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The main Hepatitis B virus (HBV) mutants resistant to nucleoside analogs are susceptible *in vitro* to non-nucleoside inhibitors of HBV replication

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ABSTRACT

Long-term treatment of chronic hepatitis B with nucleos(t)ide analogs can lead to the emergence of HBV resistant mutants of the polymerase gene. The development of drugs with a different mode of action is warranted to prevent antiviral drug resistance. Only a few non-nucleosidic molecules belonging to the family of phenylpropenamides (AT-61 & AT-130) and heteroaryldihydropyrimidines (BAY41-4109) can prevent RNA encapsidation or destabilize nucleocapsids, respectively. The sensitivity of the main nucleos(t)ide analog- resistant mutants to these inhibitors was evaluated *in vitro*. HepG2 stable cell lines permanently expressing wild type (WT) HBV or the main HBV mutants resistant to lamivudine and/or adefovir (rtL180M + rtM204V, rtV173L + rtL180M + rtM204V, rtM204I, rtL180M + rtM204I, rtN236T, rtA181V, rtA181V + rtN236T, rtA181T, rtA181T + rtN236T) were treated with AT-61, AT-130 or BAY-41 4109. Analysis of intracellular encapsidated viral DNA showed that all mutants were almost as sensitive to these molecules as WT HBV; indeed, the fold-resistance ranged between 0.7 and 2.3. Furthermore, the effect of a combination of either AT-61 or AT-130 with BAY41-4109, and the combination of these compounds with tenofovir was studied on wild type HBV as well as on a lamivudine and an adefovir-resistant mutant (rtL180M + M204V and rtN236T, respectively). These combinations of compounds resulted in inhibition of viral replication but showed slight antagonistic effects on the three HBV species. Based on this *in vitro* study, BAY-41 4109, AT-61 and AT-130 molecules that interfere with capsid morphogenesis are active against the main lamivudine- and adefovir-resistant mutants. These results suggest that targeting nucleocapsid functions may represent an interesting approach to the development of novel HBV inhibitors to prevent and combat drug resistance.

1. Introduction

Despite the availability of an effective vaccine, 350 million people around the world are chronically infected by HBV (Wright and Lau, 1993), and are exposed to major complications including cirrhosis and hepatocellular carcinoma, leading to the death of 600,000 patients per year according to the World Health Organization.

Alpha interferon, followed by its pegylated form, was the first treatment accepted against HBV infection but only one third of treated patients show a sustained virologic response. Furthermore, the beneficial antiviral effects of interferon administration have to be weighed against numerous adverse side effects (Zoulim, 2006). Over the last decade, specific inhibitors of the viral polymerase activity that belong to the family of nucleos(t)ide analogs (NA) and include lamivudine, adefovir, telbivudine, entecavir and tenofovir have been approved (Bhattacharya and Thio, 2010; Zoulim, 2006). In spite of their high efficiency in reducing viremia levels, eradication of HBV genomes from the infected liver cannot be achieved. Long term antiviral therapy is therefore necessary to control viral replication. Because of viral persistence in the liver and the error rate of the viral polymerase, prolonged antiviral administration can lead to the emergence and selection of drug-resistant mutants harboring amino acid substitutions in the viral polymerase (Zoulim and Locarnini, 2009). Because of the overlap of the surface and polymerase genes, these resistant mutants may also harbor envelope protein modifications that can result in

Abbreviations: HBV, hepatitis B virus; NA, nucleos(t)ide; HAP, heteroaryldihydropyrimidine.

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immune escape or modification of viral fitness (Torresi et al., 2002; Villet et al., 2009). The management of antiviral drug resistance remains a clinical challenge, especially in countries where patients have been exposed to sequential therapy with antivirals having a low barrier to resistance. In these patients, the risk of multiple drug resistance is significant as well as the risk of suboptimal response to drugs with a high barrier to resistance, i.e. entecavir and tenofovir (Zoulim and Locarnini, 2009).

To counter resistance to antiviral drug directed against the viral polymerase, there is a major need to develop novel compounds with a different mode of action. In this respect, one of the potentially interesting targets is nucleocapsid assembly (Prevelige, 1998). This includes the formation and stability of viral capsids as well as the encapsidation of viral pregenomic RNA (pgRNA). Capsid sub-units interact to form a stable icosahedral structure (Seifer and Standring, 1995). Only a few compounds have been shown to target efficiently HBV nucleocapsid formation and stabilization (Weber et al., 2002). BAY-41 4109 is a heteroaryldihydropyrimidine known to reduce extra- and intra- cellular HBV DNA by impairing the formation of normal icosahedral capsids *in vitro* (Deres et al., 2003; Stray et al., 2005). Its antiviral activity was further confirmed *in vivo* in transgenic mice (Shi et al., 2007; Weber et al., 2002). Other compounds, AT-61 or AT-130 that belong to the family of phenylpropenamides, were shown to affect pgRNA encapsidation, leading to the production of empty capsids (Feld et al., 2007). It has recently been shown that by favoring protein-protein interactions to the detriment of pgRNA encapsidation and by trapping HBV capsid assembly intermediates, the administration of phenylpropenamides accelerates the assembly of empty capsids not containing the viral polymerase/pgRNA complex (Katen et al., 2010).

The inhibitory activity and the mechanism of action of phenylpropenamides and heteroaryldihydropyrimidines on the replication of WT HBV have been studied in detail, but only against a few mutants, mainly lamivudine resistant mutants (Delaney et al., 2002). The objective of our study was therefore to determine the antiviral activity of these compounds against a series of lamivudine, adefovir, and multi- resistant mutants in cell lines expressing these viral genomes. Our results showed a comparable activity against these major resistant mutants as with WT HBV. The combination of these compounds as well as their combination with tenofovir showed inhibitory activities against HBV replication but did not result in a synergistic effect in our experimental conditions. However, because of their different modes of action/non-overlapping resistance profiles, these combinations may represent a novel antiviral strategy that deserves further evaluation in study models where cccDNA formation and several rounds of infection can be observed.

2. Materials and methods

2.1. Mutant cell lines

Stable cell lines were obtained by transfection of HepG2 cells with plasmids encoding HBV mutants made by site-directed mutagenesis as described (Qi et al., 2007; Yang et al., 2005). HepG2 stable cell lines permanently expressing the main HBV mutants resistant to lamivudine and adefovir were as follows: rtL180M + rtM204V, rtV173L + rtL180M + rtM204V, rtM204I, rtL180M + rtM204I, rtN236T, rtA181V, rtA181V + rtN236T, rtA181T, rtA181T + rtN236T (Table 1). The rtA181V + rtN236T and the rtA181T +rtN236T are suspected to be multidrug-resistant strains. Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum, 200 mM of L-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin, 110 μg/ml sodium pyruvate and non-essential amino acids. In addition, cell lines were grown in the presence of 400 μg per ml of geneticin to maintain the selective pressure and expression of HBV.

2.2. Antiviral compounds

BAY-41 4109, AT-61 and AT-130 were synthesized as described previously (Perni et al., 2000; Weber et al., 2002). Tenofovir (TFV) was obtained from Gilead Sciences (Foster City, CA).

2.3. Cytotoxicity testing

To assess cytotoxic effects of antivirals compounds, stable mutant cell lines were seeded into 12-well plates and treated with compounds using the same protocol as for the antiviral assay. Following treatment, cell viability was measured with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following the manufacturer's recommendation (Sigma–Aldrich, St. Louis, MO). The concentration of a compound that inhibits cell viability by 50% (CC_{50}) was determined by linear regression.

2.4. Determination of the inhibitory activity of antiviral compounds on viral DNA synthesis

Cells were seeded in 12-well plates at a density of 3×10^5 cells per well and cultured at 37 °C and 5% CO_2 for 3 days. At days 3 and 5, cell culture medium was removed and cells were treated with fresh medium containing or not increasing concentrations of drugs, *i.e.* 1.5625, 3.125, 6.25, 12.5, 25 μM of AT-61 or tenofovir, or 0.0625, 0.125, 0.25, 0.5, 1 μM of AT-130, or 0.125, 0.25, 0.5, 1, 2 μM of BAY-41 4109. On day seven, cell culture supernatant was removed and encapsidated HBV DNA was isolated by lysing cells in Tris EDTA NP40 buffer (50 mmol/L Tris–HCl [pH 8], 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 1% NP-40). The lysates were then transferred into 96 well V-bottom plates and spun at 4500 rpm for 5 min to pellet cellular nuclei. Supernatants were digested by a mix of RNase and DNase at 100 ng/ml during 2 h at 37 °C in order to eliminate non protected viral RNA and DNA. Extracts were directly loaded on a 1.2% agarose gel and were electrophoresed at 80 V in 0.5X TAE buffer (20 mM Tris acetate and 0.5 mM EDTA). After migration, HBV DNA was transferred to a nylon membrane (Hybond N+, Amersham, United Kingdom) in 20X TNE buffer (200 mM Tris; 4.0 M NaCl; 20 mM EDTA; pH 7.4). Membranes were then incubated in denaturation buffer containing 1 M NaCl and 0.2 M NaOH during 20 min, neutralized by 0.5 M Tris, pH 7.4; 1 M NaCl during 5 min and washed with a solution consisting of 0.3 M sodium chloride and 30 mM trisodium citrate (adjusted to pH 7.0 with HCl). Membranes were fixed for 2 h at 80 °C and hybridized with an HBV-specific radioactive probe. Signals were quantified using PhosphorImager analysis (GE Healthcare, Velizy, France). For each drug, the concentration inhibiting by 50% the amount of encapsidated viral DNA (IC_{50}) compared to untreated cells was calculated in at least four experiments.

2.5. Drug combination assay

Cell lines expressing WT HBV or the rtL180M + M204V and rtN236T mutants were seeded in a 24 well plate at a density of 4×10^4 cells per well. Cells were then fed with medium containing drugs with increasing concentrations as described above at day 3 and day 5. Combinations of AT-61 + BAY-41 4109, AT-130 + BAY-41 4109, AT-61 + TFV, AT-130 + TFV and BAY-41 4109 + TFV were studied. At day 7, encapsidated HBV DNA was analyzed after migration in native agarose gels as described above.

2.6. Statistical analysis of the inhibitory effect of the combined administration of the compounds

The effect of the combination of phenylpropenamides, heteroaryldihydropyrimidines and tenofovir were assessed using the Chou–Talalay model. The median-effect principle and its extension, the combination index (CI) equation, are derived from the fundamental principle of the mass-action law (Chou and Talalay, 1984). This general equation predicts either synergism (CI < 1), an additive effect (CI ∼ 1) or antagonism (CI > 1). The CI was defined as $(IC_{50A/B}/IC_{50A}) + (IC_{50B/A}/IC_{50B}) + ((IC_{50A/B} \times IC_{50B/A})/(IC_{50A} \times IC_{50B}))$, where IC_{50A} and IC_{50B} are the IC_{50}s of drug A and B used alone, respectively, and $IC_{50A/B}$ and $IC_{50B/A}$ are the IC_{50}s of A and B used in combination, respectively (Chou and Talalay, 1981).

A graph indicating the equipotent combination of various doses of two drugs, called an "isobologram", was plotted from the raw data using the concentration of compounds that would inhibit HBV replication by 50%, 75% and 90% (Fa = 0.5; 0.75 and 0.9, respectively) (Steel and Peckham, 1979). Synergism is defined when the experimental points fall below that line, whereas antagonism is defined when the points lie above it.

A plot of CI on the *y*-axis as a function of effect level (% inhibition) on the *x*-axis was performed in order to order to cover all effect levels (1–99%). Below, on or above the CI = 1 horizontal line, indicates, respectively, synergistic, additive or antagonistic effects of the combination.

3. Results

3.1. The main nucleoside analog resistant mutants are susceptible to inhibition by phenylpropenamides and heteroaryldihydropyrimidines

Results of MTT assay performed in all cell lines expressing either WT HBV or the lamivudine and adefovir resistant mutants and treated with increasing concentrations of AT-61, AT-130 and BAY-41 4109 showed the absence of cytotoxic effects, with CC_{50} > 25μM for AT-61, > 1μM for AT-130, and >2 μM for BAY-41 4109.

The inhibitory effect of phenylpropenamides and heteroaryldihydropyrimidines on viral DNA synthesis was determined in cell lines expressing the main nucleoside analog-resistant mutants (Table 1). Following four days of treatment, viral nucleocapsids isolated from the different cell lines were subjected to electrophoresis in native agarose gels and encapsidated HBV DNA was quantified after hybridization with a specific probe (Table 2). Native agarose gel analysis displayed the same results as the Southern blot analysis and the real time PCR quantification of encapsidated HBV DNA (data not shown). Depending on the cell lines, the IC_{50} of AT-61 was 11.6 μM ± 1.85 for WT HBV and ranged from 8.05 to 13.68 μM for the studied mutants, that of AT-130 was 218 nM ± 0.05 for WT HBV and ranged from 101 to 231 nM for the mutants, and that of BAY-41 4109 was 276 nM ± 0.03 for WT HBV and ranged from 187 to 323 nM for the mutants.

Table 1
Summary of the main HBV mutants that were evaluated and their amino acid substitutions in the viral polymerase and the surface proteins.

Amino acid substitutions in the viral polymerase protein	Amino acid substitutions in the envelope proteins	Mutant names
–	–	WT
rtL180M + M204V	sI195M	LMMV
rtV173L + L180M + M204V	sE164D + I195M	VLLMMV
rtM204I	sW196F	MI
rtL180M + M204I	sW196F	LMMI
rtN236T	–	NT
rtA181V	sL173F	AV
rtA181V + N236T	sL173F	AVNT
rtA181T + N236T	sW172*	ATNT
rtA181T	sW172*	AT

Table 2
Sensitivity of WT and drug-resistant variants to phenylpropenamides (AT-61 and AT-130) and the heteroaryldihydropyrimidine BAY-41 4109.

Cell line	AT-61	FR[a]	AT-130	FR[a]	BAY-41 4109	FR[a]
WT	11.60 ± 1.85	1.00	0.218 ± 0.05	1.00	0.276 ± 0.02	1.00
LMMV	9.92 ± 0.53	0.86	0.101 ± 0.03	0.47*	0.323 ± 0.02	1.17
VLLMMV	10.92 ± 2.02	0.94	0.200 ± 0.09	0.92	0.240 ± 0.01	0.87
MI	9.75 ± 1.24	0.84	0.198 ± 0.02	0.91	0.210 ± 0.02	0.76
LMMI	8.05 ± 1.99	0.69*	0.224 ± 0.10	1.03	0.215 ± 0.05	0.78
NT	10.34 ± 2.41	0.89	0.207 ± 0.09	0.95	0.213 ± 0.04	0.77
AV	8.19 ± 2.74	0.71	0.212 ± 0.05	0.97	0.247 ± 0.04	0.89
AVNT	10.41 ± 1.51	0.90	0.185 ± 0.08	0.85	0.249 ± 0.02	0.90
ATNT	12.13 ± 2.47	1.05	0.185 ± 0.08	0.85	0.187 ± 0.11	0.67
AT	13.68 ± 3.43	1.18*	0.231 ± 0.07	1.06	0.196 ± 0.01	0.71

IC_{50}s are presented in μM with standard deviation. The results represent the mean and the standard deviation of at least four independent experiments.
[a] FR -fold resistance is the ratio of the drug IC_{50} for a given mutant over its IC_{50} for WT HBV.
* Statistically significant difference ($p < 0.05$).

3.2. Study of the combination of non-nucleoside inhibitors and their combination with tenofovir

The effect of the combination of the two classes of drugs, and their combination with tenofovir was studied against WT HBV and the rtN236T and rtL180M + M204V mutant cell lines. Dual treatments included AT-61 + BAY-41 4109, AT-61 + TFV, AT-130 + BAY-41 4109, AT-130 + TFV, and BAY-41 4109 + TFV. The experimental conditions for cell culture, compound administration and HBV DNA analysis were similar to those described above. A dose-dependent inhibition of the formation of encapsidated HBV DNA following administration of tenofovir and BAY-41 4109 (Fig. 1) was observed. As described by others (Katen et al., 2010; Stray and Zlotnick, 2006), we observed that the non-nucleoside inhibitors studied here, at low concentrations, activate HBV replication.

The antiviral effect of different drug combinations was analyzed by both the methods of Chou & Talalay and the Bliss independence test (Bliss, 1956; Chou and Talalay, 1981). The first method can create isobolograms of the inhibition data (50% and 90%) for each combination (Fig. 2). The straight lines of the isobologram graphs represent additive effects and data points below this line indicate synergism while data points above the line correspond to antagonism. Almost all data points of the five combinations at IC_{50} or IC_{90} were above the lines indicating antagonistic effects. The exceptions were for the AT-130 + BAY-41 4109 combination for WT and NT viruses at IC_{50}, suggesting that only these combinations did not act antagonistically (Fig. 2). However, most of the mutant data points for the other combinations were lower (or not very different) than for the wild type (Fig. 2), suggesting that the combinations did not act antagonistically against these mutants.

The second method for analyzing the results of combination assays was by plotting combination indexes (CI) of the inhibition data (Fig. 3). CI is based on the unified theory derived from the mass-action law principle and allows quantitative determination of drug interactions (Chou and Talalay, 1984). According to the equations derived from this theory, CI < 1, =1, and >1 indicate synergism, additive effects and antagonism, respectively. CI values at each 5% interval of HBV replication inhibition were used to analyze the drug combinations. When the combinations were analyzed by the Bliss test, most combinations stayed above the level of additiv-

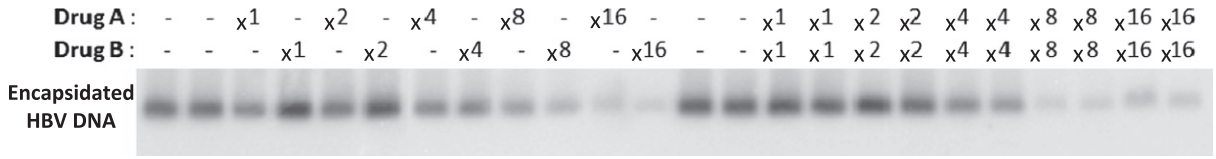

Fig. 1. General schedule illustrating the effect of drug combinations on encapsidated viral DNA. Factors x1 to x16 correspond to increased factors of each drug concentration. x1 factor of AT-61 correspond to 1.5625 μM, AT-130 to 0.0625 μM, BAY-41 4109 to 0.125 μM and x1 factor of tenofovir correspond to 1.5625 μM. A WT HBV expressing cell line was treated with BAY41-4109 (0–2 μM) (drug A) and TFV (0–25 μM) (drug B) in single and double treatment.

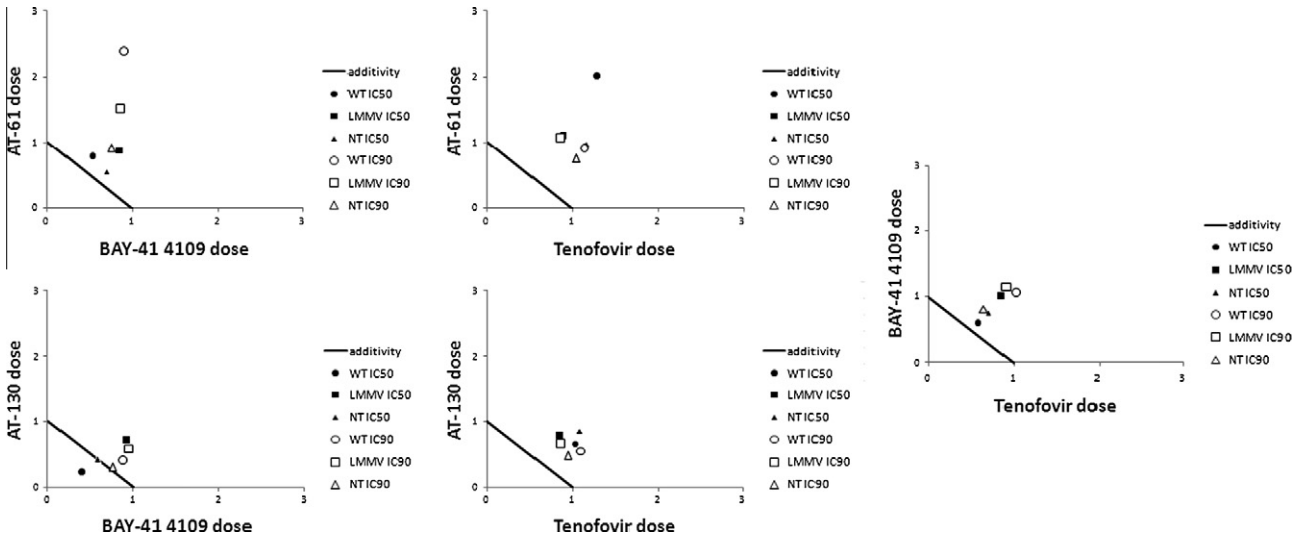

Fig. 2. Normalized isobolograms of the antiviral action between phenylpropenamides, heteroaryldihydropyrimidine and tenofovir (TFV). ● WT HBV at IC_{50}; ■ LMMV at IC_{50}; ▲ NT at $IC_{50;}$ ○ WT HBV at IC_{90}; □ LMMV at IC_{90}; Δ NT at $IC_{90.}$ If the experimental dots are below the line, the compounds are acting synergistically; if the dots are above the line, the compounds are acting antagonistically; and if they are on the line, the compounds are acting additively. The results represent the mean of at least four independent experiments.

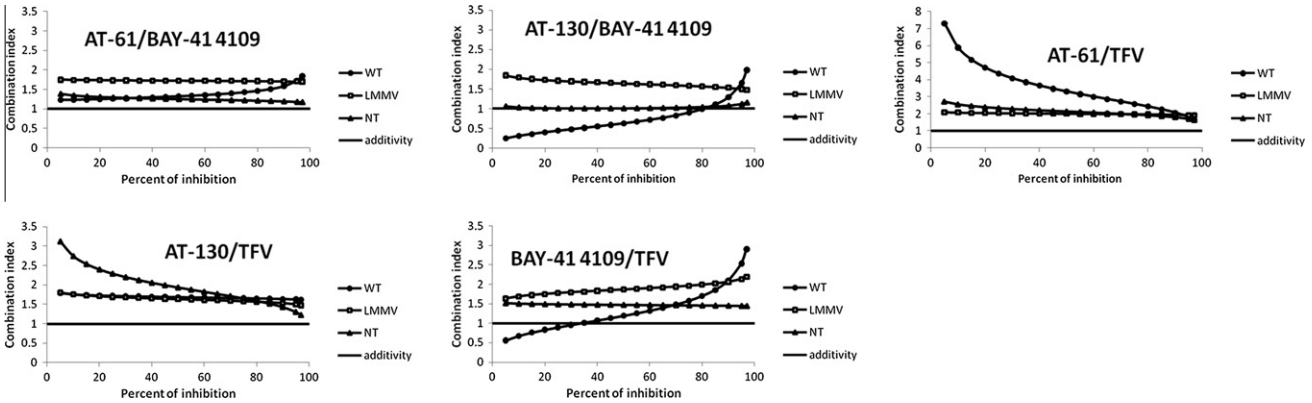

Fig. 3. Combination Index analysis of the combined antiviral activity of phenylpropenamides, heteroaryldihydropyrimidine and tenofovir (TFV). ● WT HBV; □ LMMV; ▲ NT. Combination index (CI) is a quantitative determination of drug interactions based on the unified theory for the main biochemical equations. CI values <1, =1 or >1 are indicative of synergistic, additive or antagonistic effects, respectively. The Y axis corresponds to CI values and the *X* axis indicates the percent of HBV replication inhibited by the combination tested. The results represent the mean of at least four independent experiments.

ity. BAY-41 4109 combined with AT-130 or TFV resulted at low concentrations in slight synergistic effects but in a rather antagonistic effect at higher levels (Fig. 3). The AT-61 + TFV combination proved the least antagonistic on the mutants as compared to WT HBV (Fig. 3).

4. Discussion

Currently, nucleos(t)ide analogs (NA) are the only virus specific drugs approved for the treatment of chronic HBV infection (Jafri and Lok, 2010). However, the administration of viral polymerase inhibitors can select for drug-resistant mutants that can lead to treatment failure (Zoulim and Locarnini, 2009). The search for new antiviral targets with a different mode of action is therefore warranted to develop combination strategies to combat NA drug resistance not only when it is already established but also to prevent its emergence. One of the potential targets within the viral life cycle is nucleocapsid assembly. Several compounds belonging to the family of heteroaryldihydropyrimidines and of phenylpropenamides were shown to interfere with viral pregenome packaging or with nucleocapsid assembly. These compounds were mainly stud-

ied on WT HBV and lamivudine resistant mutants. In this study, we have analyzed their activity against the main lamivudine and adefovir resistant mutants, as well as on mutants harboring resistance mutations to both drugs.

Our results confirmed observations of previous work on these compounds showing the absence of significant cellular toxicity *in vitro* in cell culture experiments (Deres et al., 2003; King et al., 1998). The results of our experiments confirm that AT-61, AT-130 and BAY-41 4109 exhibit a similar antiviral activity against lamivudine-resistant mutants and WT HBV. Our study also showed that all frequently described lamivudine-resistant mutants were sensitive to these drugs. Only rtL180M, rtM204V/I and rtL180M + M204V LAM-R mutants were previously shown to be as sensitive to phenylpropenamide compounds as WT HBV (Delaney et al., 2002; King et al., 1998), but rtV173L + L180M + M204V, rtL180M + M204I and rtA181V/T mutants were not tested. Also, these compounds had not yet been tested against adefovir-resistant mutants. Our results provide new information showing that all frequently described adefovir-resistant mutants (rtA181V/T, and rtN236T) are sensitive to these non-nucleos(t)ide drugs. Furthermore, an inhibitory activity was also found against two complex mutants combining mutations at position rt181 and rt236 that confer not only multiple resistances to both lamivudine and adefovir but also a reduced susceptibility to tenofovir (Patterson et al., 2011; Villet et al., 2008). This highlights the fact that drugs targeting steps of the viral life cycle other than the viral polymerase activity may become relevant to the inhibition of replication of complex resistant mutants.

After demonstrating that polymerase gene resistant mutants were sensitive to drugs belonging to the family of phenylpropenamides and of heteroaryldihydropyrimidines, we studied what the antiviral effect is of combination of these two classes of drugs or their combination with a nucleotide such as tenofovir. The effect of drug combinations was assessed against WT HBV, a LAM-resistant mutant (rtL180M + M204V) and an ADV-resistant mutant (rtN236T). All combinations tested displayed inhibitory effects on HBV replication (data not shown). Interestingly, combination of BAY-41 4109 with the two phenylpropenamide compounds showed the best inhibitory effect on viral DNA synthesis for all viruses tested. In our experimental conditions, none of these combinations were found to act synergistically, except for the AT-130 + BAY-41 4109 combination on WT HBV. The combination of AT-61 + TFV seemed to be antagonistic which was different in another study performed in a different cellular model (King et al., 1998). The statistical analysis performed with the Bliss independence model and the Chou & Talalay model led to the same results (data not shown). It is perhaps not surprising that in our model system we did not find additive or synergistic effects.

It cannot be excluded that the antiviral effect of the combinations may show potentiation in experimental models that allow several rounds of infection and amplification of viral cccDNA (chimeric mice colonized with human hepatocytes infected with human HBV or animal models of hepadnavirus infection) (Dandri et al., 2006)

In conclusion, our results show that phenylpropenamide and heteroaryldihydropyrimidine compounds exhibit an interesting cross-resistance profile by inhibiting the main mutants that are resistant to nucleoside analogs, including complex mutants with a multidrug resistance profile. The combination of these drugs with nucleos(t)ide analogs do not show major antagonistic effects, suggesting that the development of compounds targeting different steps of nucleocapsid assembly could be relevant to tackle resistance to polymerase inhibitors. In this drug development perspective these compounds (or derivatives thereof) should be further evaluated in experimental models.

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