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Novel rtM204 Mutations in HBV Polymerase Confer Reduced Susceptibility to Adefovir and Tenofovir

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Abstract

Background: Treatment of chronic HBV-infection is limited by selection of resistance. The rtM204I/V mutations in the YMDD motif of HBV reverse transcriptase are well documented resistance determinants against lamivudine and entecavir, but not against adefovir or tenofovir. Limited systematic phenotypic data are available for the latter two drugs.

Methods: rtM204 mutations (rtM204A/I/K/L/Q/S/T/V) were systematically introduced into replication-competent 1.1-fold HBV-overlength constructs under control of a CMV promoter. Viral replication fitness was determined by selective qPCR after normalized transient transfection. *In vitro* drug susceptibilities were evaluated by determining IC50 values of lamividune, entecavir, adefovir, and tenofovir using standardized high-throughput phenotypic assays. Infectivity was analyzed by infection of HepaRG cells.

Results: *In vitro* phenotyping showed that rtM204K conferred high-level resistance to adefovir and tenofovir but simultaneously impaired replication capacity. Its fitness could not be restored by rtL180M or rtL80I as described for rtM204I/V. rtM204L and rtM204Q conferred low-level reduced susceptibility to adefovir/tenofovir without loss of replication capacity. rtM204A/I/S/T reduced susceptibility to either drug substantially. Interestingly, the single mutation rtM204V showed significantly reduced susceptibility to both drugs but lost resistance in combination with the compensatory mutation rtL180M. By affecting the overlapping S-gene, rtM204 mutants except rtM204L showed reduced or diminished infectivity in HepaRG cells.

Conclusions: We have established a time- and cost-effective phenotypic assay and identified novel rtM204 mutations conferring cross resistance to adefovir and tenofovir *in vitro*. Despite of their low frequency in the viral population, their clinical significance should not be underestimated due to the potential selection of compensatory mutations, which may restore viral fitness.

Keywords: Hepatitis B virus; Antiviral resistance; YMDD motif; HepaRG

Abbreviations:

HBV: Hepatitis B Virus; **cccDNA:** covalently closed circular DNA; **LAM:** Lamivudine; **ADV:** Adefovir, **ETV:** Entecavir; **TFV:** Tenofovir

Introduction

Despite a potent vaccine and the possibility to produce safe blood products, the human hepatitis B virus (HBV) remains a major medical and economic burden. It is assumed that 250 million humans are chronically infected with HBV, worldwide, and thus are at risk of developing severe liver damage and hepatocellular carcinoma in the course of the chronic inflammatory disease (www.who.int).

For nearly two decades treatment of HBV with potent antivirals has been practiced, with five approved reverse transcriptase inhibitors available to date, namely lamivudine, adefovir, entecavir, telbivudine, and tenofovir [1]. Unfortunately, each newly introduced antiviral drug also faces the problem of resistance against this drug. Moreover, cross resistance between different drugs can be frequently observed. Distinct patterns of mutations emerge which are located mainly in the postulated active center of the reverse transcriptase domain of the viral polymerase gene [2]. Since fewer drugs are available for the treatment of HBV than for the treatment of HIV, which is often referred to as a model for HBV resistance development regarding the viral reverse transcriptase, the mechanisms of resistance of HBV against antivirals don't reach the same level of complexity as for HIV [3-5].

An increasing number of clinical studies and case reports have indicated that some key motifs within the reverse transcriptase domain are most crucial for antiviral resistance. The most prominent motif is the so called YMDD motif, which is frequently involved in resistance against nucleoside and nucleotide analogues. In all previously published studies in which this motif was associated with drug resistance it conferred resistance (mainly against lamivudine and entecavir) which was based on an amino-acid exchange of the methionine [6-9]. Hitherto, no resistance against adefovir was associated with a mutation of this motif, except for the clinical observation in a pediatric patient carrying an YKDD motif and clinical Adefovir resistance [10]. Besides YKDD, YVDD and YIDD motifs further mutation patterns observed were YSDD (M204S), YADD, YQDD, YTDD, and YLDD [11,12].

In order to address the hypothesis that YMDD mutations also mediate resistance against antivirals other than lamivudine we therefore analyzed the *in vitro* resistance pattern of YMDD mutations systematically for the first time and tested the replicative fitness of HBV strains bearing these mutations.

Material and Methods

Mutagenesis

The site-directed mutagenesis protocol was adapted from the QuikChange (Agilent) strategy to generate point mutations. The primers, containing the desired mutations as listed in Table 1, were extended during temperature cycling by Phusion Hot Start High-Fidelity Polymerase. The PCR products were first digested by Dpn I and then transformed into competent E. coli strain STBL3. The plasmids containing the mutated sites were then verified by sequencing.

HBV *in vitro* replication and antiviral susceptibility assay

HuH7 cells were plated into 10 cm dishes at 2.5×10^6 cells/dish. Approximately 24 h post plating, cells were co-transfected at >90% confluency with 8 µg plasmid DNA (7 µg pCH-HBV plus 1 µg pSEAP) using 24 µl FuGENE HD transfection reagent according to the manufacturer's protocol. The following day, transfected cells were separated by trypsinization and washed twice by centrifugation at 300 ×g for 3 min and resuspension with washing medium. Finally the cells were resuspended in reseeding medium (containing 5% FCS and 1% DMSO) and seeded into a 96-well plate at a concentration of 1.5×10^4 cells/well in a volume of 110 µl. After a brief centrifugation of the plate followed by incubation at 37°C for 1-2 h, the cells were fed with 110 µl/well prepared reseeding medium containing the 5 serial dilutions of each drug for treatment in triplicates (same reseeding medium without drugs as non-treatment control). 72 h after treatment, the supernatants were collected and centrifuged at 500 g for 5 min to remove the dead cells and transferred to a new plate for immediate DNA extraction or short-term storage at -20°C. For each complete phenotypic testing with 4 drugs, a total of 64 supernatants were obtained and submitted for HBV DNA extraction. A protocol for high-throughput DNA extraction using BioSprint 96 One-For-All Vet kit was optimized on Tecan's robotic platform Freedom EVO 100, with a performance comparable to that of the standard spin column-based method. The extracted DNA was then subjected to real-time qPCR for HBV DNA quantification in the supernatants, using the selective primers to distinguish HBV rcDNA from input plasmid. Each PCR well contained the following: 10 µl LightCycler 480 SYBR Green I Master, 1 µl selective forward primer (20 µM), 1 µl selective reverse primer (20 µM) and 8 µl extracted HBV DNA in a final volume of 20 µl. The qPCR was conducted on LightCycler 480 II with a sequence of conditions as: denaturation at 95°C for 5 min, followed by 40 amplification cycles of 95°C for 15, 60°C for 15 s and 72°C for 20 s, ended with a standard melting curve procedure. HBV DNA copy numbers were calculated based on a serial dilution of calibrated HBV DNA standard. The average values from 3 replicate wells at each drug concentration were used to calculate the 50% inhibitory concentration (IC50) based on dose response equations.

Phenotypic resistance assay

HuH7 cells were seeded into 10 cm^2 dishes at 1.5×10^6 cells/dish, reaching around 90% confluency before transfection the following day. Cells were transfected using FuGENE®HD (Roche, IN, USA) reagent with HBV expressing plasmid. 16-20 h post-transfection, transfected cells were washed twice and then seeded into a 96-well plate at 3×10^4 cells/well. The cells were treated with serial dilutions of four drugs in fresh medium for 3 days, including lamivudine, adefovir, entecavir and tenofovir (Sequoia Research Products Limited, UK). The supernatants were collected after centrifugation at 1500 ×g for 5 min, and then prepared for HBV DNA extraction using the BioSprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany). The extracted DNA was then subjected to real-time qPCR for HBV DNA quantification in the supernatants. Half maximal inhibitory concentrations (IC_{50}) were calculated for each construct where the resistance factor is calculated as the IC50 of mutant divided by the IC_{50} of the wt strain.

Analysis of HBV replication intermediates

HBeAg and HBsAg loads were determined using commercial immunoassays (Siemens Molecular Diagnostics, Marburg and Abbott Laboratories, Wiesbaden, Germany). Total DNA was purified from infected cells using DNA MiniElute Kit (Qiagen, Hilden, Germany). Total RNA was extracted from infected cells and transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA). HBV-DNA, pgRNA and cccDNA were detected using specific PCR primers. HBV-DNA was quantified relative to an external plasmid standard. cccDNA is quantified as normalized ratio to mitochondrial DNA, pgRNA as normalized ratio to GAPDH as previously described. Real-time PCR (qPCR) runs were performed using the LightCycler™ system (a dilution series of a calibrator was included in each PCR run) and analyzed using the second-derivative maximum method that includes both normalization to the reference gene (mitochondrial DNA for cccDNA and GAPDH for pgRNA, HNF4a and TDO) and to primer efficiency (Roche Diagnostics, Mannheim, Germany).

Production of wild-type and variant rHBV, and analysis of the infectivity *in vitro*

For production of rHBV and variant HBV, HuH7 cells were transfected with pCH-9/3091 plasmid or its derivative/s containing specific mutations or isolates in the HBV genome. Transfection was performed in 10-cm cell culture dishes at 90% cell confluence using 8 µg plasmid DNA plus 24 µl Fugene HD transfection reagent, as described before. 16-18 h post transfection, cells were washed with DMEM wash medium and fed with DMEM seeding medium (containing 5% FCS and 1% DMSO). Every two days, the virus-containing medium was collected and cell debris was removed by centrifugation at 1000 rpm for 10 min. The supernatant that contained

the viral particles was purified through Heparin HP column (GE Healthcare) and then concentrated by centrifugal filter device (Centricon Plus-70, Biomax 100, Millipore Corp.). The virus concentrates were supplemented with 10% glycerol and stored at -80°C. The titer of the produced HBV was determined as enveloped DNA-containing viral particles by dot blot analysis following a CsCl density-gradient as outlined before, or alternatively, by real time PCR quantification.

Infection of differentiated HepaRG cells with HBV was performed as described before. The preparation of differentiated HepaRG cells was performed in 12-well plate format and took 4 weeks. For the first 2 weeks, the cells were maintained in standard growth medium with medium exchange twice per week. Then the cells were maintained in differentiation medium for another 2 weeks with medium exchange twice per week as well until ready. For infection, a viral dose of 200 MOI (or viral genome equivalent per cell) was diluted with differentiation medium to a final volume 500 µl/well containing additional 5% PEG, and then applied to the cells. The incubation for infection lasted overnight and subsequently the cells were washed with PBS for 3 times and then fed with fresh differentiation medium. 8-10 days after infection, HBV markers including HBeAg, rcDNA in the supernatant and intercellular cccDNA were measured respectively to characterize the infection.

Results

In vitro phenotyping of HBV rtM204 mutations

Based on clinical observations [10] we hypothesized that mutations at amino-acid position 204 in the viral reverse transcriptase domain were associated with resistance not only to lamivudine and entecavir but also to other antiviral drugs used for therapy of chronic HBV infections (Table 1).

RT Mutation	Treatment	Patient status	Corresponding HBsAg mutation	Associated RT mutation	Reference
rtM204V	LAM | ADV	Resistance	sI195M	rtL180M	[15,16]
	TDF	Partial response			[17]
rtM204I	LAM | ADV	Resistance	sW196L	rtL180M/-	[16,18]
	TDF	Partial response			[17]
rtM204A	ADV	Resistance	sI195M +sW196R	-	[19]
rtM204K	LAM | ADV	No response	sW196R	-	[10]
rtM204L	TDF	Partial response	-	-	[20]
rtM204S	LAM	Resistance	sW196V	rtL180M	[21]
rtM204Q	ADV | TDF	Partial response	sW196R	rtA181T/-	[22]
rtM204T	ADV	Resistance	sW196R	rtI169T	[23]

-, none; |, treatment switch.

Table 1: rtM204 mutations identified in literature mining

Therefore, *in vitro* phenotyping was performed systematically for the mutants M204V, M204I, M204 S, M204L, M204A, M204Q, M204T, and M204K Introduced into a genotype D HBV genome. As the mutant M204K was expected to confer resistance to adefovir a characterized adefovir resistant mutant (N236T) as well as the wild type were used as controls. Antiviral drugs used for this screening were lamivudine (LAM), entecavir (ETV), adefovir (ADV), and tenofovir (TDF). In the first instance the inhibitory concentration 50 (IC50) and the factor of antiviral resistance (FR) were determined. The results are given in Table 2 As expected, M204V/I displayed a 45-fold to more than 100-fold resistance to lamivudine, whilst mutants M204S/L/A and M204T showed 10- to 2- fold resistance against this drug.

Phenotypic results of rtM204 variants

By site-direct mutagenesis, all M204 mutations were introduced into the WT construct. Then all the variants were phenotyped and the results are summarized in this table. It was noticed that the mutation M204K confers high level resistance to ADV and TDF with a resistance factor exceeding. For comparison, the typical ADV resistance mutation N236T only confers a resistance factor around 4-5.

Surprisingly, M204Q and M204K have an 80-fold and 500-fold higher resistances compared to the wild type, respectively. Resistance against Entecavir was highest for M204I and moderate for all other mutants ranging from resistance compared to the wild type. Similar results were obtained for phenotypic resistance against ADF and TDF which was moderate for all tested mutants (RF 1.3-10) but which was strikingly high for the M204K variant with resistance factor above the measurable nontoxic dosage of both drugs (Table 2).

Recombinant	LAM		ETV		ADV		TDF	
	IC50	RF	IC50	RF	IC50	RF	IC50	RF
WT	0.1	1.0	0.003	1.0	1.3	1.0	0.6	1.0
rtM204I	>100	>1000	0.1	30	2.0	1.5	1.1	1.8
rtM204V	4.5	45	0.005	1.6	6.5	5.0	6.0	10
rtM204S	1	10	0.003	1.0	2.6	2.0	2.4	4.0
rtM204A	1	10	0.003	1.0	1.6	1.2	0.8	1.3
rtM204T	2.0	20	0.003	1.0	2.0	1.5	1.8	3.0
rtM204Q	8.0	80	0.003	1.0	2.5	1.8	2.5	4.0
rtM204L	1.0	10	0.002	0.5	5.2	4.0	4.5	7.5
rtM204K	50	500	0.005	1.8	>128	>100	>64	>100

RF, resistance factor; IC50 in µM

Table 2: Summary of *In vitro* drug susceptibilities of tested rtM204 variants.

This latter effect of multiresistance conferred by the single point mutation resulting in rtM204K is depicted more in detail in Figure 1. Dose-relation curves showing the viral replication in relation to increasing concentrations of antiviral drugs are presented for LAM,

ADF, ETC, and TDF, respectively. Even with high doses no significant inhibition of viral replication was observed whenever TDF and ADF were added to the cell culture medium.

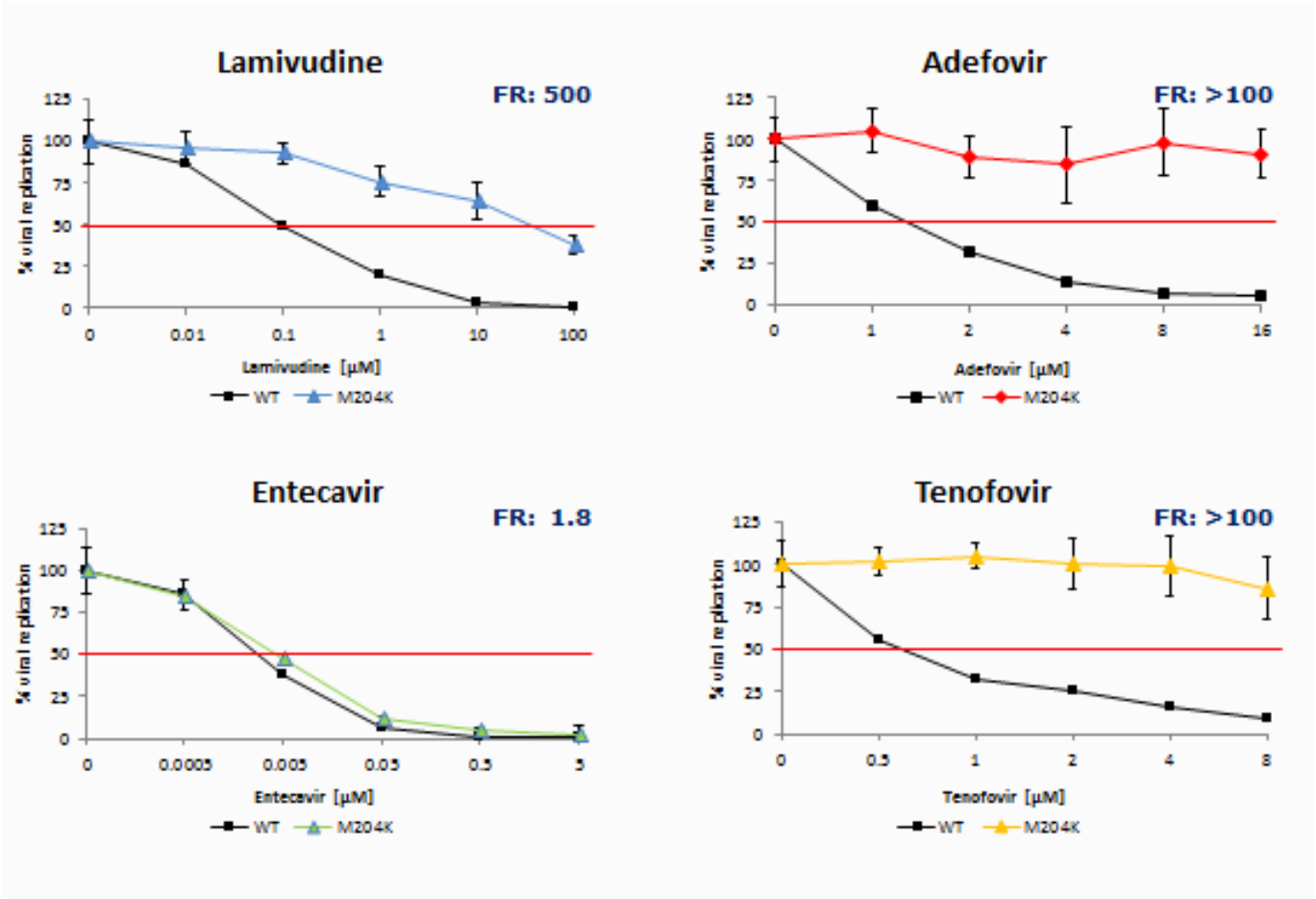

Figure 1: *In vitro* susceptibility of the rtM204K variant to four nucleos(t)ide analogues in clinical use. Huh-7 cells were transfected with wild-type (WT) or rtM204K variant HBV plasmid and treated with lamivudine, adefovir, entecavir or tenofovir in increasing concentrations as indicated. HBV DNA concentration was measured in cell culture supernatants by a selective real-time PCR and normalized for transfection efficiency to the secreted alkaline phosphatase activity to determine HBV production. HBV DNA production is indicated relative to the level of HBV DNA in the absence of drug (mean ± SD). Dose–inhibition curves for the two HBV strains were used to estimate IC50 values for each drug.

Replication capacity of rtM204 variants

In order to evaluate the effect of the rtM204 mutations on the replication capacity, normalized transient transfections in HuH-7 cells were performed. The normalization was performed relatively to the replication of the wild type strain which was set to 100% (Figure 2).

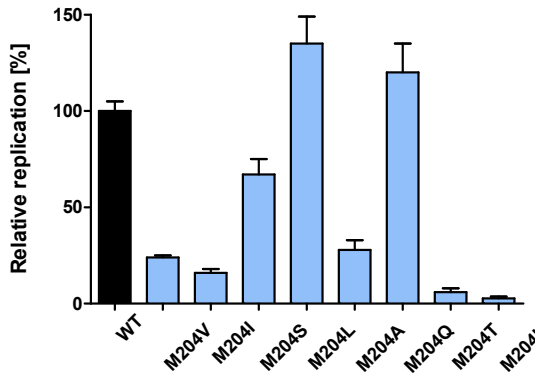

Figure 2: Replication capacity of HBV rtM204 polymerase variants. Huh-7 cells were transfected with wild-type (WT) or indicated rtM204 variant HBV plasmids. HBV replication was determined by measuring HBV DNA concentration in cell culture supernatants by a selective real-time PCR. Values were normalized for transfection efficiency to secreted alkaline phosphatase activity to determine HBV production. HBV DNA production is indicated as replication relative to the level of HBV WT. Mean ± SD of three independent transfections is shown. While rtM204K and most other variants impair the viral replication capacity, rtM204L and rtM204Q even slightly increase it.

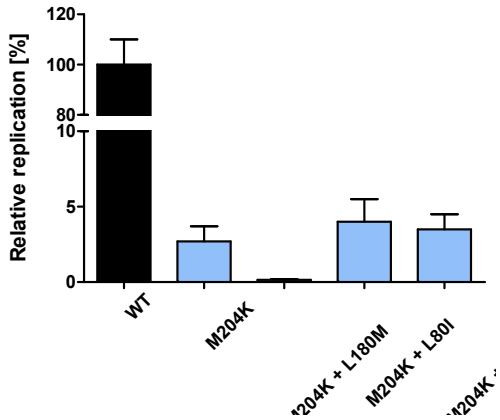

Figure 3: Replication defect of rtM204K variant HBV is not restored by compensatory mutations rtL180M, rtL80I or rtA181T. Huh-7 cells were transfected with plasmids expressing wild-type (WT) or rtM204K variant HBV or HBV containing rtM204K variant and either rtL180M, rtL80I or rtA181T mutations. HBV replication was determined by measuring HBV DNA concentration in cell culture supernatants by a selective real-time PCR. Values were normalized for transfection efficiency to secreted alkaline phosphatase activity to determine HBV replication. HBV replication is indicated relative to the level of HBV WT. Mean ± SD of three independent transfections is shown. None of the potentially compensatory mutations could restore replication capacity of rtM204K.

Remarkably, it was observed that mutation M204K (Figure 3) strongly impaired viral replication capacity, while M204L and M204Q even slightly increased it. The remaining variants also displayed a significant reduction in their replication capacity although the effect was less pronounced than in case of the M204K mutation.

Influence of putative compensatory mutations on the replication capacity of M204K

Having observed the impaired replication capacity of the M204K variant the question was addressed to what extent compensatory mutations are able to restore the replication fitness of the M204K variant. It was observed that neither the mutation L80I nor the mutation A181T were able to increase the viral replication capacity. Interestingly the mutation L180M led to a further impairment of the viral replication (Figure 4).

HBsAg and virion secretion in rtM204 variants

Taking into account that the rtM204 mutation may have a negative effect on the integrity of the three-dimensional protein structure of HBsAg which is encoded by an overlapping reading frame and which is responsible for proper secretion of itself and viral particles, the question was addressed which effect the rtM204 mutations have on the secretion and release of infectious particles.

As shown in Figures 4 and 5 no significant impairment on the HBsAg secretion was observed for the variants M204V, M204I, M204S, and M204L, respectively, whilst the secretion of HBsAg was dramatically reduced by the mutations rtM204A, rtM204Q, rtM204T, and M204K all of which lead to the sW196R mutation in the HBs antigen (Figure 4a). The fact that rtM204K is an isolated variant from a single patient we further analyzed if this variant can be "rescued" by simultaneous occurrence of wild-type genomes (Figure 4b). Indeed, although a strong inhibitory effect of the mutation rtM204K/sW196 on the secretion of both HBsAg and virions was observed, in presence of wt DNA there remains a secretion of HBsAg and viral particles. Thus, in the relevant patient who will have multiple HBV genome copies within an infected liver cell the mutation could be compensated and enable replication also of wild-type genomes thus leading to resistance against adefovir and tenofovir *in vivo* (Figure 4b).

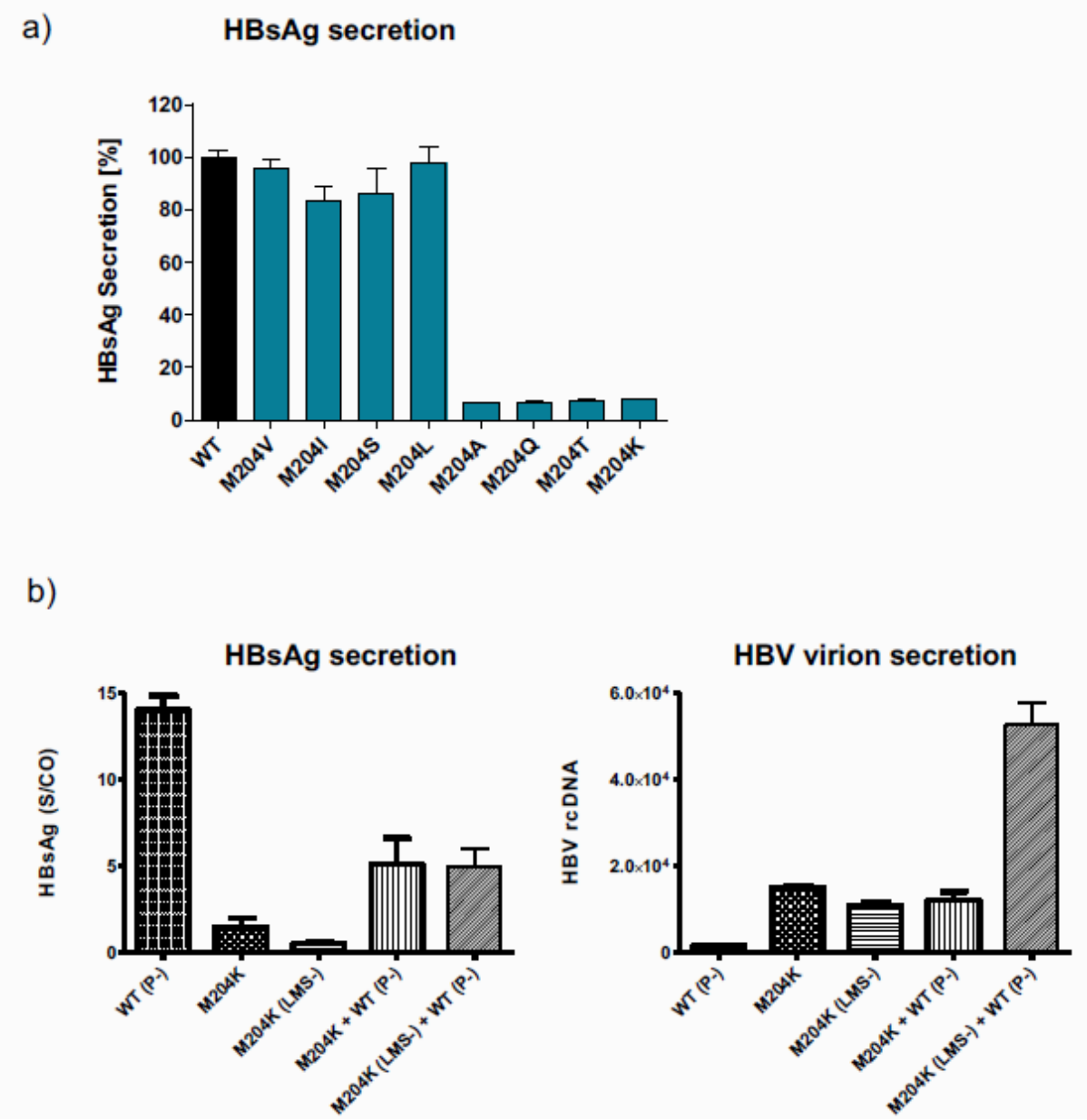

Figure 4 (a): HBsAg and virion secretion of different HBV rtM204 variants. rtM204 variant HBV constructs were transfected into Huh-7 cells and HBsAg was measured in the supernatant using an immune based HBsAg assay (AxSYM, Abbott). **(b):** Supernatants were collected from Huh-7 cells transfected with a WT HBV in which the viral polymerase was knocked out (P-), rtM204K or an HBsAg-deficient rtM204K variant HBV (LMS-), and both rtM204K variant constructs co-transfected with WT (P-) in a 1:1 ratio. Supernatants were collected and submitted to density gradient centrifugation. HBsAg and HBV DNA were measured by immunoassay or qPCR, respectively. Mean ± SD from triplicate transfection.

Infectivity of rtM204 variants on HepaRG cells

To test how far the rtM204 variants would diminish infectivity of resulting HBV, we performed infection experiments. In an *in vitro* infectivity assay based on the usage of HepaRG cells it was shown that most variants of rtM204 have a severely reduced infectivity with respect to intra- and extracellular viral DNA, cccDNA, and HBeAg measured 10 days post inoculation. The only exception observed was rtM204L, whose infectivity was comparable to that of the wild type variant with the tendency to produce more intracellular viral rcDNA than wt (Figure 5).

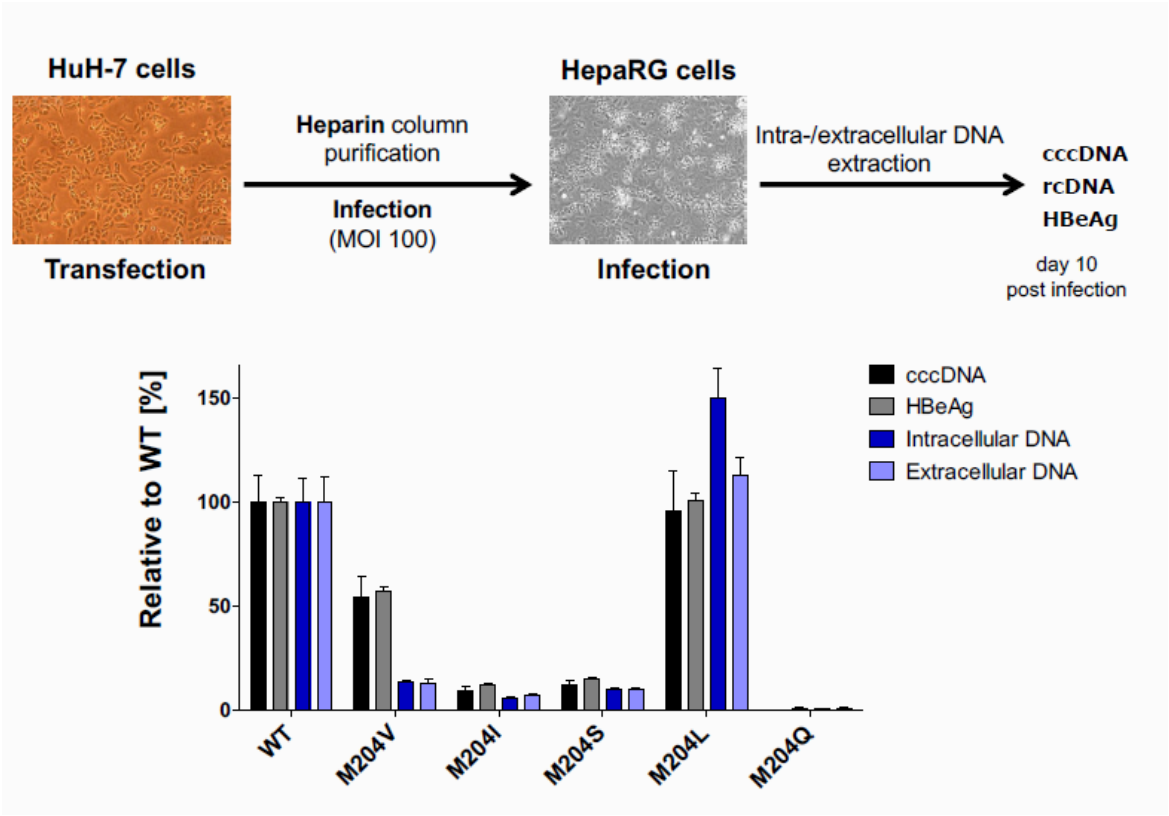

Figure 5: Infectivity of rtM204 variants on HepaRG cells. HuH-7 cells were transfected with different rtM204 variant HBV constructs, cell culture medium was collected and secreted virions were purified by heparin affinity column chromatography. Virion were then concentrated by ultrafiltration to allow infection of differentiated HepaRG cells at a multiplicity of infection (moi) of 100 virions / cell. 10 days after infection of HepaRG cells, total intra- and extracellular was extracted and total HBV-DNA as well as nuclear HBV cccDNA were determined by qPCR. Secreted HBeAg was measured by immune based assay (BEP, Siemens). HBV infection markers for each variant are given relative to that determined in parallel WT HBV infection.

Discussion

This study comprises the first systematic evaluation and comparison of rtM204 mutations in an *in vitro* setting. This allows the direct comparison of the resistance factors in a single system, for the first time.

We were able to confirm previously known resistances such as the "classical" YVDD and YIDD motif leading to lamivudine resistance. More interestingly we could clearly demonstrate that novel variants such as rtM204K display a dramatically high resistance against adefovir and cross resistances to tenofovir, although this variant leads to seriously impaired viral fitness. Due to the fact that this mutation was initially observed as a variant that occurred in a clinical case it may have indeed contributed to the development of clinical resistance despite of its reduced secretion fitness. We hypothesize that in cases where this mutation is observed liver cells doubly infected with wild type and the rtM204K variant are able to produce and secrete infectious viral particles and cross-rescue each other. Specifically, the wild type HBsAg can be produced and will be used in cis and in trans, whilst the mutated viral reverse transcriptase is used in trans, as the wild type molecule is blocked by antiviral drugs. We have shown that the defect of these variants regarding secretion of viral particle results in intracellular retention of surface proteins and has a dominant negative effect on WT virion secretion, resulting in lower extracellular viral loads hindering their detection. It is also possible that during HBV replication these mutated proteins cause cell stress and damage by their intracellular retention which might induce the unfolded protein response via ER stress, which in hepatocytes can lead to HCC, as reported for mutation rtA181T/sW172 [13,14].

In the future, similar systematic studies and testing for antiviral drugs are required. In these studies it should be analyzed if mutated variants are be present as minor populations in advance to antiviral therapies and thus could influence the outcome of the antiviral therapy in order to predict the clinical outcome. Moreover, systematic testing is required as it will enable to predict cross-resistances and thus avoid expensive and unsuccessful therapies. Finally, it is crucial to analyze if the variants are playing a significant role in clinical settings or if they are "rare flowers" in the jungle of antiviral resistances.

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