



SYSTEMATIC REVIEW

Hepatitis B virus resistance to tenofovir: fact or fiction? A systematic literature review and structural analysis of drug resistance mechanisms [version 1; peer review: 3 approved]

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V1 First published: 29 Jun 2020, 5:151
<https://doi.org/10.12688/wellcomeopenres.15992.1>
Latest published: 29 Jun 2020, 5:151
<https://doi.org/10.12688/wellcomeopenres.15992.1>

Abstract

Background: Tenofovir (TFV) is a widely used treatment for chronic hepatitis B virus (HBV) infection. There is a high genetic barrier to the selection of TFV resistance-associated mutations (RAMs), but the distribution and clinical significance of TFV RAMs are not well understood. We here present assimilated evidence for putative TFV RAMs with the aims of cataloguing and characterising mutations that have been reported, and starting to develop insights into mechanisms of resistance.

Methods: We carried out a systematic literature search in PubMed and Scopus to identify clinical, *in vitro* and *in silico* evidence of TFV resistance. We included peer-reviewed studies presenting original data regarding virological TFV breakthrough, using published methods to assess the quality of each study. We generated a list of RAMs that have been reported in association with TFV resistance, developing a 'long-list' (all reported RAMs) and a 'short-list' (a refined list supported by the most robust evidence). We assessed the potential functional and structural consequences by mapping onto the crystal structure for HIV reverse transcriptase (RT), as the structure of HBV RT has not been solved.

Results: We identified a 'long-list' of 37 putative TFV RAMs in HBV RT,

Open Peer Review

Reviewer Status

Invited Reviewers

	1	2	3
version 1			
29 Jun 2020	report	report	report

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occurring within and outside sites of enzyme activity, some of which can be mapped onto a homologous HIV RT structure. A 'short-list' of nine sites are supported by the most robust evidence. If clinically significant resistance arises, it is most likely to be in the context of suites of multiple RAMs. Other factors including adherence, viral load, HBeAg status, HIV coinfection and NA dosage may also influence viraemic suppression.

Conclusion: There is emerging evidence for polymorphisms that may reduce susceptibility to TVF. However, good correlation between viral sequence and treatment outcomes is currently lacking; further studies are essential to optimise individual treatment and public health approaches.

Keywords

Hepatitis B virus, HBV, Tenofovir, TDF, TAF, TFV, resistance, RAMs

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Mokaya J:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **McNaughton AL:** Writing – Review & Editing; **Bester PA:** Visualization, Writing – Review & Editing; **Goedhals D:** Writing – Review & Editing; **Barnes E:** Supervision, Writing – Review & Editing; **Marsden BD:** Formal Analysis, Visualization; **Matthews PC:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Wellcome Trust [110110; to PCM], the Leverhulme Mandela Rhodes Scholarship to JM, the Medical Research Council UK to EB, the Oxford NIHR Biomedical Research Centre to EB. EB is an NIHR Senior Investigator. The views expressed in this article are those of the author and not necessarily those of the NHS, the NIHR, or the Department of Health. ALM is funded through NIHR Research Capability Funding.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Mokaya J, McNaughton AL, Bester PA *et al.* **Hepatitis B virus resistance to tenofovir: fact or fiction? A systematic literature review and structural analysis of drug resistance mechanisms [version 1; peer review: 3 approved]** Wellcome Open Research 2020, 5:151 <https://doi.org/10.12688/wellcomeopenres.15992.1>

First published: 29 Jun 2020, 5:151 <https://doi.org/10.12688/wellcomeopenres.15992.1>

Abbreviations

3TC, lamivudine; ADV, adefovir; ART, antiretroviral therapy; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B virus infection; ETV, entecavir; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LdT, telbivudine; NA, nucleos(t)ide analogue; RAM, resistance-associated mutation; RT, reverse transcriptase; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; TFV, tenofovir (the active component of both TAF and TDF); YMDD, tyrosine methionine aspartate aspartate motif in HBV RT.

Introduction

Nucleot(s)ide analogues (NA) are the most widely used antiviral treatments for chronic hepatitis B virus (HBV) infection¹, with tenofovir (TFV) being a safe, cheap, and widely available agent. NA agents inhibit the action of HBV reverse transcriptase (RT), acting as DNA chain terminators. NA therapy can be effective in suppressing HBV viraemia, thus reducing the risks of inflammation, fibrosis and hepatocellular carcinoma (HCC) as well as lowering the risk of transmission¹. However, NAs are not curative due to the persistent intracellular hepatic reservoir of HBV covalently closed circular DNA (cccDNA). Long-term administration is therefore typically required², with a potential risk of selection of resistance-associated mutations (RAMs) in the virus^{3,4}. RAMs are mostly likely to arise in the context of high viral replication, arising as a result of the error prone RT enzyme¹.

Lamivudine (3TC), telbivudine (LdT) and adefovir (ADV) have been phased out of use in HBV management, mainly due to the predictable selection of RAMs over time¹. The best recognised 3TC RAM arises at RT-M204, representing the second position of the tyrosine-methionine-aspartate-aspartate ('YMDD') motif in viral RT^{1,4}. Resistance to TFV, formulated either as tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF) (Suppl Fig 1, *Extended data*⁵), remains controversial. Unlike other NAs, TFV has a high genetic barrier to resistance¹, corroborated by studies that report no resistance after many years of treatment⁶. An on-line tool, '[geno2pheno hbv](#)', lists only one position (RT N236T) in association with reduced TFV susceptibility, while other reports also include A181T/V⁷. However, there are emerging reports of a wider range of amino acid substitutions that are associated with reduced TFV sensitivity, described in both treatment-experienced and treatment-naïve individuals with chronic HBV infection (CHB)^{8,9}.

There is some degree of homology between the sequence, structure and function of HIV and HBV RT enzymes, explaining why certain NAs are active against both viruses¹⁰. Although no crystal structure has been resolved for HBV RT, some studies have modelled this enzyme based on the HIV crystal structure¹⁰⁻¹², suggesting that insights into HBV drug resistance mechanisms might be inferred from what is known about HIV.

A better understanding of the role of NA therapy in driving HBV elimination at a population level is crucial to underpin efforts to move towards international targets for elimination by

the year 2030^{3,13}. For populations in which HIV and HBV are both endemic, as exemplified by many settings in sub-Saharan Africa, there are particular concerns about drug resistance in HBV, given the widespread population exposure to TDF as a component of first-line antiretroviral therapy (ART) for HIV³. In order to progress towards these targets, many more people will need to be treated in the decade ahead.

We have therefore undertaken a systematic approach to assimilate the current evidence for the development of clinical or virological HBV breakthrough during TFV therapy. The evidence on this topic is not currently sufficiently advanced to underpin definitive conclusions regarding specific genetic signatures that underpin TVF resistance, or the extent to which these are significant in clinical practice. However, we add to the field by providing a comprehensive summary of relevant publications, together with a quality appraisal of the evidence. We used this process to assimilate a 'long-list' (all putative TFV RAMs) and a 'short-list' (a refined catalogue containing only the polymorphisms most robustly supported by existing data). We highlight gaps in the existing data and the urgent need for more research.

Methods

Search strategy and quality appraisal

We undertook a systematic search of PubMed and Scopus in February 2019, using PRISMA criteria (Suppl Fig 2, *Extended data*⁵). Data extraction was performed independently using the search terms ("Hepatitis B virus" [Mesh] OR "hepatitis b" OR HBV) AND (Tenofovir OR TDF OR TAF OR "Tenofovir alafenamide" OR "Tenofovir Disoproxil Fumarate") AND (resista* OR drug muta* OR DRMs OR RAMs OR viremia OR replica*). We reviewed the titles and abstracts matching the search terms and included those reporting virological HBV breakthrough after exposure to TFV, only including studies that presented original data and had undergone peer review. All retrieved articles were in English, therefore no exclusion in relation to language was required. For each study, we extracted information on type of study, characteristics of study participants, sequencing method, genotype, HBV treatment used, mutations associated with TFV resistance and method used to define TFV resistance. We used the Joanna Briggs Institute Critical Appraisal tool checklist to assess for quality of case reports¹⁴. For assessment of quantitative studies, we used the BMJ adapted Quality Assessment Tool for Quantitative Studies¹⁵.

Appraisal of putative sites of drug resistance

Recognising that there is sparse and varied evidence to support TDF RAMs, we divided our data into two categories. First, we generated a 'long list' of all polymorphisms that have been reported in association with TFV resistance, to summarise all the available data in the most inclusive way. We then refined this into a 'short list' including just those sites supported by the most robust evidence, based on reporting in ≥ 2 studies and a combination of *in vivo* and *in vitro* evidence.

Sequence analysis

To assess similarity between HIV and HBV RT, we downloaded HIV (HXB2 - [K03455](#)); and HBV reference sequences (Geno

A – [FJ692557](#), Geno B – [GU815637](#), Geno C – [GQ377617](#), Geno D – [KC875277](#), Geno E – [GQ161817](#)) from publicly available repositories: HIV sequence database¹⁶ and Hepatitis B Virus Database¹⁷. We aligned amino acid RT sequences using MAFFT version 7¹⁸. The alignment illustrates regions of similarity and differences between HIV and HBV RT. We obtained RAMs associated with HIV resistance to TFV published in the Stanford University HIV drug resistance database under drug resistance summaries for nucleoside RT inhibitors.¹⁹

Structural analysis

The crystal structure for HBV RT has not been solved. However, the enzyme is homologous to HIV RT. In order to further assess the relevance of sites included in a ‘long list’ of all polymorphisms, we therefore considered evidence for a mechanistic influence by mapping HBV RAMs onto a previously solved crystal structure of HIV RT (Protein Data Bank (PDB) code [3dlk](#))²⁰ using ICM-Pro platform, which provides a direct link to the PDB²¹

Results

Nature and quality of the evidence identified

We identified 15 studies that met our search criteria. Although some studies used hybrid methods, we classified them broadly as seven studies arising from clinical case reports^{8,9,22–26}, (one of these also presented *in vitro* evidence for drug resistance⁹), four from *in vitro* studies^{27–29} and four from longitudinal studies of CHB (with or without HIV coinfection)^{30–33}. Studies were from Asia^{8,9,22,24,29}, Australia³³, Europe^{23,26,28,30–32} and USA^{25,27,34}. Despite the high prevalence of HBV infection in Africa, and the widespread use of TFV for HIV across this continent, it is striking that no African data have been published to date. Eight studies reported HBV genotypes, representing genotypes A–G^{9,22,25–27,30–32,34}. Metadata for individual studies are provided in Suppl Table 1 (see *Extended data*⁵).

A detailed quality assessment of each individual reference is included in Suppl Table 2 (see *Extended data*⁵). Among the case reports, three were of high quality as they clearly described patients’ characteristics, clinical details, diagnosis, treatment and follow-up, and concluded with take away lessons^{22–24}. Three further case reports did not describe diagnostic or assessment methods^{8,25,26}, and one study did not describe post-intervention follow-up⁹. The overall quality rating for four cohort studies was strong because participants selected represented the target population, characteristics of participants were clearly described, there was a clear hypothesis for the study, and inclusion/exclusion criteria were specified^{30–33}. Two studies had a weak rating because there was no description of the characteristics of participants and it was not clear to what extent participants were representative of the target population^{27,34}. Two natural experimental studies were not rated because the quality assessment questions were not applicable^{28,29}.

Approach to defining resistance

Resistance was studied based on exposure to TDF in 13 studies and TAF in two studies; we therefore refer to TFV

throughout the results section. TFV resistance was determined using a range of strategies, which can be summarised as follows:

- i A sequencing approach to identifying possible RAMs in HBV sequence isolated individuals in whom viraemia was not suppressed by TFV therapy, undertaken in seven studies^{8,22,24–26,30,33};
- ii *In vitro* assays to measure the effect of TFV on viral replication in cell lines, reported by three studies^{27,28,34};
- iii Approaches (i) and (ii) in combination, applied in four studies^{9,23,31,32};
- iv Approach (ii) combined with an animal model, described by one study²⁹.

In 12/15 studies, HBV mutations were reported in association with TFV resistance (suggesting complete virologic escape from the impact of a drug) or reduced TFV susceptibility (evidenced by incomplete suppression of viraemia, but not complete drug resistance)^{9,22,23,26–32,34}. In the remaining three studies, persistent viraemia was reported among individuals with chronic HBV infection while on TFV but either no RAMs were identified^{25,33} or viral sequencing was not performed due to low viral load²⁴.

Location and number of RAMs associated with TFV resistance

We generated a ‘long-list’ of 37 different sites of polymorphism, arising both within (n=15) and outside (n=22) enzymatically active sites in RT ([Figure 1](#); Suppl Table 3, *Extended data*⁵). HBV mutations outside active sites of the enzyme occurred in combination with RAMs located within active sites, with the exception of A194T. Only two studies reported TFV resistance arising from the selection of a single mutation, S78T and A194T^{23,28}. S78T was defined by sequencing HBV from two individuals in whom viraemia was not suppressed by TDF, combined with *in vitro* assays²³, while A194T was only defined *in vitro*²⁸. In all other studies, ≥ 2 RAMs were required to confer TFV resistance (2 RAMs in four studies^{27,29,30,34}, 3 RAMs in one study³², 5 RAMs in one study⁹, and ≥ 8 RAMs in a further four studies^{8,22,26,31}). This pattern supports the high genetic barrier to selection of TFV resistance.

We narrowed the list of 37 sites down to compile a ‘short-list’ of TFV RAMs that have been identified in ≥ 2 studies, regarding these sites as having the strongest evidence base (nine sites; [Table 1](#)). The most frequently described RAMs were L180M^{22,31,32}, A181T/V^{27,30,31,34}, M204I/V^{9,22,31,32}, and N236T^{27,30,34}, which were all identified through sequencing and tested in *in vitro* assays to measure the effect of TDF on viral replication in cell lines. Among these, the M204 mutation (within the ‘YMDD’ motif) is well established in association with 3TC resistance, commonly arising in combination with substitutions at positions V173, L180 and A181, while N236 substitutions appear to be more specifically associated with reduced susceptibility to ADV and TFV⁷. Mutations at sites 177, 194 and 249 may also be more

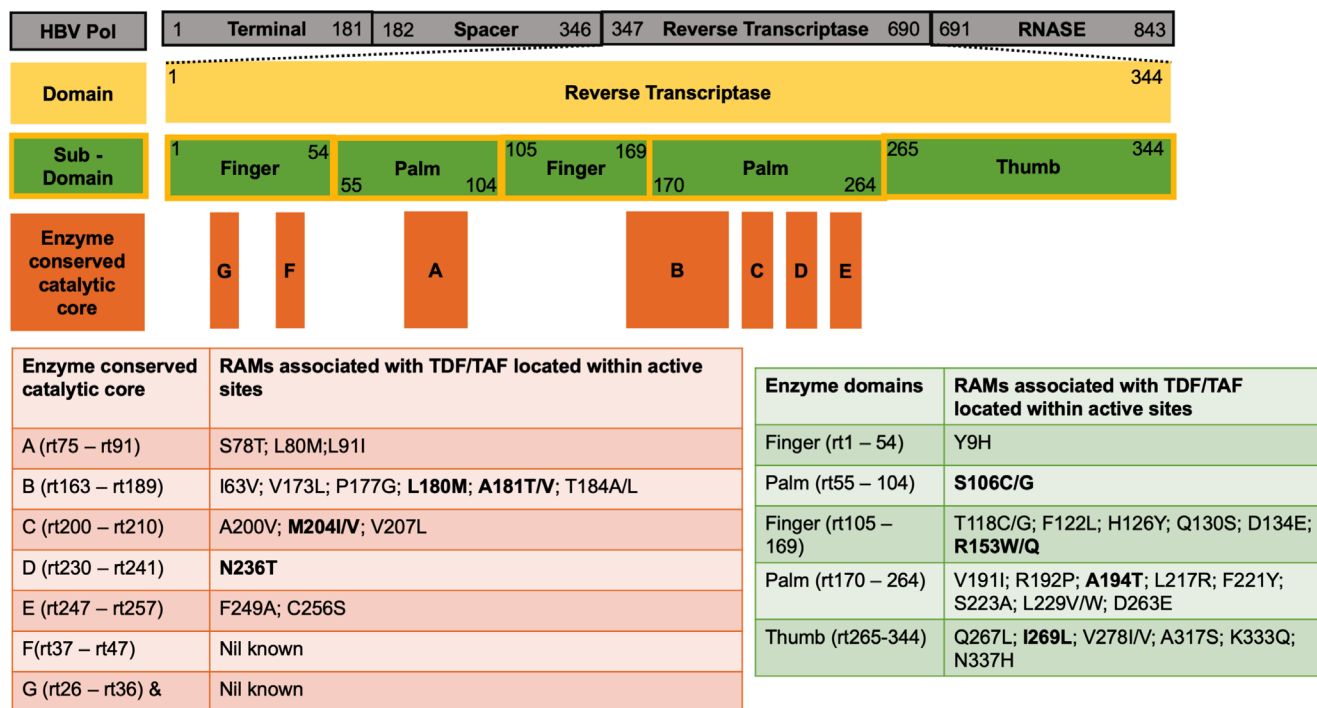


Figure 1. Mutations associated with TFV resistance located within and outside the active sites of the HBV RT enzyme. Polymerase numbering shown in the grey bars is based on genotype A sequence (accession number X02763). Yellow bar represents RT; green bars represent subdomains which are designated finger, palm and thumb; orange rectangles represent active sites of the enzyme referred to as regions A-G. Mutations associated with TFV resistance (n=37 sites) are listed according to their location within active sites of the enzyme (orange table) or outside active sites (green table). The sites shown in bold represent the nine mutations in our short-list with best literature support (evidence summarised in Table 1). Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥ 2 polymorphisms. TFV, tenofovir; HBV, hepatitis B virus; RT, reverse transcriptase; RAM, resistance-associated mutation; TDF, tenofovir disoproxil fumarate; TAF, tenofovir alafenamide.

specific to TFV resistance, having been less clearly reported in association with resistance to other agents^{3,35}. Polymorphisms at positions 80, 173 and 184 have been described as compensatory changes to allow the virus to accommodate the primary drug escape substitution⁷. These mutations on their own may not be sufficient to mediate TFV resistance but may be a necessary compensatory contribution to combinations of mutations that underpin resistance.

Some reported polymorphisms associated with drug resistance represent wild type sequence in some genotypes (Y9H, F122L, H126Y, R153W/Q, F221Y, S223A, C256S, D263E, V278I/V and A317S), and our assimilation shows more resistance in genotype D (Suppl Table 4, *Extended data*⁵). Most of these polymorphisms are located outside the active site of the enzyme, with the exception of position 256. The barrier to selection of TFV resistance may therefore be lower in certain genotypes, (Figure 1; Suppl Table 4, *Extended data*⁵). The same phenomenon has been described for HCV resistance, in which certain sub genotypes are predicted to be intrinsically resistant to certain direct acting antiviral agents due to the presence of resistance associated polymorphisms in the wild type sequence^{36,37}.

One study assessed the replication competence and susceptibility to TFV of mutated HBV clones *in vitro* and *in vivo* using mice models. The introduction of P177G and F249A mutations (substitutions in active sites of the RT enzyme) in HBV clones resulted in a reduction in their susceptibility to TFV²⁹.

RAMs occurring as minor quasispecies

There is limited evidence for the significance of TFV RAMs occurring as minor quasispecies. One study that performed ultra-deep pyrosequencing enrolled HIV/HBV co-infected individuals on (or about to start) TFV-containing ART, reporting minor variants present at <20% with mutations (V173, L180M, A181T/V and M204V) in 2/50 TFV naïve samples and 1/14 sample obtained from a TDF-experienced individual³³. One other study performed deep sequencing using Illumina on HBV clones⁹, revealing that RAMs S106C, H126Y, D134E, M204I/V & L269I were predominant. Only one study reported sequencing the whole HBV genome, but this was undertaken following *in vitro* introduction of RAMs into a clinically isolated virus³⁴, so does not provide any evidence of the association between TFV RAMs and other polymorphisms that might arise on the same viral haplotype.

Table 1. Mutations most commonly reported in association with TFV resistance in HBV, identified from a systematic literature review. This table reports a 'short-list' of mutations identified in two or more different studies^{8,9,22,26-28,30-32,34}. The symbols *, ** and *** indicate the method(s) used to determine drug resistance. All positions are in HBV RT, listed in numerical order. The 'long-list' of 37 mutations identified in 15 included studies is reported in Suppl Table 3 (see *Extended data*).

Mutations	Park et al. 2019	Cho et al. 2018	Lee et al. 2014	Mikulska et al. 2012	Liu et al. 2014	Amini-Bavil-Olyaei et al. 2009	Van Bommel et al. 2012	Lada et al. 2012	Sheldon et al. 2005	Liu et al. 2017	Total number of studies	Associated with 3TC resistance	Associated with ETV resistance
S106C/G	***	*									2	No	No
R153W/Q			*					***			2	No	No
V173L				*				***			2	Yes	Yes
L180M			*	*				***	***		4	Yes	Yes
A181T/V					**		*	***		**	4	Yes	Yes
A194T						**			***		2	No	No
M204I/V	***		*	*				***	***		5	Yes	Yes
N236T					**		*			**	3	No	No
I269L	***	*									2	No	Yes
KEY:	Method used to identify TFV resistance												
*	Sequencing HBV sequence from individuals in whom viraemia was not suppressed by TDF												
**	<i>In vitro</i> assays to measure the effect of TDF on viral replication in cell lines												
***	Two above approaches (* and **) in combination												

HBV, hepatitis B virus; RT, reverse transcriptase; TFV, tenofovir; 3TC, lamivudine; ETV, entecavir; TDF, tenofovir disoproxil fumarate.

Duration of therapy and treatment compliance prior to detection of tenofovir resistance

Five studies reported the duration in which individuals were on TFV prior to treatment failure, with virological breakthrough occurring between 48 weeks and 48 months of therapy (48 weeks²², 18 months²⁴, 20 months³¹, 26 months⁹, and 48 months^{8,23}). Compliance was assessed in six studies, among which virological breakthrough despite good treatment compliance was reported in five^{9,22,24,31,32}, and one reported concerns with compliance²⁶. Quantification of drug levels in plasma supported good compliance in two studies^{9,31}.

Comparison between HIV and HBV RT

HBV RT has been classified into subdomains which are further divided into regions A – G³⁸, which form the main catalytic core of the enzyme (Figure 1). Alignment of sequences of HBV and HIV RT demonstrates 25-27% homology between HBV and the HXB2 HIV reference sequence (Figure 2). Comparing

sites that have been reported in association with drug resistance in HBV vs HIV (Figure 3; Suppl Table 5, *Extended data*⁵), we found that among our long-list of 37 HBV RAMs, two sites had identical substitutions in HIV RT (M204 and L229 in HBV^{10,12}, corresponding to M184 and L210 in HIV, respectively; Stanford University HIV drug resistance database; Figure 3). Other sites reporting an association with TVF resistance in HBV have substitutions that overlap with HIV RAMs, but not all of these are associated with TFV resistance (Suppl Table 6, *Extended data*⁵).

Six established TFV RAMs in HIV RT (M41L, K65R, K70E, Y115F, Q151M and T215F/Y)¹⁹ do not correspond to an equivalent mutation in HBV RT, although three of these HIV RAMs have an HBV RAM within three amino acids up- or down-stream in the equivalent sequence, suggesting there may be homology in the mechanism through which drug resistance is mediated.

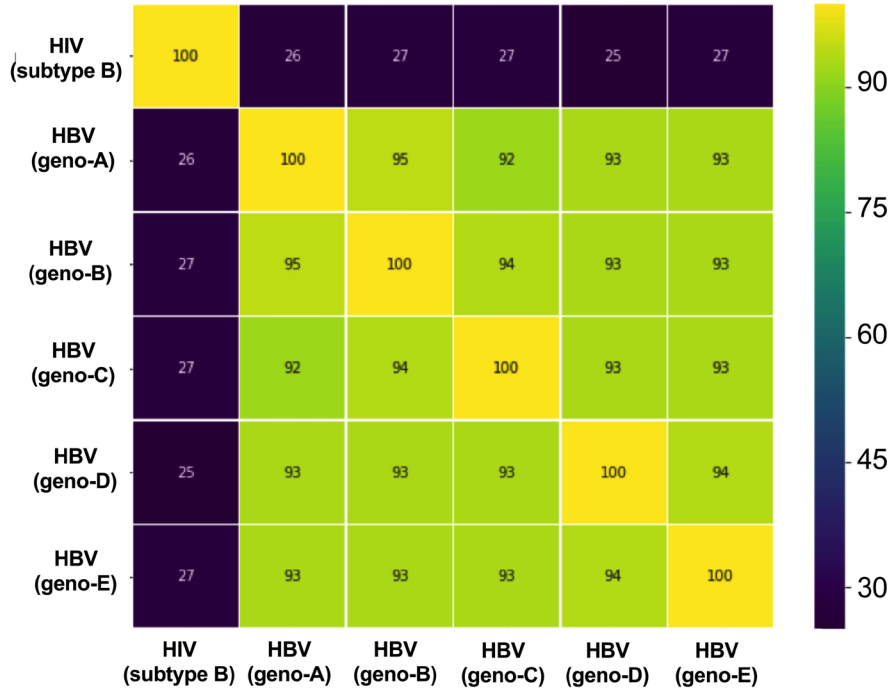


Figure 2. Heatmap showing identity comparison matrix of reference sequence alignment of HBV RT and HIV RT. Chart shows a comparison based on sequences downloaded from [HIV sequence database](#) and [Hepatitis B Virus Database](#) and aligned using MAFFT version 7. HIV reference sequence is HIV HXB2 (K03455). HBV reference sequences are Genotype A – FJ692557, Genotype B - GU815637, Genotype C – GQ377617, Genotype D - KC875277, Genotype E - GQ161817. HBV, hepatitis B virus; HIV, human immunodeficiency virus; RT, reverse transcriptase.

HBV SEQUENCE POSITION	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233			
HBV_Geno_A1	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	T	A	V	T	N	F	L	L	S	L	G	I		
HBV_Geno_B2	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	A	V	T	N	F	L	L	S	L	G	I			
HBV_Geno_C2	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	F	T	S	I	T	N	F	L	L	S	L	G	I		
HBV_Geno_D1	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	F	T	A	V	T	N	F	L	L	S	L	G	I		
HBV_Geno_E	F	S	Y	M	D	D	V	V	L	G	A	K	-	S	V	Q	H	L	E	S	L	Y	T	S	V	T	N	F	L	L	S	L	G	I		
HBV TDF mutation hotspots				M204I/V			V207L										L217R				F221Y		S223A										L229V/W			
HIV SEQUENCE POSITION	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214		
HIV_Subtype_B	Y	Q	Y	M	D	D	L	Y	V	G	S	D	L	E	I	Q	G	H	R	T	K	I	F	E	L	R	Q	H	L	L	R	W	G	L		
HIV TDF mutation hotspots				M184I/V																															L210W	
Sites with same AA when comparing HIV and HBV RT after alignment			*	*	*	*				*																			*	*			*			

Figure 3. A section of the reference sequence alignment of HBV RT and HIV RT. Sequences downloaded from [HIV sequence database](#) and [Hepatitis B Virus Database](#). Sequences were aligned using MAFFT version 7. HIV subtype B reference sequence is shown in light green (accession number K03455). HBV reference sequences are shown in yellow (Genotype A: FJ692557; Genotype B: GU815637; Genotype C: GQ377617; Genotype D: KC875277; Genotype E: GQ161817). Sites of TFV resistance are highlighted in red, based on the data assimilated in this study. HIV tenofovir RAMs were obtained from the online [Stanford University HIV drug resistance database](#). Sites marked * have the same amino acid in HIV and HBV RT after alignment, and those coloured blue also share TFV resistance mutations. This section is shown as it contains the only two homologous TFV RAMs that we have identified using this approach. Sequence alignments and RAMs throughout the whole RT protein is shown in Suppl Table 5 (see *Extended data*). Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥ 2 polymorphisms. HBV, hepatitis B virus; HIV, human immunodeficiency virus; RAM, resistance-associated mutation; RT, reverse transcriptase; TFV, tenofovir.

We mapped HBV RAMs onto the crystal structure of the likely structurally-related HIV RT (PDB code 3dlk) in order to visualise their approximate 3D locations and infer possible functional consequences (Figure 4; Suppl Table 6, *Extended data*⁵). The RAMs are primarily located within the ‘fingers’, ‘palm’, ‘thumb’ and ‘connection’ subdomains of the p66 polymerase domain of HIV RT, with the majority within the ‘palm’. A number of RAMs (e.g. V207, M204, F249) are spatially adjacent to the catalytically critical (and highly conserved) residues D110, D185 and D186 in HIV RT (D83, D204 and D205 in HBV), suggesting that these RAMs are highly likely to affect catalytic competency. The 22 HBV RAMs that map to HIV RT residue positions are likely to cause polymerase structure destabilisation if mutated, suggesting that many of these RAMs are likely to impact upon resistance.

Discussion

Summary of key findings

TFV is a safe and effective treatment choice for CHB in the majority of cases, and large case series have not raised significant concerns about clinically significant drug resistance. However, it is important to consider the potential for the emergence of

resistance, demonstrated by persistent viraemia on therapy and/or reduced virologic suppression *in vitro*. Based on existing evidence, TFV resistance seems likely to depend on the selection of suites of mutations (most commonly including L180M, A181V/T, M204I/V and/or N236T), overlapping with RAMs that allow escape from other NA drugs. There is also a suggestion that, rarely, single mutations can confer TFV resistance, best demonstrated for S78T.

Notably, the literature to date is limited and heterogenous, and there remains a lack of evidence about the frequency and likely impact of proposed TFV RAMs, either within individual patients or at population level. At present, we have tackled this uncertainty by dividing our catalogue of polymorphisms into a ‘long-list’ (all reported RAMs) and a ‘short-list’ (RAMs with the best evidence-base of support).

Tools that have been designed to identify drug resistance may bias against detection of relevant mutations if they do not scrutinise all relevant sites that contribute to reducing TFV susceptibility. For example, ‘TRUGENE’ a commercially available HBV drug resistance interpretation system, captures

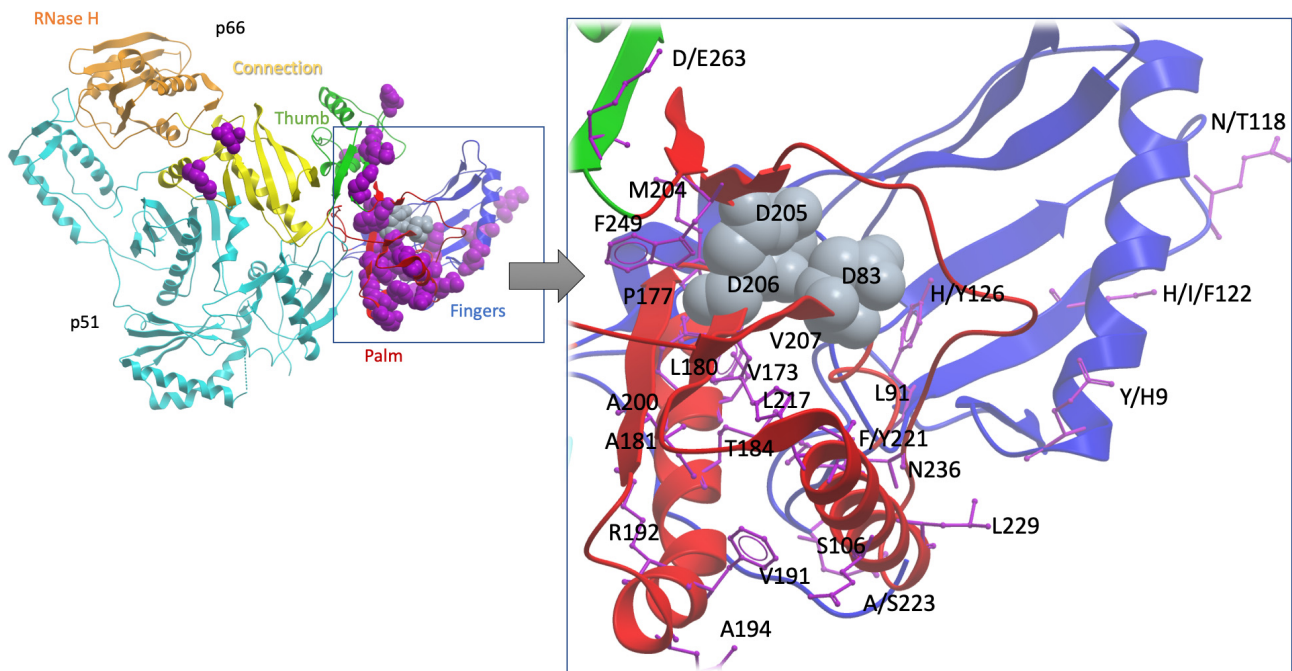


Figure 4. Cartoon to show the sites of TFV drug resistance polymorphisms, using the homologous crystal structure of HIV RT as a model. The sequence alignment of HBV was extended with HIV RT’s p66 domain and then projected onto a high-resolution HIV RT structure (PDB code 3dlk). Sub-domains of the HIV RT are coloured and annotated. Positions associated with resistance are scattered primarily throughout the finger and palm subdomains of the p66 domain (purple space-filled representations, left whole-molecule view, purple stick representation on the zoomed-in view on the right). Three aspartate residues, D83, D205 and D206 (indicated by grey space-filled representation) form the catalytic triad of the enzyme and are shown as a point of reference. Of the 37 sites identified as potential TFV RAMs, 24 residues which are visible in the structure are labelled (using HBV numbering). This excludes seven putative HBV mutations at sites which do not have a homologous site in the HIV structure (sites 78, 80, 130, 134, 153, 163 and 256), and six sites which are beyond the end of the sequence of the solved crystal HIV structure (267, 269, 278, 317, 333 and 337). Figure produced using the ICM platform. Note that in most cases, individual mutations are unlikely to be sufficient to mediate resistance, and a resistant phenotype arises only as a result of combinations of ≥ 2 polymorphisms. HBV, hepatitis B virus; HIV, human immunodeficiency virus; RAM, resistance-associated mutation; RT, reverse transcriptase; TFV, tenofovir.

common HBV RAMs but does not include positions 78, 177, or 249 which may be pertinent to TFV resistance³⁹ and ‘*geno2pheno hbv*’ only lists one TFV mutation at position 236.

Overlap of TFV RAMs with RAMs to other NA agents

RAMs L180M, M204I/V and A181T/V have been associated with resistance to 3TC, telbivudine (LdT) and entecavir (ETV)^{3,40–43}; their reported association with TFV resistance is of concern in suggesting that prior NA exposure can increase the likelihood of cross-resistance to TFV. A study of HIV/HBV co-infected individuals demonstrated a decreased likelihood of HBV DNA suppression with TDF among individuals exposed to prolonged 3TC treatment, possibly due to accumulation of such mutations⁴⁴. A large study in China reported A181 and/or N236 substitutions in 11% of the population⁴², which may underpin reduced susceptibility to TFV. The structural similarities between ADV and TFV, and similar interaction with HBV polymerase^{1,2} explain why the ADV RAMs A181T/V and N236T are also reported to confer resistance to TFV^{1,45}.

Although TFV has been considered effective in the context of resistance to other NAs⁴⁶, the current evidence suggests that there may be common pathways to resistance⁴⁷. There is some evidence showing co-location of RAMs conferring resistance to different antiviral agents on the same viral haplotype⁴⁸. These findings suggesting cross-resistance are of concern, especially for settings in which there has been widespread use of NA therapy as a component of ART for HIV³.

Sites of TFV RAMs in HBV RT

Resistance to TFV can be explained by RAMs both within and outside the active site of the RT enzyme, some of which may have similar mechanisms to those described in HIV^{10,38}. The mechanism of resistance in most of these polymorphisms remains unknown, but may interfere with drug access to sites of activity through steric hindrance. Mutations within active sites of the enzyme may be associated with a higher fitness cost to the virus than mutations at other locations in the RT sequence, as they are more likely to interfere with the RT function. Some polymorphisms listed as RAMs may in fact represent compensatory mutations, which are co-selected in the presence of primary RAMs. For example, substitution at position 269 has been previously described as a compensatory mutation that restores impairments to RT function⁴⁹.

Currently, HBV genotyping is not routinely undertaken in clinical practice, so it is difficult to amass data for any potential relationship between resistance and viral genotype. However, there are some clues that genotype may be relevant. For example, C256S has been linked to TFV resistance, but S256 is wild type in genotype C (Suppl Table 4, *Extended data*⁵), suggesting that the genetic barrier to TFV resistance in genotype C might be lower than in other genotypes. However, a study of >1000 individuals in China found no differences in drug resistance rates between genotype B vs genotype C infection⁴². The identification of Y9H as a TFV RAM should be viewed with caution as H9 is frequently the wildtype residue, irrespective of genotype.

Other factors associated with persistent viraemia

In addition to RAMs, there are other explanations for incomplete suppression of HBV viraemia on therapy^{25,33}, including a higher baseline HBV DNA level, positive baseline HBeAg status, history of 3TC exposure, a lower nadir CD4+ T cell count in the context of HIV coinfection, and high serum HBV RNA levels^{44,50,51}. Given that HBV DNA is inhibited in a dose-dependent manner², it is also possible that insufficient drug delivery to the infected hepatocyte could be the cause of persistent viraemia even in the absence of specific RAMs.

Incomplete adherence to therapy can also contribute to virological breakthrough⁵². Two studies included in our review assessed treatment compliance by measuring drug concentration in plasma^{9,31}. Assessment of adherence in chronic HBV has been through the use of questionnaires⁵³, but these are subject to self-reporting bias. Evidence of potential TFV resistance may emerge when individuals with HIV/HBV coinfection are treated with a TFV-containing regimen leading to suppression of HIV but with sustained HBV viraemia⁵⁴.

It has been reported to take three years for 90% of HBV infected individuals to reach viraemic suppression on therapy⁵⁵, in contrast to HIV, in which 88% of patients suppress the virus within the first year of TDF-based treatment⁵⁶. In the studies we have reported in this review, persistent HBV viraemia on therapy could be due to the prolonged timeline for viraemic suppression; however, in most studies there was a reduction in viral load when TDF was initiated, with subsequent virological breakthrough that is more in keeping with the selection of resistance.

Implications for patient management

There are not currently sufficient data about TFV RAMs to underpin robust universal guidelines for clinical practice. However, the evidence that we have gathered here can underpin some practical recommendations:

- i There is an urgent need for more HBV sequencing data, together with contemporaneous viral load measurements and clinical metadata to advance understanding of the relationship between viral sequence and treatment outcomes. Sequence repositories, databases and tools for sequence analysis should regularly review the evidence for TDF RAMs in order to highlight all sites that may be significant in mediating resistance.
- ii In the context of failure of viraemic suppression in a patient prescribed therapy, assessing and supporting drug compliance is crucial, ideally together with viral sequencing.
- iii Therapeutic failure of TFV – whether in the presence or absence of known RAMs – should lead to an expert clinical decision about switching therapy or combining agents, as long as adherence has been optimised and supported. The presence of recognised RAMs, especially when in combination, may support a change of therapy, considering ETV or combination therapy, although guidance and options are currently limited.

- iv If there is an ongoing emergence of data to suggest TFV resistance, there will be a need for expert guidelines to include practical recommendations in order to unify clinical approaches. HIV guidelines should take an active stance on incorporating recommendations for those with HBV co-infection, particularly if dual therapy regimens are adopted.
- v Evidence of TFV resistance highlights the need for development of robust novel direct acting antivirals and immune therapies for HBV.

Caveats and limitations

There is sparse literature on HBV resistance to TFV, and studies are of varying quality. While there is a high genetic barrier to selection of TFV resistance, it is likely that there is under-reporting of cases of resistance, particularly in low/middle income settings in which routine monitoring of HBV viral load on treatment is not undertaken. It can be difficult to infer the impact of common polymorphisms on drug resistance phenotype; for example, it is plausible that M204I/V may be enriched among TFV resistant strains simply as a 'footprint' of prior exposure to 3TC.

Most studies to date have used Sanger sequencing, and it is possible that significant minority variants may be under-represented, as suggested by one report in which phenotypic TFV resistance was associated with RAMs in <20% of minor variants³³. Low HBV DNA viral loads are a further barrier to sequencing, and bias existing data towards samples from individuals with high viral loads, in which the full spectrum of relevant RAMs may not occur. It is therefore important to invest in deep sequencing platforms that offer the opportunity to explore the full landscape of HBV variants isolated from an infected individual, and to improve sensitivity of sequencing methods including both Sanger and 'next-generation' approaches. Some sequencing methods, such as Oxford Nanopore Technologies, can generate long reads that allow reconstruction of complete viral haplotypes, providing improved certainty about linkages between sites⁵⁷. To be able to undertake an appropriate haplotypes analysis, datasets with robustly phenotyped patients (displaying clinical evidence of drug resistance), together with full length viral sequence data, would be required; such datasets have not been generated to date but are an important long-term aim.

We recognise the limitations of drawing direct comparisons between HIV and HBV RT, given the limited (<30%) sequence homology between the two enzymes, and the finding that only 2/37 sites associated with TVF resistance in HBV are homologous RAMs in HIV. This highlights a need for future work to solve the crystal structure of HBV RT.

Conclusions

We have assimilated emerging evidence for HBV polymorphisms that reduce susceptibility to TFV, also acknowledging

the potential influences of other viral and host factors in cases of persistent viraemia on therapy. While the genetic barrier to resistance is high, evidenced by the large number of mutations that typically have to be selected to produce resistance, of concern is the overlap with other NA resistance mutations, and the instances in which individual amino acid polymorphisms may be sufficient to produce phenotypic resistance. Enhanced studies representing larger numbers of patients, tracking longitudinal viral sequence changes, and monitoring viral suppression over time are needed. In addition, the evolution of better *in vitro* models will support experiments to investigate the effect of individual and combined RAMs. In order to optimise the use of NA therapy as a tool in driving advancements towards elimination at a population level, improved insights into drug resistance are essential. If resistance emerges as a substantial clinical problem, there will be a need for consideration of synergistic drug regimens, new agents that inhibit a target other than viral RT, and for the development of new therapeutic strategies that can bring about cure.

Data availability

Source data

HIV HXB2 reference sequence on GenBank, Accession number [K03455](#)

HBV Geno A reference sequence on GenBank, Accession number [FJ692557](#)

HBV Geno B reference sequence on GenBank, Accession number [GU815637](#)

HBV Geno C reference sequence on GenBank, Accession number [GQ377617](#)

HBV Geno D reference sequence on GenBank, Accession number [KC875277](#)

HBV Geno E reference sequence on GenBank, Accession number [GQ161817](#)

Extended data

Figshare: Tenofovir resistance in HBV. <https://doi.org/10.6084/m9.figshare.8427746.v2>⁵

This project contains the following extended data:

- Suppl Fig 1 (Pharmacokinetics of tenofovir disoproxil fumarate (TDF), tenofovir alafenamide (TAF) and adefovir dipivoxil (ADV), in PDF format)
- Suppl Fig 2 (PRISMA flow diagram in PDF format)
- Suppl Table 1 (Metadata for 15 studies reporting TDF resistance in HBV infection, in XLSX format)
- Suppl Table 2 (Quality assessment for 15 studies reporting TDF resistance in HBV infection, in XLSX format)

- Suppl Table 3 (List of 37 tenofovir RAMs in HBV Reverse Transcriptase (RT) reported in 15 studies identified through a systematic literature review, in XLSX format)
- Suppl Table 4 (List of TFV RAMs in HBV, showing consensus residue at each position according to HBV genotype, and genotype-specific resistance where this has been reported, in PDF format)
- Suppl Table 5 (Reference sequence alignment of HBV RT and HIV RT, in XLSX format)
- Suppl Table 6 (Mapping of RAMs to the HIV RT structure, in XLSX format)

Reporting guidelines

Figshare: PRISMA checklist for ‘Tenofovir resistance in HBV’. <https://doi.org/10.6084/m9.figshare.8427746.v2>

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](#).

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Reviewer Report 14 April 2021

<https://doi.org/10.21956/wellcomeopenres.17540.r43245>

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Suzane K Ono 

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This is an excellent systematic review on clinical, *in vitro* and *in silico* TNF resistance. The authors reviewed and listed polymorphisms associated to TNF resistance and analyzed the existence of polymorphisms according to HBV genotypes. In addition, compared the polymorphisms in the HBV and HIV RT gene sequence alignments.

Comments:

Figure 1. The green box is named within active site? Is it correct? Please verify.

Figure 1. As mentioned by the authors in the text, polymorphisms at position 80, 173 and 184 are described as compensatory changes, therefore the title "RAMs associated with TDF/TAF ..." this could mislead the readers to believe they are all confirmed resistant mutations.

The urgent need for more HBV sequencing data is important for research and better understanding of the topic; however, it is important to first check patient adherence to treatment (feasible and lower cost) and then look for RAMs. Therefore, I would suggest to change position of item "i" There is urgent need for more HBV sequencing... with item "ii"

Are the rationale for, and objectives of, the Systematic Review clearly stated?

Yes

Are sufficient details of the methods and analysis provided to allow replication by others?

Yes

Is the statistical analysis and its interpretation appropriate?

Not applicable

Are the conclusions drawn adequately supported by the results presented in the review?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Hepatology - hepatitis B.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 08 April 2021

<https://doi.org/10.21956/wellcomeopenres.17540.r43247>

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TVF Abbreviation was not explained in full.

This is an excellent systematic review. There is still however limited data regarding HBV primary resistance against TFV or TDF. The data is even sparse in low/middle income settings such as sub Saharan Africa where routine monitoring of HBV viral load on treatment is rarely undertaken despite high usage of TDF as part of HIV/AIDS management. While some resistance mutations have been reported, phenotypic studies are lacking regarding primary TDF resistance mutation. In a recent Gilead HBV clinical studies, all the patients harbouring rtA194T or CYE/CYEI (S106C, H126Y, D134E and L269I) at baseline achieved viral suppression by week 96 after TDF or TAF treatment. No patients developed an rtA194T mutation or > 1 substitution of CYEI, and the number of patients losing any substitutions of CYEI (n = 17) was similar to the number who developed a single substitution of CYEI (n = 32) during treatment. Phenotypic evaluation of the site-directed mutant panel containing these mutations with or without other resistance mutations did not demonstrate a significant shift in TDF and TAF potency *in vitro*. No evidence of rtA194T and CYEI conferring resistance to TDF or TAF was observed based on the treatment responses to TDF or TAF in patients with mutations at baseline, the lack of selection of mutations after starting TDF or TAF treatment and no change in susceptibility to TDF or TAF *in vitro*. Overall, rtA194T and CYEI did not appear to confer resistance in any patient on a TDF or TAF regimen.

Are the rationale for, and objectives of, the Systematic Review clearly stated?

Yes

Are sufficient details of the methods and analysis provided to allow replication by others?

Yes

Is the statistical analysis and its interpretation appropriate?

Not applicable

Are the conclusions drawn adequately supported by the results presented in the review?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 16 December 2020

<https://doi.org/10.21956/wellcomeopenres.17540.r41653>

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This is an interesting systematic review. Whether HBV may develop primary resistance to tenofovir (TFV or TDF) remains controversial. Some resistance mutations early reported have been denied by other investigators, such as for rtA194T. Recently, a Korean group reported that a quadruple mutation pattern could confer virus resistant to TFV. However, the results were also questioned for this mutation pattern was also reported being detected in TFV-naive patients. In fact, TFV and adefovir (ADV) had similar structure of nucleotide analogue with similar viral suppression ability. TFV is superior to ADV just due to much lighter side-effect. As a result, the daily dose of TFV is 30 fold higher over the dose of ADV (300 mg for TFV vs. 10 mg for ADV). Therefore, since ADV resistance mutation (rtN236T, rtA181V) which confer at least 2-fold increase of EC50 could lead to resistance, a primary TFV-resistance mutation or mutation pattern should at least confer 60-fold increase of EC50. It is a pity that no convincing data are available to confirm that a mutation or a mutation pattern could certainly confer such an increase of EC50 to TFV. From this view, currently reported HBV mutations are lack of full evidence from phenotypic analysis to be a primary TFV-resistance mutation. For my part, only a few reported mutation(s) such as the quadruple mutation pattern could be termed as TFV-resistance-associated mutations. The mutations could apparently increase EC50 (but less than 60 fold) and thus decrease the effect of TFV. With some other influencing factor, the resistance is likely to occur. In general, this systemic review comprehensively collected and analyzed relevant literatures. The analysis conclusion was adequate with novelty. My only concern is whether it is appropriate to term all mutations listed in Figure 1 as "mutations associated with TFV", because supportive evidence for most of them to be a TFV-associated mutation is weak or even lack. Probably they should be described as "mutations potentially associated with TFV".

Are the rationale for, and objectives of, the Systematic Review clearly stated?

Yes

Are sufficient details of the methods and analysis provided to allow replication by others?

Yes

Is the statistical analysis and its interpretation appropriate?

Not applicable

Are the conclusions drawn adequately supported by the results presented in the review?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious Diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
