments is a major clinical concern. One alternative to delay the occurrence of drug-resistant mutants is to combine drugs that do not share the same resistance profile. We therefore asked whether an HBV mutant with a polymerase gene harboring both 3TC- and ADV-resistance mutations would have the capacity to escape the combination of 3TC+ADV given as a *de novo* or an add-on therapy for 3TC-resistance. We constructed this mutant and investigated its replicative capacity and susceptibility to drugs in hepatoma cell lines.

Compared to wt, L180M+M204V and N236T laboratory strains, the L180M+M204V+N236T mutant exhibited the lowest level of replication *in vitro*. This effect was more pronounced in HepG2 than in Huh7 cell lines. Previous studies showed that polymerases harboring mutations in the YMDD motif are less active than wt polymerase (12, 39) and require higher dNTP concentrations to reach maximum efficiency (38). Under conditions of dNTPS depletion in transiently transfected HEK 293 cells, we observed that the replication of L180M+M204V+N236T and L180M+M204V mutants are both significantly reduced compared to wt and N236T HBV. Thus, the concentration of dNTPs within HBV-producing cells may be involved in the decreased replication efficiency of the L180M+M204V polymerase mutant, as suggested previously by Melegari *et al.* (11). With respect to the N236T mutant, the replication of its genome is inhibited by less than 30 % when dNTPs pool is depleted, as well as the replication of wt HBV. Thus, variations in the intracellular dNTPs pool concentration affected weakly the replication of ADV-resistant mutant, which may explain the similarity of its

replicative capacity in Huh7 and HepG2 cells. However, the addition of the N236T mutation seemed to increase the impairment of the polymerase processivity of the L180M+M204V mutant, the triple mutant replicating its genome about 3 fold less efficiently than the double mutant. According to the HBV RT model based on homology with HIV RT, the N236 amino acid is adjacent to the active site of the RT and the N236T mutation may change the conformation of the dNTPs binding site, thus conferring resistance to ADV (40). The N236T mutation might also affect the binding of dNTP and thus impair their affinity for HBV RT harboring mutations in the YMDD motif. The low genome replication capacity of the L180M+M204V+N236T mutant may have different consequences in terms of viral fitness, i.e. reduced virion production (data not shown) and/or decreased infectivity. It was suggested that the lower replication level of the L180M+M204V mutant may result in a lower virion production (41). Until now, studies of the fitness of HBV drug-resistant strains have been hampered by the lack of easy to handle tissue culture or animal models for infectivity studies. It would be interesting to see if the low viral genome replicative capacity of the L180M+M204V+N236T mutant may result in a significant decrease in virus infectivity, which could therefore result in delayed emergence of such mutant during combination therapy *in vivo*.

According to our results, combining 3TC- and ADV-resistance mutations did not restore some level of susceptibility to nucleos(t)ide analogs. This differs from what was observed with HIV where the 3TC-resistant M184V mutation, the counterpart of HBV M204V, restore some susceptibility to HIV strains carrying thymidine analog mutations (TAM) (42). With respect to HBV, the addition of L180M+M204V mutations to N236T mutation seemed to increase the level of resistance to ADV although 3TC-resistant

strains are sensitive to this drug *in vitro* and *in vivo* (12, 16, 17, 18, 14, 15). Indeed, the triple mutant was 2 fold less susceptible to ADV than the N236T mutant (Table 1). At the molecular level, the steric hindrance due to the L180M+M204V mutations (43) may worsen the binding of the ADV in a dNTPs binding site already altered by the N236T mutation (40) and thus decreased the susceptibility to this drug. These *in vitro* findings suggest that, *in vivo,* the selection of the triple mutant could induce viral resistance during a prolonged 3TC+ADV combination therapy.

With respect to the susceptibility of the triple mutant to other drugs, the L180M+M204V mutations confer resistance to L-pyrimidine analogs, ie -FTC, L-FMAU and β-L-FD4C (12, 27) and probably accounted for the absence of response of the triple mutant to these drugs. The 3TC-resistance mutations also decrease the susceptibility to ETV *in vitro* (12, 28) which explains that the triple mutant was 6.25 fold less sensitive to ETV than wt HBV. Finally, L180M+M204V+N236T and N236T mutants were 4.5 fold less susceptible to PMPA than wt HBV. The mechanism of resistance conferred by the N236T mutation probably accounts for this decreased antiviral effect of PMPA since this drug and ADV share common structural features.

Phenotypic analysis of the L180M+M204V+N236T mutant also indicated that IFN-α inhibit equally its replication and these of L180M+M204V, N236T and wt laboratory strains *in vitro* (Table 3). This was foreseeable since IFN-α exhibit at least two HBV-specific antiviral activities independent of the viral polymerase sequence: one reduces the levels of core protein and replicative intermediates, the other leads to posttranscriptionnal degradation of HBV RNA (44).

The therapeutic potency of a drug depends on different factors including its antiviral capacity and its toxicity. Consequently, the selectivity index (IC50 / CC50) of a drug may prevent the use of optimal dose to reach a more potent antiviral effect. As triple mutant susceptibilities to ADV and ETV are decreased by about 6 fold *in vitro*, the treatment of patients infected with this HBV strain may require higher doses of drugs to overcome this decreased susceptibility. However, increasing the ADV daily dose from 10 to 30 mg leads to renal adverse effects (5) which precludes its use in the treatment of this multiresistant strain infection. Clinical studies showed that ETV is safe and inhibits viral replication more potently than 3TC when administered at 0.5 mg/ day (45, 46). Although L180M+M204V mutant displays a decreased susceptibility to ETV *in vitro* (12, 28), ETV used at 1 mg-dose was shown to decrease viral load in patients infected with 3TC-resistant HBV strains (29) and may be efficient against infection by the triple mutant since its susceptibility to ETV *in vitro* is similar to the L180M+M204V mutant counterpart (Table 3). However, viral strains with a decreased ETV susceptibility have been observed and correspond to the selection of complex mutants harboring specific polymerase mutations in addition to 3TC-resistant ones (29). PMPA is also interesting for the treatment of patients who may be infected with 3TC+ADV-resistant HBV strain. Indeed, PMPA was slightly more potent than ADV and ETV to inhibit the triple mutant replication *in vitro* and by contrast to ADV, PMPA is not associated with renal side-effect at doses that suppress significantly the viral load in patients (32). Moreover, PMPA is efficient for the treatment of HIV/HBV co-infected patients infected with 3TC-resistant HBV strains (32, 33, 34).

In conclusion, the combination of ADV- and 3TC-resistance mutations impaired the replication of the HBV genome in hepatoma cell lines, which suggests that the emergence of an HBV strain harboring these two mutations may be delayed compared to either 3TC- or ADV-resistant HBV strains in patients treated with a 3TC+ADV bitherapy. Our *in vitro* findings also showed that this combination of mutations conferred resistance to both 3TC and ADV. Consequently, non optimal strategies based on the sequential use of nucleos(t)ide analogs could lead to the selection of multiple drug-resistant strains. Interestingly, the triple mutant retains susceptibility to IFN α, ETV and PMPA *in vitro*, suggesting that these drugs could be evaluated to rescue resistance in patients infected with this multidrug-resistant HBV strain. These *in vitro* data also provide useful information for the rationale evaluation of *de novo* combination therapies in clinical trials (47).

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Legends to Figures

Fig. 1. Replicative ability of wt and RT HBV mutants in Huh7 and HepG2 cells.

(A) Autoradiograms of Southern Blots showing the replication of wt, N236T, L180M+M204V, and L180M+M204V+N236T HBV laboratory strains. Transiently transfected Huh7 and HepG2 cells were grown for one week post-transfection and Southern Blot analysis of intracellular encapsidated DNA was performed. Lin. HBV: linear HBV DNA. RC: relaxed circular DNA. RI: replication intermediates. (B): Relative replicative abilities of RT HBV mutants. After phosphorimager analysis of A, values obtained for each mutants were divided by wt HBV value that was assumed to represent 100 % of viral replication. Each value is the mean of at least 3 independent experiments, each performed in duplicate.

Fig. 2. Effect of dNTPs pool depletion on the replicative ability of wt and RT HBV mutants in HEK 293 cell lines.

(A) Autoradiogram of Southern Blot showing the replication of wt, N236T (NT), L180M+M204V (LMMV), and L180M+M204V+N236T (LMMV+NT) HBV laboratory strains in absence or in presence of 0.1 mM of hydroxyurea. Transiently transfected HEK 293 cells were treated for 2 days post-transfection with 0 or 0.1 mM of hydroxyurea. Southern Blot analysis of intracellular encapsidated DNA was performed. RC: relaxed circular DNA. RI: replication intermediates. (B): Inhibition of the viral replication due to hydroxyurea treatment. After phosphorimager analysis of A, values obtained for each HBV laboratory strains in presence of 0.1 mM of hydroxyurea were divided by the

corresponding values obtained in absence of treatment that were assumed to represent 100 % of viral replication. Each value is the mean of at least 3 independent experiments, each performed in duplicate. The Mann-Whitney test was used to determine whether the level of replication of the different HBV strains were significantly different ($p < 0.05$).

Fig. 3. Susceptibility of wt HBV and RT mutants to PMPA.

(A) Representative autoradiograms of Southern Blots showing the *in vitro* PMPA-susceptibility profiles of wt HBV, L180M+M204V+N236T (LMMV+NT), N236T (NT) and L180M+M204V (LMMV) mutants. Transiently transfected Huh7 cells were treated for five days with increasing concentrations of PMPA (indicated on the top of each autoradiogramm). Southern Blot analysis of intracellular encapsidated DNA was performed. RC: relaxed circular DNA. RI: replication intermediates. Note: The autoradiograms for the N236T (NT) and L180M+M204V (LMMV) mutants came from different set of experiments. (B) Graphs obtained after quantitative analysis of Southern Blots by phosphorimager, and used to determine the PMPA IC50 for wt HBV and RT mutants (Table 3). Each value is the mean of at least 3 independent experiments, each performed in duplicate.

Tables

Table 1. *In vitro* suceptibility of the L180M+M204V+N236T laboratory strain to 3TC, ADV and 3TC+ADV bitherapy.

HBV	3TC		ADV		3TC + ADV[c]	
	[a]IC50 (µM)	[b]Fold resistance	[a]IC50 (µM)	[b]Fold resistance	[a]IC50 (µM)	[b]Fold resistance
wt	2.48 ± 0.67	1	15.8 ± 1.9	1	1.7 ± 0.36	1
N236T	2.65 ± 0.52	1.06	50.3 ± 11	3.2	2.68 ± 0.35	1.58
L180M+M204V	>100	> 40	15.5 ± 1.8	0.98	18.75 ± 5.7	11
L180M+M204V +N236T	>100	> 40	100 ± 20	6.3	100 ± 20	58.8

[a] : Values represent the mean of 3 independent experiments, each performed in duplicate.

[b]: Fold resistance = (mutant IC50) / (wt IC50)

[c]: In the combination therapy experiment, both drugs were administrated at the same concentrations.

Table 2. *In vitro* efficacy of antivirals and IFN-α to inhibit the genome replication of HBV carrying 3TC-, ADV- or 3TC+ADV-resistance mutations.

Compound	Tested dose	Inhibition of the replication compared to no drug (%)			
		Wild-type[a]	N236T[a]	L180M+M204V[a]	L180M+M204V +N236T[a]
-FTC	**15 µM**	76.2 ± 2.5	73.8 ± 2.8	4.0 ± 10.8	7.7 ± 9.3
β-L-FD4C	**1.25 µM**	85.7 ± 1.2	67.0 ± 5.7	7.8 ± 7.7	4.2 ± 14.5
L-FMAU	**100 µM**	73.0 ± 5.6	63.1 ± 6.8	13.2 ± 11	15 ± 2.3
ETV	**5 µM**	84.6 ± 1.8	77.1 ± 6.0	48.9 ± 2.6	48.9 ± 7.0
PMPA	**100 µM**	82.3 ± 1.3	74.7 ± 4.1	43.9 ± 7.2	70.8 ± 7.4
IFN-α	**10^3 IU/ml**	46.9 ± 9.2	54.8 ± 6.2	58.8 ± 5.8	51.3 ± 5.4

[a]: Values represent the mean of at least 3 independent experiments, each performed in duplicate.

Table 3. Susceptibility of wild-type and mutant HBV to PMPA and ETV.

HBV	PMPA		ETV	
	[a]IC50 (µM)	[b]Fold resistance	[a]IC50 (µM)	[b]Fold resistance
Wild type (wt)	10.3 ± 1.3	1	0.8 ± 0.1	1
N236T	46 ± 6	4.5	0.7	0.88
L180M+M204V	35.2 ± 5.1	3.4	5 ± 0.25	6.25
L180M+M204V +N236T	45.5 ± 6.1	4.4	5 ± 0.7	6.25

[a] : Values represent the mean of at least 3 independent experiments, each performed in duplicate.

[b]: Fold resistance = (mutant IC50) / (wt IC50)