Original article

Entecavir allows an unexpectedly high residual replication of HBV mutants resistant to lamivudine

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Background: Entecavir is an efficient inhibitor of HBV reverse transcriptase (RT) and widely used for therapy of chronic hepatitis B. Entecavir treatment of HBV patients with lamivudine-resistant viral strains, however, often fails, but the mechanism of cross-resistance development is not fully understood.

Methods: Using non-linear regression models, dose–response curves of cloned HBV strains from patients pre-treated with RT inhibitors were established in human hepatoma cell lines after transfection with HBV genomes containing HBV polymerase genes from patient isolates. 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) and corresponding antiviral resistance factors (RF_{50} and RF_{90}) were calculated.

Results: The entecavir dose–response curve of lamivudine-resistant HBV RT mutants rtM204 for the replication of HBV decreased less than expected with increasing drug dose. Remarkably, due to the flat dose–response curves, RF_{90} values against entecavir of samples with rtM204 substitutions were up to 30× higher than their RF_{50} values.

Conclusions: The unexpectedly high IC_{90} indicates a strong residual replication capacity of lamivudine-resistant HBV rtM204 variants under entecavir therapy, although IC_{50} values are initially within the therapeutic range of entecavir. This characteristic favours rapid selection of additional mutants with overt resistance against entecavir. Thus, the current phenotypic resistance assays should include determination of IC_{90}.

Introduction

Infection with HBV is still a major health problem. An estimated 240 million patients are chronically infected with HBV [1] and have an increased risk for developing liver cirrhosis and/or hepatocellular carcinoma. Since this risk is directly related to the viral load in the serum of chronic carriers [2], a sustained suppression of viral replication with low to non-detectable viral load in plasma is the major aim of antiviral therapy. Treatment of hepatitis B patients with interferon-α leads to a sustained virological response in only 30% to 40% [3], but is not applicable in patients with advanced liver disease and has severe side effects. Inhibitors of HBV reverse transcriptase (RT), which interfers with viral replication, do not eliminate the pre-existing viral genomes from the liver and need to be given for life. Three nucleoside analogues (lamivudine, telbivudine and entecavir) and two nucleotide analogues (adefovir and tenofovir) are approved for treatment of chronic hepatitis B but only entecavir and tenofovir are recommended due to their superior efficacy and high genetic barrier against resistance [4].

Antiviral resistance is caused by drug-resistant variants, but the rate of development is different for each drug and needs to be addressed carefully. Replication of HBV is error-prone, due to the lack of proofreading during reverse transcription of the viral RNA pregenome and during plus strand DNA synthesis, resulting in high mutation rates [5]. Together with high levels of viral replication, this can lead to a broad spectrum of viral variants that are selected during antiviral treatment. Resistance rates of up to 80% are common for lamivudine after 5 years of treatment [6], while entecavir and tenofovir show a much higher resistance-barrier in clinical

practice [7]. Patients carrying lamivudine-resistant HBV strains, showed a highly increased entecavir-resistance rate (51% compared to 1.2% in treatment-naive patients after 5 years of entecavir therapy) [6,8]. This is surprising because entecavir is considered a very strong inhibitor of the HBV RT and should not allow a level of replication, necessary for frequent generation and selection of new resistant variants.

The complete clinical resistance to entecavir is based on amino acid exchanges rtL180M and rtM204V, caused by mutations within the viral genome that are frequently selected during lamivudine therapy, but needs at least one further exchange at positions rtI169, rtS184, rtS202 or rtM250 (Table 1). We therefore investigated the influence of lamivudine resistance mutations on entecavir resistance by testing HBV clones from patients reflecting the typical lamivudine resistance patterns (rtL180M+rtM204V, rtL80V/I ±rtM204I and rtA181V), as described in a study based on 492 sequences from the SeqHepB database [9,10].

For quantitative description of antiviral resistance against nucleoside/nucleotide analogues, usually the resistance factor (RF_{50}) is determined by dividing the 50% inhibitory concentration (IC_{50}) of a virus variant by the IC_{50} of the wild type [11–15]. A limitation of this approach is that at IC_{50}, 50% of replication still occurs, which would be an optimal condition for development of resistant variants. Thus, we extended our study and obtained data at more efficient suppression of replication, for example, at IC_{90}. Using this method we have analysed resistant HBV strains from different patients and obtained unexpected results for antiviral efficiency of entecavir which help to explain the weakness of entecavir against lamivudine resistant variants.

Methods

Isolation of HBV DNA, amplification and cloning of HBV polymerase ORFs

Nearly the entire polymerase open reading frame (ORF; amino acids 9 to 816) including the primer and RNaseH domain of the respective viral strains was amplified and cloned into a vector that allows HBV replication after transfection to human hepatoma cell lines. HBV DNA from plasma of chronic HBV carriers was purified with the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. None of the chronic hepatitis B patients had been treated with entecavir, but with lamivudine (numbers 1–8) and adefovir (numbers 9 and 10; Table 2). The respective HBV polymerase ORF region was amplifyed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol using primers pol-fw 5′-GTCACTTCCGGAGACTACTGTTGTTAGACGACG-3′ and pol-rev: 5′-GCGCATCGGTCCGGCAGATGAGAAGGC-3′, which amplify the polymerase region between the restriction sites for BspEI and RsrII unique within the polymerase ORF (Figure 1, left). These sites were used to insert the polymerase gene fragments from patient HBV strains by replacing the polymerase gene fragment of vector pCH9-3091 containing a replication competent 1.1-fold overlength HBV genome (genotype D, ayw) under the control of the human cytomegalovirus immediate early promoter (Figure 1, right) [16].

Phenotypic *in vitro* resistance assay

Human hepatoma cell lines HuH7 and HepG2 were maintained in DMEM with 5% (vol/vol) FCS (Invitrogen). For transient transfection of plasmids, FuGene HD (Roche) was used at 80% cell confluency according to the manufacturer's protocol. For phenotypic analysis of HBV drug resistance, 10 cm dishes of HuH7 or HepG2 cells were transfected with either pCH9-3091 (wild-type control) or pCH9-3091 derivatives containing polymerase sequences from HBV patient isolates. One day after transfection, cells were trypsinized and washed 3× with 30 ml DMEM by centrifugation at 60 g for 6 min and resuspension in DMEM to remove residual plasmid DNA. Using these extensive washing

Table 1. Relevant HBV resistance mutations and their cross-resistance pattern

Amino acid substitutions in the HBV RT domain	ADF	TDF	LdT	LMV	ETV	Selected during therapy with
Wild type	S	S	S	S	S	–
A181V/T	R	I	R	R	S	ADF
N236T	R	I	S	S	S	ADF
M204I/V	S	S	R	R	I	LdT, LMV
L180M +M204V/I ±I169 ±T184 ±S202 ±M250	S	S	R	R	R	ETV
Dosage, mg/day	10	300	600	100	0.5	

Adapted from [6] with permission from Elsevier. Primary resistance mutations are shown in bold. The cross-resistance related to the most important amino acid changes is shown for reverse transcriptase (RT) inhibitors currently used for the treatment of chronic hepatitis B (adefovir [ADF], tenofovir [TDF], telbivudine [LdT], lamivudine [LMV] and entecavir [ETV]). For a complete resistance against ETV, a complex pattern of at least three mutations is needed. S, sensitive; I, intermediate; R, resistant; based on clinical and *in vitro* data.

Table 2. Phenotypic resistance data of isolates from different patients, harbouring mutations relevant for lamivudine resistance

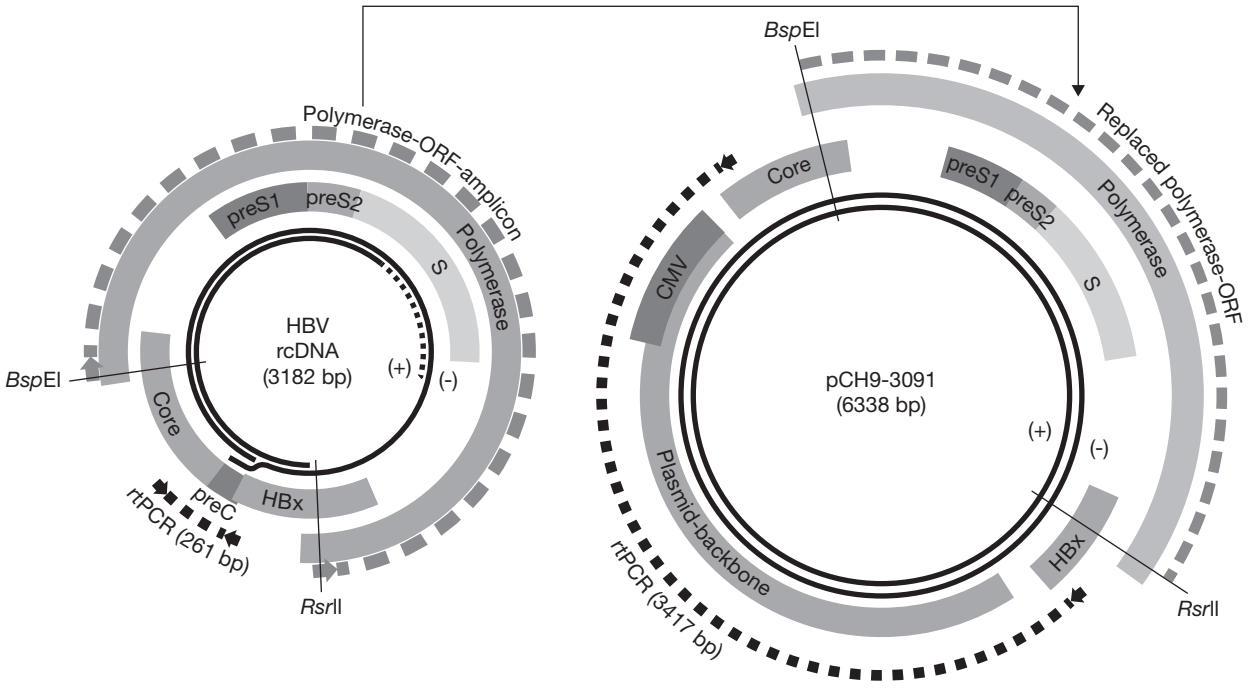
Drug	Number	Genotype	Mutations in RT-domain	C	IC_{50}	IC_{90}	RF_{50}	RF_{90}	R^2	Sign
Adefovir	WT	D	Wild type	1.03	0.9	7.6	1.0	1.0	1.00	NA
	C-1	A	Wild type	0.99	1.7	15.4	1.8	2.0	1.00	NS
	C-2	B	Wild type	1.11	1.0	6.9	1.1	0.9	0.99	NS
	C-3	D	Wild type	0.93	2.7	28.2	2.9	3.7	0.98	NS
	1	A	L180M, M204V	0.69	1.6	39.8	1.7	5.2	0.99	NS
	2	B	L180M, M204V	1.13	1.9	13.0	2.1	1.7	0.99	NS
	3	D	L80V, M204I	1.36	2.2	10.6	2.3	1.4	0.99	NS
	4	D	L80V, M204I	1.29	1.4	7.8	1.5	1.0	1.00	NS
	5	A	L80V, M204I	1.04	0.8	7.3	0.9	1.0	0.99	NS
	6	D	L80I, M204I	0.92	0.8	8.3	0.8	1.1	1.00	NS
	7	D	L80I, M204I	1.65	0.9	3.4	1.0	0.5	0.99	NS
	8	A	M204I	1.32	0.9	4.6	1.0	0.6	0.97	NS
	9	D	A181V	1.50	3.0	12.7	3.2	1.7	0.98	[a]
	10	A	A181V	1.14	4.2	27.1	4.4	3.5	0.99	[a]
Tenofovir	WT	D	Wild type	1.31	1.0	5.1	1.0	1.0	1.00	NA
	C-1	A	Wild type	0.84	1.4	18.7	1.4	3.6	0.99	NS
	C-2	B	Wild type	1.04	1.3	10.9	1.7	2.1	1.00	NS
	C-3	D	Wild type	0.95	2.0	20.0	2.1	3.9	0.98	NS
	1	A	L180M, M204V	1.56	2.2	9.0	2.3	1.8	1.00	NS
	2	B	L180M, M204V	0.82	1.2	18.0	1.2	3.5	0.98	NS
	3	D	L80V, M204I	1.00	1.0	9.2	1.0	1.8	1.00	NS
	4	D	L80V, M204I	1.48	1.2	4.9	1.2	1.0	0.95	NS
	5	A	L80V, M204I	1.63	1.6	6.0	1.6	1.2	1.00	NS
	6	D	L80I, M204I	1.62	0.7	2.6	0.7	0.5	1.00	NS
	7	D	L80I, M204I	1.52	0.5	2.0	0.5	0.4	1.00	NS
	8	A	M204I	0.83	0.7	9.7	0.7	1.9	0.99	NS
	9	D	A181V	1.65	3.8	14.1	3.9	2.8	0.99	[a]
	10	A	A181V	1.39	4.7	21.0	4.8	4.1	0.96	[a]
Entecavir	WT	D	Wild type	1.77	0.9	3.1	1.0	1.0	1.00	NA
	C-1	A	Wild type	0.80	0.88	13.84	1.0	4.5	1.00	NS
	C-2	B	Wild type	1.59	0.45	1.8	0.5	0.6	1.00	NS
	C-3	D	Wild type	1.00	1.1	10.3	1.3	3.3	1.00	NS
	1	A	L180M, M204V	0.39	31.8	7,946.1	35.3	2,572.9	0.97	[a]
	2	B	L180M, M204V	0.40	30.4	6,581.0	33.7	2,130.9	0.94	[a]
	3	D	L80V, M204I	0.63	65.9	1,826.9	73.0	591.6	0.94	[a]
	4	D	L80V, M204I	0.52	12.3	704.6	13.6	228.2	0.94	[a]
	5	A	L80V, M204I	0.45	4.7	575.9	5.2	186.5	0.98	[a]
	6	D	L80I, M204I	0.64	18.0	497.6	19.9	161.1	0.96	[a]
	7	D	L80I, M204I	1.01	2.9	25.8	3.2	8.4	1.00	[a]
	8	A	M204I	0.72	8.3	147.4	9.2	47.7	0.90	[a]
	9	D	A181V	1.65	1.0	3.3	1.1	1.1	0.95	NS
	10	A	A181V	1.65	0.7	2.5	0.7	0.8	1.00	NS

The calculation of the slope C, 50% inhibitory concentration (IC_{50}), 90% inhibitory concentration (IC_{90}), the resulting resistance factors RF_{50} and RF_{90} is based on the regression model for the dose–response curves described in Figure 2. Triplicate measurements of 6 different concentrations per isolate and drug were performed and compared to 10 independent assays of a wild-type (WT) consensus genome (genotype D). Additional WT controls (C1-C3) of genotypes A, B and D from chronic carriers are shown. The accuracy of the regression is indicated by the R^2 value. The column 'Sign' indicates significant differences to WT ([a]$P<0.001$, Bonferroni corrected; NS, not significant; NA, not applicable). The table shows the measurements for the drugs adefovir, tenofovir and entecavir.

steps and virion-specific detection methods (see below), a detection limit of <1% replication relative to the uninhibited control was achieved. Afterwards, cells were resuspended in DMEM with 2% (vol/vol) FCS and equally distributed on a 96-well cell culture plate containing a dilution series of the respective inhibitors in triplicates.

Three days after transfection, the amount of virion-associated HBV DNA was determined in the supernatants. 100 µl of cell supernatant was used for automatic DNA purification with a Freedom Evo 100 4/D (Tecan, Crailsheim, Germany) using the sample preparation system DNA Kit from Abbott (Wiesbaden, Germany) according to the manufacturer's protocol. Quantification

Figure 1. Cloning of the HBV DNA polymerase region and discriminative detection of newly replicated viral DNA



The relaxed circular form of HBV DNA (HBV rcDNA, left) and the plasmid pCH9-3091 carrying the respective HBV genome are shown. Grey arrows: binding sites of primers for amplification of polymerase fragments (grey broken line), including the restriction sites for cloning (*Bsp*EI and *Rsr*II), black arrows: binding sites of primers for discriminative real-time PCR (rtPCR) with indicated length of the respective amplicons (black broken lines). bp, base pair; CMV, human cytomegalovirus immediate early promoter; (+) and (-) indicate the orientation of the DNA strands, the variable end of the (+) strand of the viral genome is represented by a dotted line.

of viral DNA was done via SYBR-green real-time PCR with the LightCycler LC 480 (Roche) and the Absolute SYBR Capillary Mix (Thermo Scientific, Dreieich, Germany). To correct for plasmid-derived background, a PCR with the primers HP-fw: 5′-ACTAGGAGGCTGTAGGCATA-3′ and HP-rev: 5′-AGACTCTAAGGCTTCCCG-3′ was used, that discriminates between newly synthesized HBV DNA and plasmid associated input DNA. The natural relaxed circular DNA form of the HBV genome has only 261 basepairs (bp) between the primer binding sites, whereas the plasmid has 3417 bp between the primer binding sites (Figure 1). With short elongation phases very little plasmid-derived HBV DNA is amplified while the short PCR product is efficiently generated.

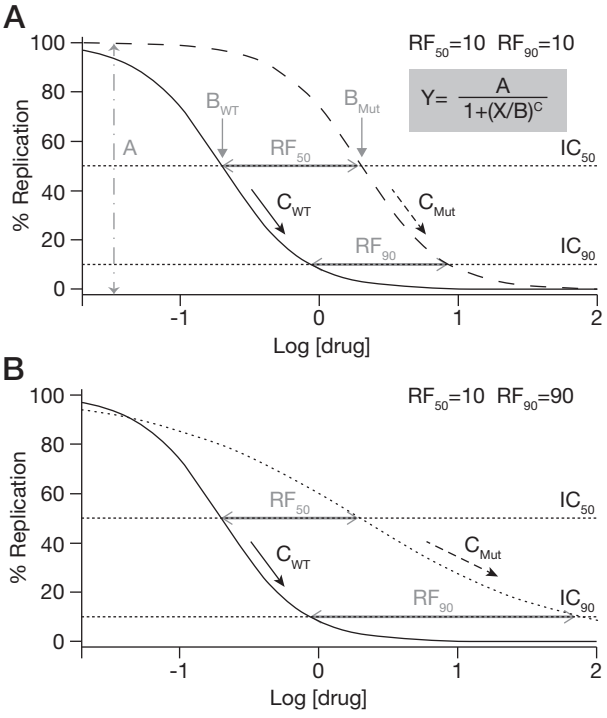
Statistical analysis of resistance data

The amounts of newly synthesized HBV DNA in the cell culture supernatants were plotted against the drug concentrations and a sigmoid regression curve was fitted as shown in Figure 2. This regression model allows calculation of the HBV replication at any drug concentration and provides the IC_{50} and IC_{90} values (Figure 2). SigmaPlot (Systat Software, Erkrath, Germany) was used for regression and SPSS (IBM, Ehningen, Germany) for the univariate ANOVA. The non-linear regression model based on a three parameter logistic dose–response equation of the form $y=A/(1+[x/B]^C)$ was used as described previously [17]. The meaning of the parameters A–C is explained in the figure legend. Individual equations were calculated for each dose–response curve and used to determine the IC_{50}, the IC_{90} and the correlation coefficient R^2. The univariate ANOVA was used to determine whether the data set of the values of the respective mutant is significantly different to the consensus data set of the wild type or not. The significance threshold was adjusted to the number of comparisons by using the Bonferroni correction.

Results

HBV polymerase variants with mutations typical for antiviral resistance were isolated and cloned from plasma samples of 10 chronic HBV patients (Table 2). Samples 1 to 8 have amino acid exchanges at position rtM204 with or without additional exchanges

Figure 2. Determination of 50% and 90% inhibitory concentrations and resistance factors using a sigmoid regression function

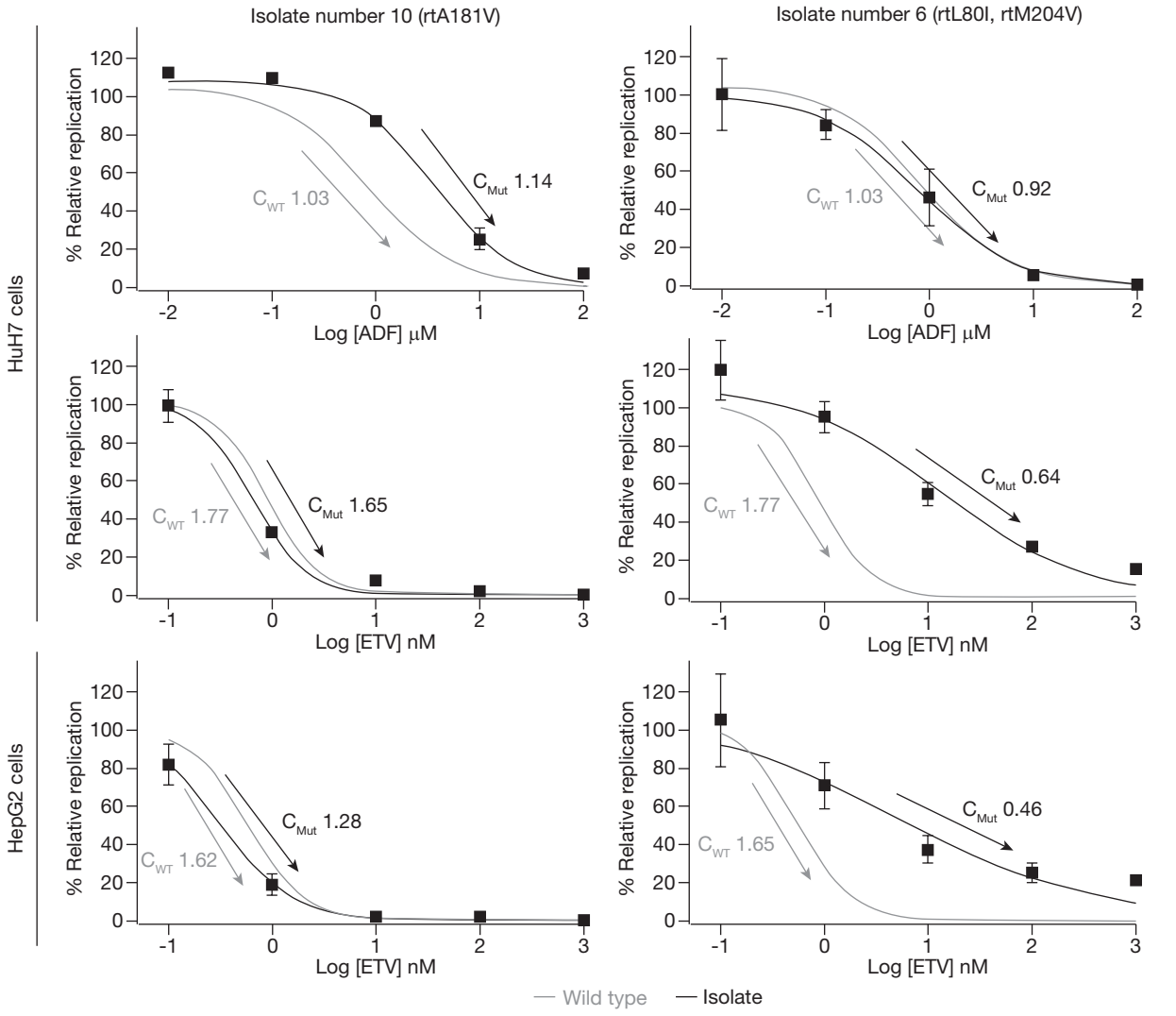


(A) The regression curve is based on an equation (grey box) with three different parameters: the amplitude A defines the difference between absent and maximum inhibition. Parameter B specifies the inflexion point. Parameter C is the transition width of the regression curve (distance on the *x*-axis between 1/3A and 2/3A), and defines the slope of the inhibition curve. If C is identical for wild type and variant, 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) are changed by the same factor, and respective resistance factors RF_{50} and RF_{90} are identical. **(B)** With a decreased C-value (C_{Mut}), an extrapolation of the RF_{90} based on the RF_{50} value underestimates the drug concentration required to suppress replication to 10% (IC_{90}).

at position rtL80 or rtL180M, representing the most common lamivudine resistance patterns following primary mutations at rtM204 [9]. These exchanges lead to very high lamivudine resistance and to reduced, but clinically still acceptable, entecavir efficacy [12]. Samples 9 and 10 carry the exchange rt181V that is associated with lamivudine and adefovir resistance and an intermediate but clinically tolerable resistance to tenofovir [18] (Table 1). All samples were compared to a control plasmid (WT) containing an HBV genotype D wild-type (WT) genome, and the IC_{50} and IC_{90} values for each drug were determined as shown in Figure 2. The accuracy of the regression curves is shown by the coefficient of correlation (R^2) which ranged between 0.9 and 1.0 in all calculations. The WT HBV strain showed IC_{50} values for the RT inhibitors comparable to those described in the literature (Table 2) [15]. Since the viral isolates clustered into three different HBV genotypes (A, D and one B genotype), we also included three wild-type sequences from patients of the respective genotypes as controls (C-1, C-2, C-3, Table 2). With all three control isolates we observed similar IC_{50} values for the RT inhibitors compared to the WT control of genotype D (Table 2). The RF can either be based on the IC_{50} (RF_{50}) or the IC_{90} (RF_{90}), respectively (Figure 2 and Table 2), provided the dose–response curves of wild type and variant run parallel. The three parameters A–C defining the curve are listed for each of the 10 samples in Table 2. As expected, isolates 1 to 8 showed no significant resistance to adefovir and tenofovir and had low RF_{50} values between 0.5 and 2.3 (ADF mean ±SD: 1.40 ±0.57, TDF mean ±SD: 1.15 ±0.58), confirming the sensitivity of these variants to nucleotide analogues (Table 2). For adefovir and tenofovir the inhibition curves resulted in RF_{90} values comparable to the corresponding RF_{50} values. The only exception was the adefovir-RF_{90} value of isolate 1 that was larger due to a slightly decreased slope C (0.69 versus 1.03 of the wild type); but this did not cause significant resistance as proven by univariate ANOVA testing (Table 2). For all other isolates, inhibition curves showed RF_{90} values that were comparable to the corresponding RF_{50} values but with a larger variation.

Isolates 9 and 10 showed a significant resistance to adefovir and tenofovir, due to the mutation at rtA181V. An example of the adefovir inhibition curve is shown for sample 10 (Figure 3). The curve of the resistant mutant parallels the curve of the WT because their C-values (that is, slopes) are similar; thus the resistance is represented by the shift of the inhibition curve to higher concentrations, that is parameter B increases (Figure 2A and Table 2). In contrast, the antiviral resistance towards entecavir for isolates 1 to 8 does not only shift the curves to the right but also decreases the slope C of the curves (WT C-value ±SD of 1.77 ±0.35 versus C-values of 0.39 to 1.01 with a mean ±SD of 0.60 ±0.21 for samples 1 to 8). Importantly, parameter C is not in linear but in exponential relation to the concentrations of the drug used. Examples for the different inhibition curves generated by adefovir and entecavir are shown in Figure 3. While the inhibition curve of isolate 6 (rtL80I, rtM204I) also runs parallel to the curve of WT HBV for adefovir, the entecavir inhibition curve represents a much smaller decline with increasing drug levels. These findings were not limited to HuH7 cells, but were reproducible in another human liver cell line, HepG2 (Figure 3). The consequences of the smaller parameter C for the resistance pattern against entecavir are dramatic. The replication of WT HBV was completely (<1%) suppressed at 12 nM entecavir. With an RF_{50} of 19.9 for isolate 6, an entecavir concentration of 239 nM (RF_{50} × 12 nM) would inhibit HBV replication of this isolate to the detection limit of 1%, if the resistance would only depend on the increase of parameter B. However, we

Figure 3. Selected examples of inhibition curves of HBV replication *in vitro*



The inhibition curves of HBV strain 6 (right panel) and 10 (left panel) for adefovir (ADF) and entecavir (ETV) are shown (black) together with the inhibition curve of the corresponding wild type (WT; grey). The C-values for the WT (C_{WT}) and the strain (C_{Mut}) are also indicated. Experiments with ETV were done with HuH7 and HepG2 cells.

detected a relative HBV replication of 14% even at a concentration of 1,000 nM (Figure 3). According to the fitted dose–response curve, an entecavir concentration of >21,200 nM would be needed for >99% suppression of HBV replication.

Slope C in the entecavir inhibition curves is even less for the isolates 1 and 2 with two pre-existing exchanges (rtL180M and rtM204V). C was greater in those cases with the primary exchanges rtM204I ±rtL80I/V or rtM204I only. Importantly, in most samples harbouring an exchange at position rtM204, a replication above 10% was still detected even with entecavir concentrations 1,000× higher than the IC_{50} of the WT. An exception was isolate 7 that showed the steepest slope C with 3% residual replication. As expected, isolates 9 and 10 (rtA181V) showed no entecavir resistance and no change of parameters B and C (Figure 3, left side, Table 1). Consistent with the unaltered slope C in the resistance curve, treatment of isolates 9 and 10 with adefovir always resulted in complete inhibition of replication. As a control, relevant amino acid changes were also introduced into the wild-type sequence by site-directed mutagenesis (SDM) of the 1.1 expression construct (WT, Table 3). We observed a similar pattern of a decreased slope C for SDM-clones harbouring amino acid exchange at position M204 ±L80 or ±L180M for entecavir, including an unaltered slope C for the A181V variant. However, in contrast to the

Table 3. Phenotypic resistance data of cloned variants with defined amino acid changes obtained by site-directed mutagenesis of the consensus wild-type genome

Mutations in RT-domain	Entecavir						
	C	IC_{50}	IC_{90}	RF_{50}	RF_{90}	R^2	Sign
Wild type	0.96	1.2	11.3	1.0	1.0	1.00	NA
L180M, M204V	0.58	137.3	5,450.9	116.7	482.5	0.96	[a]
V173L, L180M, M204V	0.49	62.2	4,482.5	52.9	396.8	0.90	[a]
M204I	0.39	125.5	27,641.4	106.7	2,446.7	0.92	[a]
L80I, M204I	0.59	32.4	1,141.6	27.6	101.0	0.94	[a]
L180M, M204I	0.59	216.6	7,045.5	184.1	623.6	0.86	[a]
A181V	1.24	1.1	6.6	1.0	0.6	1.00	NS

The calculation of the slope C, 50% inhibitory concentration (IC_{50}), 90% inhibitory concentration (IC_{90}), respective resistance factor RF_{50} and RF_{90} is based on the regression model for the dose–response curves described in Figure 2. Relevant amino acid substitutions were introduced by site-directed mutagenesis of the wild-type consensus genome (genotype D). Measurement and calculation was done according to Table 2. The column 'Sign' indicates significant differences to WT ([a]$P<0.001$, Bonferroni corrected; NS, not significant; NA, not applicable).

isolates from patients, we did not observe an overall difference in slope C between clones harbouring M204I or M204V.

Discussion

Phenotypic resistance assays for HBV are not well standardized and limited by the problem of obtaining sufficiently accurate and sensitive measures of HBV replication (for review see [19]). A major problem is the distinction between the cloned input HBV DNA used for transfection of the hepatoma cells and the newly replicated HBV DNA. In this study, sensitivity and accuracy was improved by trypsin-treatment and washing of the cells after transfection and a quantitative real-time PCR of the cell culture supernatants which detects virion-associated HBV DNA over plasmid DNA with high selectivity. This allowed for detection of residual replication at levels lower than 1% of the uninhibited control and has very recently helped to clarify a described controversial resistance pattern against adefovir [20].

The resistance of a viral variant against a specific antiviral drug is usually expressed by the ratio between the IC_{50} of the WT and the mutant strain (Figure 2A) resulting in the resistance factor RF_{50}. For the prediction of clinical resistance it is generally assumed that resistance shifts the dose–response curve to higher drug concentrations on the x-axis, thus only changing parameter B (Figure 2A). This study using an improved phenotypic *in vitro* assay confirms in most cases the published resistance profiles based on IC_{50} values [12] (Table 1), but allowed in addition determination of IC_{90} and RF_{90} values. Remarkably, RF_{90} values against entecavir of samples with rtM204 substitutions were up to 30× higher than their RF_{50} values (Table 1). Thus, the antiviral potential of at least entecavir can no longer be predicted by the IC_{50} or RF_{50} value only, since the corresponding RF_{90} value can be unexpectedly high even if the RF_{50} is low as shown here (Figure 2B). The most intriguing consequence from the altered dose–response curve is that 10 to 15% residual replication of such mutants was detectable even at entecavir concentrations several magnitudes higher than the IC_{50} value. Although extrapolating to the *in vivo* situation should be carried out cautiously, in clinical practice, this residual replication capacity would allow the HBV variant to rapidly acquire additional mutations. This could result in complete entecavir resistance even in the presence of entecavir doses exceeding the therapeutic levels.

According to the '*Global policy report on the prevention and control of viral hepatitis*' by WHO in 2013 [21], lamivudine is still widely used, but less than half of WHO member states reported inclusion of drugs with superior efficacy, like entecavir and tenofovir as recommended key medications for treatment of hepatitis B. Usually, a treatment switch of lamivudine-resistant patients to tenofovir is recommended by many clinical guidelines worldwide. Unfortunately, among WHO member states that have currently reported inclusion of lamivudine and entecavir (but not tenofovir) in their national essential medicines list or subsidized by the government are those with a high burden of chronic hepatitis B, like Bulgaria, Jordan, Pakistan and the Russian Federation [21]. In this setting, our phenotypic assay for HBV variants would help to predict the danger of entecavir failure in lamivudine-experienced patients.

As noted above, the RF_{50} values are not helpful in calculating the risk of entecavir therapy failure. Instead, the steepness of the slope (C-value) of the dose–response can be regarded as a useful additional parameter, determining the relative risk factor for a rapid development of resistance to entecavir in patients with lamivudine resistance.

In this study a change of the slope was only seen in the case of entecavir for amino acid exchanges at

position rtM204 but not for the drugs adefovir and tenofovir based on the exchange rtA181V. One possible explanation for that could be the unusual inhibitory mechanism of entecavir. Most nucleoside/nucleotide analogues are efficiently incorporated by the RT into the growing DNA strand and terminate elongation because they lack a 3′-OH group. In contrast, entecavir contains a 3′-OH group but causes steric hindrance within the HBV polymerase during further dNTP incorporation steps and thus secondary chain termination [13]. This mode of action is unique for inhibitors of the HBV RT but shared with some other antiviral drugs, for example, the herpes simplex virus inhibiting penciclovir that also allows limited chain elongation [22]. This mechanism might provide a possible explanation for the very low IC_{50} of entecavir, 2 to 3 magnitudes below that of all other HBV RT inhibitors, but also for the leakiness of inhibition observed with several mutants in this study. Furthermore, entecavir triphosphate is an analogue to deoxyguanosin triphosphate (dGTP) and thus can compete with the protein-priming process of the HBV polymerase [23,24] in contrast to lamivudine. However, elongation after priming with dGTP involves addition of two deoxyadenosin-nucleotides (dATP) to complete the 3-nucleotide long viral minus-stranded DNA primer (dGAA) covalently attached to the terminal protein (TP) of the HBV polymerase [25]. While tenofovir, and probably also the closely related adefovir (two AMP-analogues) are able to interfere with generation of the dGAA-primer [24], we did not observe any change of the slope for the variant rtA181V (Table 2). This study casts some doubts on the reliability of certain previous resistance assays. Until now only IC_{50} was taken into account for the quantification of HBV resistance and identification of relevant resistance mutations. This method may be valid for the majority of variants, but it is insufficient to estimate the strength of relevant amino acid exchanges that alter not only the IC_{50} but also the slope of a resistance curve as was already described for tenofovir and the rtM184V mutation of the HIV reverse transcriptase [26].

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: Additional members of the HOPE consortium can be found at http://www.intmedpress.com/uploads/documents/3302_Geipel_Add_file_1.pdf

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