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The amino acid substitutions rtP177G and rtF249A in the reverse transcriptase domain of hepatitis B virus polymerase reduce the susceptibility to tenofovir

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ABSTRACT

Long term antiviral therapy with nucleoside/nucleotide analogs have been routinely used to treat chronic hepatitis B virus (HBV) infection but may lead to the emergence of drug-resistant viral mutants. However, the HBV resistance mutations for tenofovir (TDF) remain controversial. It is speculated that the genetic barrier for TDF resistance may be high for HBV. We asked whether selected amino acid substitutions in HBV polymerase may reduce susceptibility to TDF. A series of amino acids in HBV polymerase were selected based on bioinformatics analysis for mutagenesis. The replication competence and susceptibility to TDF of the mutated HBV clones were determined both *in vitro* and *in vivo*. nineteen mutations in HBV polymerase were included and impaired the replication competence of HBV genome in different degrees. The mutations at rtL77F (sS69C), rtF88L (sF80Y), and rtP177G (sR169G) also significantly affected HBsAg expression. The HBV mutants with rtP177G and rtF249A were found to have reduced susceptibility to TDF *in vitro* with a resistance index of 2.53 and 12.16, respectively. The testing in *in vivo* model based on the hydrodynamic injection revealed the antiviral effect of TDF against wild type and mutated HBV genomes and confirmed the reduced the susceptibility of mutant HBV to TDF.

1. Introduction

Patients with chronic hepatitis B (CHB) have been successfully treated with interferon and nucleoside/nucleotide analogs including lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF) (Zoulim and Locarnini, 2009). The nucleoside/nucleotide analogs inhibit reverse transcription of the hepatitis B virus (HBV) polymerase, but do not directly interfere with the formation of covalently closed circular DNA (cccDNA). Therefore, long term antiviral therapy is necessary, which usually induces the emergence and selection of drug-resistant mutations in the viral polymerase (Zoulim and Locarnini, 2009). Since the HBV polymerase gene is overlapped by the surface protein gene, the mutations in HBV polymerase may also result in amino acid (aa) substitutions in HBsAg that potentially result in immune escape or modification of viral fitness (Torresi, 2002; Villet et al., 2009).

Several HBV polymerase gene mutations have been reported to account for drug resistance. In particular, mutations at rtM204I or rtM204V in the YMDD motif within the reverse transcript (RT) domain of the HBV polymerase lead to LMV and LdT resistance (Brunelle et al., 2005). These mutations are usually accompanied by compensatory mutations of rtL180M and/or rtV173L which restore HBV replication capacity (Pallier et al., 2006). In addition to the substitutions at position rt204, a combination of mutations in the B, C, or D domain of HBV-RT could result in resistance to ETV (Zoulim and Locarnini, 2009).

TDF is widely used to treat HBV patients in the US and Europe. To date, only a few aa substitutions like rtA194T, rtV214A, and rtQ215S associated with TDF resistance have been reported and still need to be further confirmed (Liu et al., 2009; Zoulim and Locarnini, 2009). It has been shown rtA181V+rtN236T double mutants are resistant to TDF *in vitro*; however, clinical data suggested patients with rtA181 or rtN236T remain susceptible to TDF (Qi et al., 2007). On the other hand, TDF, as a first-line antiretroviral drug for human immunodeficiency virus (HIV) since 2001 (Soler-Palacin et al., 2011), does induce resistant mutations in HIV RT with the K65R mutation of HIV RT reducing TDF susceptibility about 2-fold

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(Whitcomb et al., 2003). Substitution K70E within HIV-RT was selected in HIV patients with virological failure under TDF therapy (Delaugerre et al., 2008). Co-mutations M41L, L210W, and T215Y reduce TDF susceptibility about 4-fold. However, one or two of them retain partial TDF susceptibility (Miller et al., 2004). The crystal structures of two related complexes of HIV-1 RT with template primer and TDF were determined (Tuske et al., 2004). The availability of HIV RT structural information makes it possible to determine the relative position of the aa residues with respect to the active center of the enzyme as well as the distance to the substrate if TDF is used for the modeling.

In the present study, we used another strategy to assess the potential of HBV to develop TDF resistance. Given the homology between HIV RT and HBV polymerases (Bartholomeusz et al., 2004), we aligned their primary aa sequences and identified corresponding positions of aa residues in HBV polymerase and HIV RT. Based on this alignment, the distances of a given aa residue in HBV polymerase to the TDF substrate could be estimated by comparison with HIV RT. Therefore we selected a number of aa residues according to their distances to TDF, predicted on the basis of a bioinformatics approach. We assumed that an aa substitution at a given position, especially with a change to an aa residue with a large or complex side chain, may influence the binding of the natural substrates and nucleoside/nucleotide analogs and leads to changed replication competence and nucleoside/nucleotide analogs resistance of mutant HBV genomes in some cases. To test this hypothesis, a series of replication-competent HBV constructs harboring different mutations were then constructed according the predictions. The replication capacity and resistance phenotype were analyzed both *in vitro* and *in vivo*. The results demonstrated that two mutations, rtP177G and rtF249A, significantly reduced HBV susceptibility to TDF and could be potentially used as a reference for TDF-resistance. Interestingly, the positions rtP177 and rtF249 may be not in direct contact with TDF molecule but make contacts to the template and primer oligonucleotides, respectively. Our approach could generally contribute to the understanding of HBV drug resistance.

2. Materials and methods

2.1. Plasmid constructs

For construction of HBV mutant plasmids with aa substitutions, the plasmid pHBV1.3 containing a replication-competent wild-type (WT) HBV 1.3-fold over-length genotype A genome (GenBank accession No. U95551, ayw) was used as a backbone (Gan et al., 1987; Lei et al., 2006). Mutations were introduced into the HBV polymerase gene by PCR-based mutagenesis using the primer pairs Primer-F, Primer-R and primers with the specific mutations listed in Table S1. Plasmids pHBV1.3-rtXs (X stands for the mutation, for example "P177G") were produced and the aa substitutions are presented in Fig. 1B. For *in vivo* assay, pAAV-HBV-1.3 and pAAV-HBV1.3-rtXs were constructed based on plasmid pHBV1.3 and pHBV1.3-rtXs respectively. The plasmids pHBV1.3/pHBV1.3-rtXs and pAAV were digested by Sac I, then end-filled with T4 and Klenow DNA polymerase, respectively. The recovered products were digested by HindIII and ligated by T4 ligase to generate pAAV-HBV-1.3 or pAAV-HBV1.3-rtXs.

2.2. Cells and mice

Hepatoma cell line Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM/L of glutamine, 100 IU/mL of penicillin and 100 IU/mL of streptomycin at 37 °C in a 5% CO_2 atmosphere. Female BALB/c mice (6–8 weeks of age; H-2^d) and female C57BL/6 (6–8 weeks of age; H-2^b) were held under specific pathogen-free conditions in the Central Animal Laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences and were handled following the guidelines of the animal ethical standards (Meng et al., 2008).

2.3. Nucleoside analogs

Lamivudine (LMV) (Glaxo Smith Kline), adefovir dipivoxil (ADV) (Gilead Sciences), entecavir (ETV) (Bristol-Myers Squibb Co), telbivudine (LdT) (Novartis), and tenofovir (TDF) (Gilead Sciences) were dissolved in appropriate solution according to the manufactures' instructions and used at the indicated concentrations.

2.4. Enzyme-linked immunosorbent assay (ELISA)

HBsAg and HBeAg in mouse sera and culture supernatants of Huh7 cells transfected with the plasmids pHBV1.3 and pHBV1.3-rtXs were detected by using a commercial routine diagnostic assay for HBsAg and HBeAg (Kehua, Shanghai) according to the manufacturer's instructions.

2.5. Western blot analysis

Huh7 cells transfected with indicated plasmids were harvested at 72 hour post transfection (hpt). The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). Total cell lysates (50 μg) were submitted to Western-blot assay by probing with anti-HBcAg antibody (Dako) and anti-β-actin (Beyotime), with appropriate secondary antibody. Densitometry assays were performed simultaneously.

2.6. Immunohistochemical (IHC) staining

Liver tissue was collected from mice sacrificed at the indicated time points. Intrahepatic HBcAg was detected by IHC staining of formalin-fixed paraffin-embedded liver tissue sections using rabbit anti-HBc antibodies (DAKO) with appropriate HRP-conjugated secondary antibody, and visualized by the Envision System (Huang et al., 2006). The liver sections were also stained with hematoxylin and eosin.

2.7. Detection of encapsidated HBV DNA

Huh7 cells (1×10^6) were transfected with 2 μg of the plasmids pHBV1.3 and pHBV1.3-rtXs by using lipofectamine 2000 (Invitrogen) and cultured in the presence of the different nucleoside/nucleotide analogs at the indicated concentrations. To control the transfection efficiency, a SEAP expression vector was cotransfected to monitor the transfection efficiency. Encapsidated HBV replicative intermediates were purified and subjected to Southern blot analysis as described previously (Meng et al., 2008; Qiu et al., 2011).

HBV DNA was quantified by real-time PCR using the primers (RC-sense and RC-antisense, Table S1) specially designed for the detection of HBV relaxed circular (rc) genomes in Sybr green reaction mix (Roche). Plasmid pAAV-HBV1.3 was used as a standard. All samples were analyzed in triplicate.

2.8. HBV challenge by hydrodynamic injection (HI) in mice

C57BL/6 mice were challenged by hydrodynamic injection of replication-competent pAAV-HBV1.3 and pAAV-HBV-rtXs respectively, as described previously (Huang et al., 2006). Ten micrograms of each plasmid in a volume of 0.9% NaCl solution

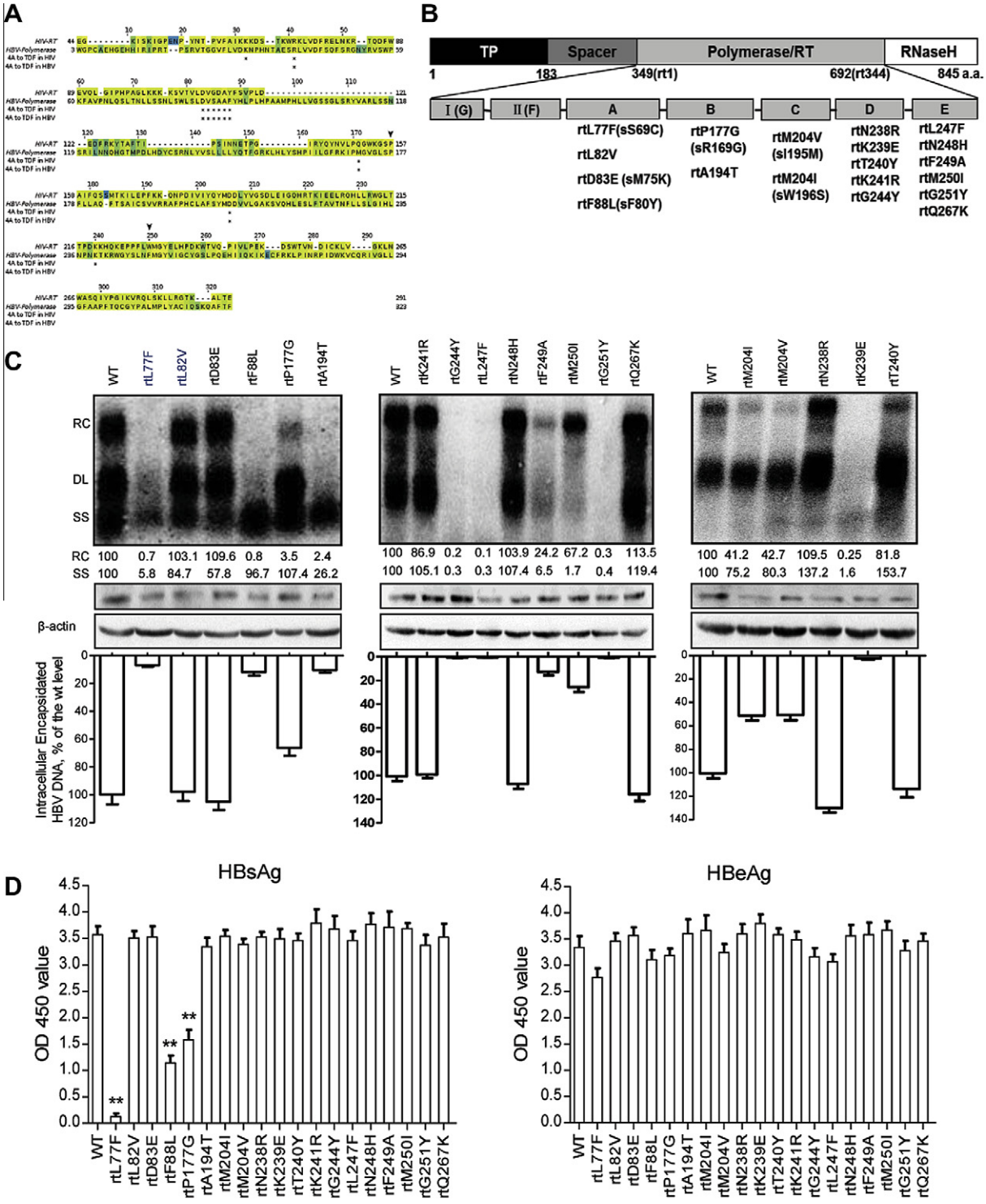

Fig. 1. (A) Alignment of amino acid sequences of HBV-RT genotypes A–H and HIV-RT. Coloring indicates sequence conservation across all nine sequences (see Section 2). For clarity, only the HBV-RT wild type sequence (V01460.1) is shown together with the HIV-RT sequence. The two sites P177 and F249 where TDF resistance mutations have been found in HBV-RT are marked by arrowheads, and asterisks mark residues with distances of up to 4 Å from any heavy atom of TDF in the X-ray structure of HIV-RT or the corresponding homology model of HBV-RT. (B) Structure of HBV reverse transcriptase and location of substitution sites. HBV P protein is divided into four regions, terminal protein, spacer, reverse transcriptase and RNaseH. Reverse transcriptase is subdivided into seven regions, G, F, A, B, C, D, and E. All the amino acid substitutions examined in this present study are located in A–E. (C) Detection of HBV replication intermediates by Southern blot. HBV replication intermediates relaxed circular (RC), double stranded linear (DL) and single stranded (SS) HBV DNAs are indicated (upper panel). The HBcAg was detected using Western blot with mouse polyclonal antibody (Dako, Carpinteria, CA). Beta-actin was used as a loading control (Middle panel). The intensity of RC and SS form of HBV replication intermediates were analyzed and compared with the wild type (set as 100, the numbers below the Southern blot). Intracellular encapsidated HBV DNA levels of each construct were compared with that of wild type genome (set as 100%, lower panel). (D) The expression of HBsAg and HBeAg were measured by commercial ELISAs (Kehua, Shanghai). Each value is the mean of three independent experiments. The error bars represent the standard deviation. Statistically significant differences between the groups are displayed as $^{*}(p < 0.05)$ or $^{**}(p < 0.01)$.

equivalent to 0.08 mL/g of the mouse body weight were injected into the tail veins of mice within 8 s. After 1 day post hydrodynamic injection (dphi), every mouse of the treatment group was treated with 500 μg TDF daily through intraperitoneal injection.

2.9. Multiple alignment of HIV RT and HBV polymerase sequences

Sequences were taken from GenBank, and translated from nucleotide to amino acid sequences using UGENE. For pairwise sequence alignments, the EMBOSS program "water" was used. Multiple sequence alignments were performed with T-COFFEE (Taly et al., 2011) and MUSCLE (Edgar, 2004). In all alignment procedures, default settings were employed. A homology model of HBV-RT, the reverse transcriptase domain of HBV polymerase, based on the complex of HIV-RT with TDF (PDB entry 1T03:B), and the mentioned multiple sequence alignment was prepared with modeller (Eswar et al., 2008) and visualized with pymol (Bramucci et al., 2012). Figures showing alignment and homology model, respectively, were consistently color-coded according to the degree of sequence conservation in each column of the alignment from yellow (=100% identity, i.e. complete conservation in column) to blue (=13% identity, i.e. all residues in column different). This sequence conservation was computed with R-package bio3d (Grant et al., 2007).

2.10. Statistical analysis

The statistical analysis was carried out using GraphPad (GraphPad Software). Differences in multiple comparisons were determined for statistical significance using the Student's *t*-test. $p < 0.05$ was considered as statistically significant. Results were presented as Means ± S.D.

3. Results

3.1. The estimation of the distances of the aa residues of HBV polymerase to TDF bound to the active center

So far, TDF-resistance-associated aa substitutions of HBV-RT remain elusive and controversial. Therefore, we attempted to answer the question whether specific aa substitutions may lead to a reduced susceptibility of HBV to TDF.

Sequences of HBV-RT of genotypes A through H and HIV-RT were submitted to pairwise and multiple sequence alignments (Fig. 1A). The alignment of the RT domains of both enzymes as shown in Fig. 1A was essentially the same for all alignment algorithms used. In particular the catalytic YMDD motif and several positions known to interact with TDF in HIV-RT were conserved between both enzymes. Based on the sequence alignment of HBV polymerase (GenBank accession No. CAA48354.1) and HIV-RT (HIV-1 polymerase protein gene, GenBank accession No. HQ718313), 19 aa residues in the HBV polymerase were selected to test their potential role in the development of TDF-resistance. These aa residues are distributed in the RT domains A, B, C, D, and E of the HBV polymerase (Fig. 1B). However, when matched to their counterparts in the HIV RT, they may have different distances to the bound substrate TDF according to the structural information of HIV RT (Fig. S1).

For the introduction of aa substitutions, three criteria were considered: (1) the side chain of the mutated aa residues should be significantly different to the wild type if possible; (2) the introduced aa substitution in HBV polymerase should have no or little influence in the coding sequence of HBsAg; (3) The number of nucleotide need to be mutated should be as less as possible. A series of point mutations on HBV replication-competent pHBV1.3 were designed and constructed using fusion PCR (Fig. 1B). Among these substitutions, rtL77F, rtD83E, rtF88L, rtP177G, rtM204I and rtM204I resulted in the aa substitutions sS69C, sM75K, sF80Y, sR169G, sI195M and sW196S in HBsAg, respectively.

3.2. Replication of pHBV1.3-rtXs in Huh7 cells

To determine the replication competence of HBV genomes with aa substitutions in HBV-RT, pHBV1.3-rtXs were transfected into Huh7 cells and the intracellular encapsidated viral genomes were extracted and subjected to Southern-blot analysis (Fig. 1C, upper panel).

For pHBV1.3-rtL77F, -rtK239E, -rtG244Y, -rtL247F, and -rtG251Y, no HBV replication intermediates were detected in Southern blot (Fig. 1C), indicating that the substitution mutants resulted in a complete loss of replication competence. These aa residues may be functionally essential for both RT and DNA polymerase activities of HBV polymerase or the aa substitutions prevented binding of the substrates, considering the large chains of the aa substitutions. For pHBV1.3-rtF88L, rtP177G, rtA194T, -rtM204I and -rtM204V, single-stranded (SS) DNA bands were synthesized, whereas the relaxed circular (RC) DNA bands were relatively weak or invisible. Compared with pHBV1.3, the levels of intracellular encapsidated HBV DNA were about 11.9%, 66.3%, 10.4%, 51.2%, and 50.8% for pHBV-rtF88L, -rtP177G, -rtA194T, -rtM204I, and -rtM204V, respectively, indicating a reduced replication competence. In contrast, pHBV-rtF249A and -rtM250I only produced RC DNA bands and their levels were 12.9% and 25.8% as compared to that of pHBV1.3, respectively (Fig. 1C, lower panel), which suggest that these two aa substitutions may impair the RT activity of HBV polymerase. The remaining 7 aa substitutions, rtL82V, rtD83E, rtN238R, rtT240Y, rtK241R, rtN248H, and rtQ267K, had no obvious effect on HBV replication capacity.

In all cases, the HBcAg expression levels were comparable (Fig. 1C, middle panel), indicating that HBcAg expression is not associated with HBV replication *in vitro*. Thus, the failure of the detection of HBV replication intermediates was not related to HBcAg expression.

The levels of HBsAg and HBeAg in culture supernatants of transfected Huh7 cells were measured by ELISA. HBsAg was absent from the supernatant of pHBV1.3-rtL77F transfected cells, while pHBV-rtF88L and pHBV-rtP177G produced significantly lower amounts of HBsAg compared to pHBV1.3 (Fig. 1D). The aa substitutions rtL77F, rtF88L, and rtP177G in HBV polymerase lead to the corresponding aa substitutions sS69C, sF80Y and sR169G in HBsAg which significantly affected HBsAg expression and/or secretion. The remaining mutated HBV genomes expressed similar levels of HBsAg compared with the wild type, although some of them exhibited changes in the HBsAg sequence. Nevertheless, in all cases, HBeAg expression levels were comparable, indicating that the plasmid transfection efficiency was the same.

3.3. TDF-resistance assay in vitro

To analyze the influence of the aa substitutions in HBV polymerase described above on the susceptibility to nucleoside/nucleotide analogs, we first determined the concentration range of nucleoside/nucleotide analogs that inhibited the replication of wild type HBV in Huh7. Huh7 cells were transfected with pHBV1.3 and treated with different concentration of the nucleoside/nucleotide analogs 6 h later. Encapsidated HBV DNA was then extracted at 96 h and detected by Southern blot. All tested drugs LMV, ADV, LdT, ETV, and TDF inhibited HBV DNA replication and the half maximal effective concentration (EC_{50}) of these drugs were 1.15, 1.38, 11.56, 0.79, and 0.19 μM, respectively (Fig. S2A–E). As reported previously, all nucleoside/nucleotide analogs did not affect HBcAg, HBsAg and HBeAg expression (Figs. S2 and S3).

Subsequently, the sensitivity to TDF of the replication competent HBV genomes with aa substitutions was tested in Huh7 cells. Southern blot and subsequent densitometry analysis demonstrated that pHBV-rtP177G and -rtF249A displayed reduced sensitivity to TDF, as manifested by sustained viral replication upon TDF treatment (Fig. 2A). This phenotype is in contrast to pHBV1.3, for which viral DNA production decreased sharply as TDF concentration increased. Other mutants, including pHBV-rtL82V, -rtD83E, -rtN238R, -rtT240Y, -rtK241R, -rtN248H, -rtM250I, and -rtQ267K exhibited a similar TDF sensitivity to pHBV1.3 (Fig. S4). HBV RC DNA was quantified by real-time PCR and the results showed that the antiviral effect of TDF to pHBV-rtP177G and -rtF249A were significantly compromised compared to pHBV1.3 (Fig. 2B), as manifested by the observation that EC_{50} of TDF to pHBV-rtP177G and -rtF249A were 0.48 and 2.31 μM, respectively, which were significantly higher than that for pHBV1.3 (0.19 μM) (Fig. 2C). The resistance indexes of the HBV genomes with rtP177G and rtF249A substitutions to TDF were 2.53 and 12.16, respectively.

In addition, pHBV-rtP177G and -rtF249A remained sensitive to LMV, ADV, ETV, and LdT (Fig. 2D).

3.4. rtP177G and -rtF249A compromise antivirus effect of TDF in mice

The aa substitutions rtP177G and -rtF249A in HBV polymerase conferred a certain degree of resistance to TDF *in vitro*. However, it is not clear whether such HBV mutants are able to replicate *in vivo* and show a different susceptibility to TDF compared with the wild type HBV genome. Then on, we explored the mouse model based on HI to characterize HBV replication upon TDF treatment. C57BL/6 mice were respectively challenged with pAAV-rtP177G, -rtF249A, -rtA194T, or -HBV1.3 by HI and treated with 500 μg TDF or PBS daily. HBV DNA in the serum and liver from mice was measured by real-time PCR targeted to RC at the indicated time points.

HBV DNA and HBsAg became detectable in mouse sera after HI with pAAV-HBV1.3 (Fig. 3), comparable with the previous published data (Yin et al., 2011). Serum HBV DNA reached the peak

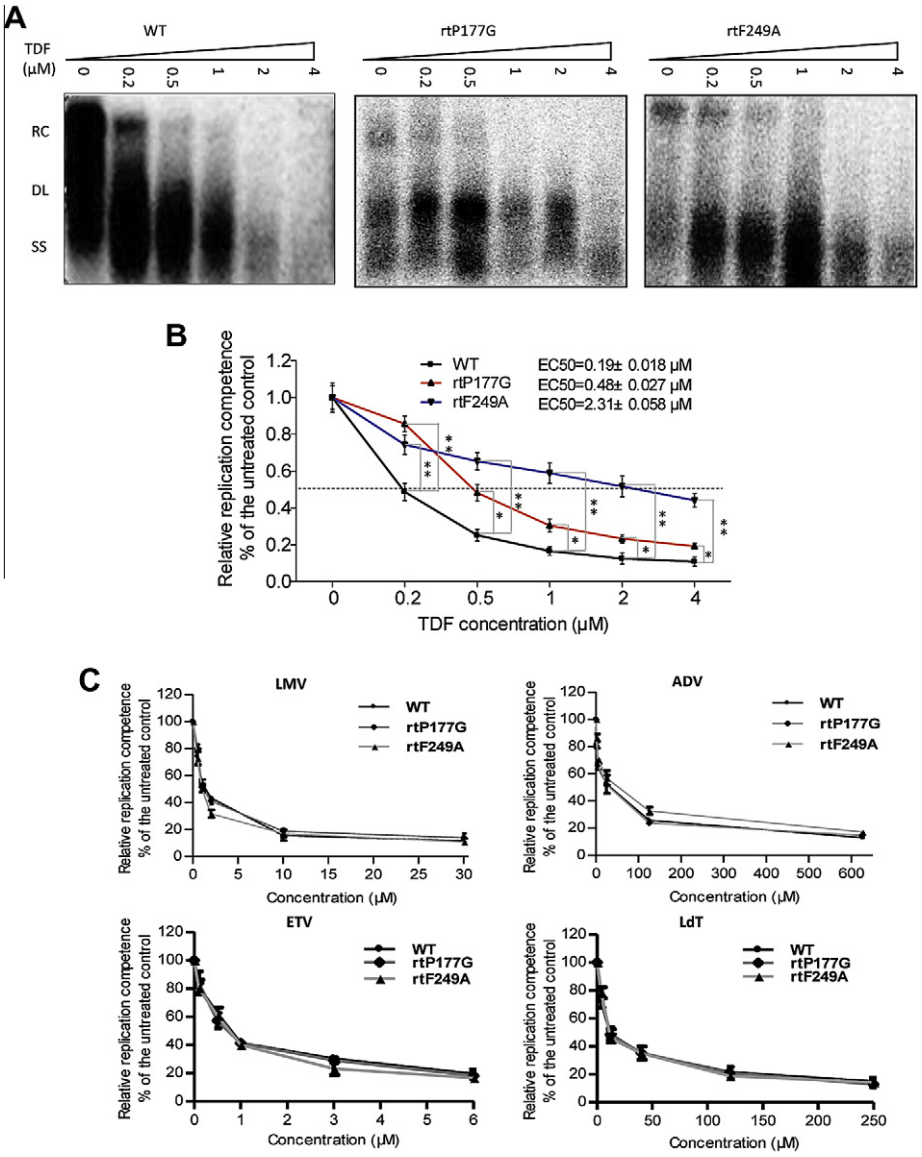

Fig. 2. TDF-resistance assay *in vitro*. Huh7 cells were transfected with pHBV1.3, -rtP177G, and -rtF249A, respectively, and then treated with the different concentrations of TDF. Encapsidated HBV DNAs were purified from intracellular core particles for Southern-blot and real-time PCR analysis. (A) Southern blot for detection of HBV replication intermediates: wt (left), rtP177G (middle), rtF249A (right). (B) Analysis of HBV RC DNA by specific real-time PCR. The relative replication competence pHBV1.3, -rtP177G, and -rtF249A was given as the percentage of the RC DNA compared with the untreated control. The EC50s are indicated. (C) Real-time PCR to analyze the susceptibility of pHBV1.3, rtP177G, and rtF249A to LMV, ADV, ETV and LdT. Each value is the mean of at least 3 independent experiments. The error bars represent the standard deviation. Statistically significant differences between the groups are displayed as $^{*}(p < 0.05)$ or $^{**}(p < 0.01)$.

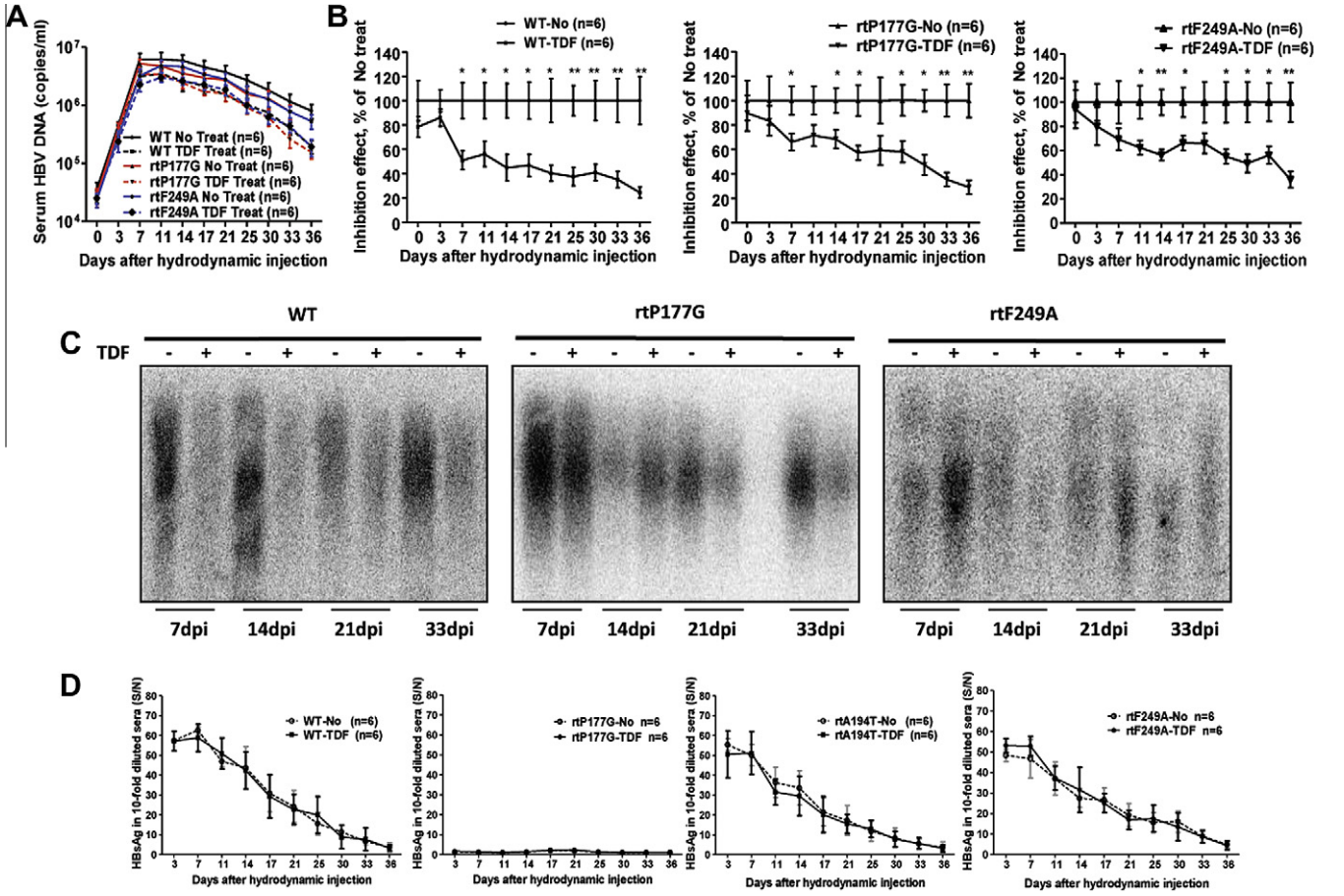

Fig. 3. TDF-resistance assay *in vivo*. C57BL/6 (H-2b) mice were challenged with pAAV-HBV1.3, -rtP177G, and -rtF249A by tail vein HI. After 1 d, mice were treated with 500 μg TDF per day. At the indicated time points, HBV DNA and proteins in the sera and liver were measured by qRT-PCR targeted to HBV RC DNA, Southern blot, ELISA, and IHC, respectively. (A) The analysis of HBV DNA in mouse sera of HBV1.3, -rtP177G, and -rtF249A treated with TDF or vesicle by real time RT-PCR. (B) The antiviral effect of TDF *in vivo* was shown as the inhibition rate of serum HBV DNA in TDF-treated mice compared with that of untreated control mice. The average HBV DNA copy numbers of the untreated control group at each indicated time points were set as 100%. (C) To detect the HBV DNA in the liver, mice from each group were killed at the indicated time points. Total DNA was isolated from the liver tissue and subjected to Southern blot. (D) Kinetics of HBsAg expression. The HBsAg level was determined by a commercial ELSA and given as optical density value (OD 450). S/N means samples/Negative.

level at 7 dphi, decreased gradually thereafter, but remained positive for the complete observation period of 5 weeks (Fig. 3A). Daily treatment with TDF led to a significant reduction of the HBV DNA levels from 7 dphi on and suppressed the HBV DNA level below 50% of the control on 13 dphi (Fig. 3B). The serum HBsAg titers in mice were determined by ELISA. Results showed that HBsAg in all the mice injected with pAAV-HBV1.3 was detected at 3 dphi, and declined gradually from 7 to 36 dphi (Fig. 3D). HBsAg levels were comparable in mice irrespective of TDF treatment. We further detected intrahepatic HBcAg by IHC staining with specific antibody (Fig. S5). HBcAg was strongly expressed in hepatic cells in pAAV-HBV-1.3 injected mice for 3 weeks and persisted weakly thereafter, at least up to 32 dphi. TDF treatment significantly decreased the hepatic HBcAg expression compared with the corresponding untreated mice, indicating TDF inhibits the formation of HBV nucleocapsids. Taken together, the antiviral activity of TDF could be demonstrated in the mouse model based on HI.

Similarly, serum HBV DNA reached the highest level at 7 dphi in pAAV-HBV1.3-rtP177G injected mice, in contrast to rtF249A that displayed a delayed viral DNA maximum level at 11 dphi (Fig. 3A). The result of Southern blot with the liver tissue also confirmed pAAV-HBV1.3 possessed a higher replication potential than pAAV-rtP177G and -rtF249A *in vivo* (Fig. 3C), in accordance with *in vitro* results (Fig. 1C). Despite the reduced replication competence of HBV genomes with the aa substitution in HBV polymerase, high serum HBV DNA levels could be established and maintained in mice after HI with both mutant HBV genomes for the experimental period (Fig. 3A). In pAAV-rtP177G and -rtF249A injected mice, TDF suppression of HBV DNA replication appeared to be less effective. HBV DNA levels in TDF treated mice were lower than that in control mice however and remained over the 50% marker for a prolonged time over 20 d (Fig. 3A), consistent with the *in vitro* data obtained in the previous experiments.

In mice receiving injections with pAAV-HBV1.3-rtF249A, HBsAg levels were comparable in mice irrespective of TDF treatment, comparable with previous findings. In contrast, HBsAg from pAAV-rtP177G injected mice, either TDF treated or not, was always undetectable (Fig. 3D), and was consistent with the *in vitro* assay (Fig. 1D). HBcAg expression was not detected in liver sections of mice that received HI with pAAVHBV1.3-rtA194T, -rtP177G, and -rtF249A (data not shown), most likely due to the decreased replication competence of mutated HBV genomes in mice.

3.5. The aa substitution rtP177G and rtF249A in the structure model of HBV RT

A homology model of the HBV-RT structure was built based on the multiple sequence alignment of HIV-RT with eight HBV-RT sequences representing HBV genotypes A through H. Fig. 4 shows that the sequence in the putative TDF-binding region of HBV-RT

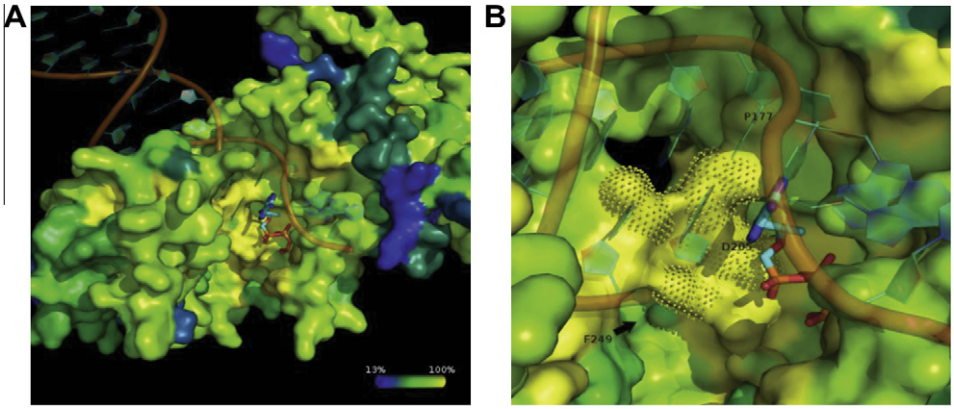

Fig. 4. A homology model of HBV-RT based on HIV-RT. Coloring according to the degree of conservation as described in Section 2 from yellow (complete conservation) to blue (high diversity). (A) The overview showing HBV-RT with bound primer and template oligonucleotides, and TDF molecule (sticks). (B) Close-up around TDF with the catalytic YMDD motif indicated by dots above molecular surface. The central catalytic D205 and the two resistance relevant positions P177 and F249 are labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is well-conserved and similar to that of HIV-RT. In the model, the two positions rtP177 and rtF249 are close to the bound TDF molecule though not in direct contact with it (Fig. 4A). However, rtP177 and rtF249 apparently do make contacts to the template and primer oligonucleotides, respectively (Fig. 4B).

4. Discussion

Viral mutants with RT mutations resistant to nucleoside/nucleotide analogs may be selected during long-term antiviral therapy. However, to date, TDF-associated resistance mutations have seldom been found in patients (Zoulim and Locarnini, 2009). For the first time, we combined the bioinformatics analysis, phenotypic assay for drug resistance, and *in vivo* analysis based on HI. Such an approach may be generally useful to understand the structure–function relationship of aa substitutions involved in drug resistance and their biological relevance. Interestingly, the mutated HBV genomes with aa substitutions rtP177G and rtF249A in HBV RT showed reduced replication capacity but enhanced resistance to TDF in both *in vitro* and *in vivo* analysis. The alignment of HBV RT with HIV RT and bioinformatics analysis suggested that rtP177 and rtF249 may not in directly interact with TDF molecule but with the template and primer oligonucleotides. Thus, the accurate molecular mechanisms leading to TDF resistance by these aa substitutions remain to be determined. The important implication of our results is that the aa substitutions in HBV RT may reduce the susceptibility to TDF. It needs to be investigated whether such or similar aa substitutions may play a role in long term TDF treatment.

HBV polymerase is a multi-functional protein with the activity of RNA- and DNA-dependent DNA polymerization. The conserved RT domain catalyzes reverse transcription using the TP domain as a protein primer to initiate the process, and is also involved in the packaging of pgRNA into viral nucleocapsids (Beck and Nassal, 2007; Lee, 1997). Here, the aa substitutions rtL77F, rtK239E, rtG244Y, rtL247F, and rtG251Y may strongly disturb the polymerase function and led to the complete loss of replication capability of HBV mutants. The YMDD catalytic motif is shared by both HBV RT and HIV RT (Zoulim, 2004) and the substitutions rtM204V and rtM204I result in HBV resistance to LMV and in the reduction of HBV replication competence, due to the lower affinities of the YMDD-mutant polymerases for the natural dNTP substrates (Gaillard et al., 2002). Southern blot analysis indicated that beside rtM204V and rtM204I, HBV with rtF88L, rtP177G, and rtA194T substitutions also produced significantly less RC forms of HBV replication intermediates. The effect of the substitution rtA194T on HBV replication competence has been documented in an earlier publication, whereas the rtF88L and rtP177G substitutions have not been found in patients so far. Notably, substitutions rtF249A and rtM250I lead to no or less SS DNA, but still produced RC DNA, likely due to a change of the relative activities of the RT and DNA-dependent DNA polymerase. Thus, the different aa substitutions affected HBV replication through very different molecular mechanisms. Further experiments are needed to reveal the mechanisms associated with the rtF88L, rtP177G, and rtA194T substitutions. The aa residues that are not located at the catalytic pocket or substitutions at sites which do not impact HBV polymerase conformation may maintain similar replication capacity as the wild type; these aa substitutions comprise rtL82V, rtD83E, rtN238R, rtT240Y, rtK241R, rtN248H, and rtQ267K.

It has been reported that the rtA194T mutation is associated with TDF resistance found in two HBV-HIV-coinfected patients (Sheldon et al., 2005), and this was confirmed in cell lines (Amini-Bavil-Olyaee et al., 2009), although a subsequent report failed to confirm this finding (Delaney et al., 2006). Here, we also failed to detect sufficient replication capacity of HBV in the presence of the rtA194T substitution both *in vitro* and *vivo*, so the susceptibility to TDF cannot be evaluated. Drug-resistance mutations often result in reduction of replication capacity and adaptive mutations occur in an attempt to restore the replication capacity, like the compensatory mutations rtL80I and/or rtV173L and/or rtL180M for rtM204V/I. This could be the reason for the low replication capacity of the HBV genome with mutation rtA194T in our study. Consistently, the aa substitutions rtP177G and rtF249A impaired the replication competence of HBV *in vitro*. In the background of a potential adaptive co-substitution mutant HBV genomes with rtP177G and rtF249A may recover their replication capacity.

It is desirable to establish *in vivo* models with prolonged persistence of drug-resistant HBV genomes, mimicking chronic HBV infection in patients. The HBV mouse model based on HI has been used to study HBV replication (Giladi et al., 2003). Here, we demonstrated the usefulness of the HI mouse model to study drug resistant HBV mutants. The HBV genomes with rtP177G and rtF249A substitutions, which are resistant to TDF *in vitro*, also have reduced sensibility to TDF in C57BL/6 mice. Thus, this model could be refined and better standardized in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.007.

References

Amini-Bavil-Olyaee, S., Herbers, U., Sheldon, J., Luedde, T., Trautwein, C., Tacke, F., 2009. The rtA194T polymerase mutation impacts viral replication and susceptibility to tenofovir in hepatitis B e antigen-positive and hepatitis B e antigen-negative hepatitis B virus strains. Hepatology 49, 1158–1165.

Bartholomeusz, A., Tehan, B.G., Chalmers, D.K., 2004. Comparisons of the HBV and HIV polymerase, and antiviral resistance mutations. Antivir. Ther. 9, 149–160.

Beck, J., Nassal, M., 2007. Hepatitis B virus replication. World J. Gastroenterol. 13, 48–64.

Bramucci, E., Paiardini, A., Bossa, F., Pascarella, S., 2012. PyMod: sequence similarity searches, multiple sequence-structure alignments, and homology modeling within PyMOL. BMC Bioinformatics 13 (Suppl. 4), S2.

Brunelle, M.N., Jacquard, A.C., Pichoud, C., Durantel, D., Carrouee-Durantel, S., Villeneuve, J.P., Trepo, C., Zoulim, F., 2005. Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. Hepatology 41, 1391–1398.

Delaney, W.E., Ray, A.S., Yang, H., Qi, X., Xiong, S., Zhu, Y., Miller, M.D., 2006. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. Antimicrob. Agents Chemother. 50, 2471–2477.

Delaugerre, C., Flandre, P., Marcelin, A.G., Descamps, D., Tamalet, C., Cottalorda, J., Schneider, V., Yerly, S., LeGoff, J., Morand-Joubert, L., Chaix, M.L., Costagliola, D., Calvez, V., 2008. National survey of the prevalence and conditions of selection of HIV-1 reverse transcriptase K70E mutation. J. Med. Virol. 80, 762–765.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113.

Eswar, N., Eramian, D., Webb, B., Shen, M.Y., Sali, A., 2008. Protein structure modeling with MODELLER. Methods Mol. Biol. 426, 145–159.

Gaillard, R.K., Barnard, J., Lopez, V., Hodges, P., Bourne, E., Johnson, L., Allen, M.I., Condreay, P., Miller, W.H., Condreay, L.D., 2002. Kinetic analysis of wild-type and YMDD mutant hepatitis B virus polymerases and effects of deoxyribonucleotide concentrations on polymerase activity. Antimicrob. Agents Chemother. 46, 1005–1013.

Gan, R.B., Chu, M.J., Shen, L.P., Qian, S.W., Li, Z.P., 1987. The complete nucleotide sequence of the cloned DNA of hepatitis B virus subtype adr in pADR-1. Sci. Sin. B 30, 507–521.

Giladi, H., Ketzinel-Gilad, M., Rivkin, L., Felig, Y., Nussbaum, O., Galun, E., 2003. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol. Ther. 8, 769–776.

Grant, B.J., McCammon, J.A., Caves, L.S., Cross, R.A., 2007. Multivariate analysis of conserved sequence-structure relationships in kinesins: coupling of the active site and a tubulin-binding sub-domain. J. Mol. Biol. 368, 1231–1248.

Huang, L.R., Wu, H.L., Chen, P.J., Chen, D.S., 2006. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc. Nat. Acad. Sci. U.S.A. 103, 17862–17867.

Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337, 1733–1745.

Lei, Y.C., Hao, Y.H., Zhang, Z.M., Tian, Y.J., Wang, B.J., Yang, Y., Zhao, X.P., Lu, M.J., Gong, F.L., Yang, D.L., 2006. Inhibition of hepatitis B virus replication by APOBEC3G in vitro and in vivo. World J. Gastroenterol. 12, 4492–4497.

Liu, Y., Wang, C.M., Cheng, J., Liang, Z.L., Zhong, Y.W., Ren, X.Q., Xu, Z.H., Zoulim, F., Xu, D.P., 2009. Hepatitis B virus in tenofovir-naive Chinese patients with chronic hepatitis B contains no mutation of rtA194T conferring a reduced tenofovir susceptibility. Chin. Med. J. (Engl.) 122, 1585–1586.

Meng, Z., Xu, Y., Wu, J., Tian, Y., Kemper, T., Bleekmann, B., Roggendorf, M., Yang, D., Lu, M., 2008. Inhibition of hepatitis B virus gene expression and replication by endoribonuclease-prepared siRNA. J. Virol. Methods 150, 27–33.

Miller, M.D., Margot, N., Lu, B., Zhong, L., Chen, S.S., Cheng, A., Wulfsohn, M., 2004. Genotypic and phenotypic predictors of the magnitude of response to tenofovir disoproxil fumarate treatment in antiretroviral-experienced patients. J. Infect. Dis. 189, 837–846.

Pallier, C., Castera, L., Soulier, A., Hezode, C., Nordmann, P., Dhumeaux, D., Pawlotsky, J.M., 2006. Dynamics of hepatitis B virus resistance to lamivudine. J. Virol. 80, 643–653.

Qi, X., Xiong, S., Yang, H., Miller, M., Delaney, W.E., 2007. In vitro susceptibility of adefovir-associated hepatitis B virus polymerase mutations to other antiviral agents. Antivir. Ther. 12, 355–362.

Qiu, J., Qin, B., Rayner, S., Wu, C.C., Pei, R.J., Xu, S., Wang, Y., Chen, X.W., 2011. Novel evidence suggests Hepatitis B virus surface proteins participate in regulation of HBV genome replication. Virol. Sin. 26, 131–138.

Sheldon, J., Camino, N., Rodes, B., Bartholomeusz, A., Kuiper, M., Tacke, F., Nunez, M., Mauss, S., Lutz, T., Klausen, G., Locarnini, S., Soriano, V., 2005. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. Antivir. Ther. 10, 727–734.

Soler-Palacin, P., Melendo, S., Noguera-Julian, A., Fortuny, C., Navarro, M.L., Mellado, M.J., Garcia, L., Uriona, S., Martin-Nalda, A., Figueras, C., 2011. Prospective study of renal function in HIV-infected pediatric patients receiving tenofovir-containing HAART regimens. AIDS 25, 171–176.

Taly, J.F., Magis, C., Bussotti, G., Chang, J.M., Di Tommaso, P., Erb, I., Espinosa-Carrasco, J., Kemena, C., Notredame, C., 2011. Using the T-Coffee package to build multiple sequence alignments of protein, RNA, DNA sequences and 3D structures. Nat. Protoc. 6, 1669–1682.

Torresi, J., 2002. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. J. Clin. Virol. 25, 97–106.

Tuske, S., Sarafianos, S.G., Clark Jr., A.D., Ding, J., Naeger, L.K., White, K.L., Miller, M.D., Gibbs, C.S., Boyer, P.L., Clark, P., Wang, G., Gaffney, B.L., Jones, R.A., Jerina, D.M., Hughes, S.H., Arnold, E., 2004. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. Nat. Struct. Mol. Biol. 11, 469–474.

Villet, S., Billioud, G., Pichoud, C., Lucifora, J., Hantz, O., Sureau, C., Deny, P., Zoulim, F., 2009. In vitro characterization of viral fitness of therapy-resistant hepatitis B variants. Gastroenterology 136 (168–176), e162.

Whitcomb, J.M., Parkin, N.T., Chappey, C., Hellmann, N.S., Petropoulos, C.J., 2003. Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. J. Infect. Dis. 188, 992–1000.

Yin, Y., Wu, C., Song, J., Wang, J., Zhang, E., Liu, H., Yang, D., Chen, X., Lu, M., Xu, Y., 2011. DNA immunization with fusion of CTLA-4 to hepatitis B virus (HBV) core protein enhanced Th2 type responses and cleared HBV with an accelerated kinetic. PLoS One 6, e22524.

Zoulim, F., 2004. Mechanism of viral persistence and resistance to nucleoside and nucleotide analogs in chronic hepatitis B virus infection. Antiviral Res. 64, 1–15.

Zoulim, F., Locarnini, S., 2009. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 137 (1593–1608), e1591–1592.