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Hepatitis B virus genetic diversity has minimal impact on sensitivity of the viral ribonuclease H to inhibitors

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Abstract

Hepatitis B virus (HBV) causes hepatitis, cirrhosis, liver failure, and liver cancer, but the current therapies that employ either nucleos(t)ide analogs or (pegylated)interferon α do not clear the infection in the large majority of patients. Inhibitors of the HBV ribonuclease H (RNaseH) that are being developed with the goal of producing anti-HBV drugs are promising candidates for use in combination with the nucleos(t)ide analogs to improve therapeutic efficacy. HBV is genetically very diverse, with at least 8 genotypes that differ by ~8% at the sequence level. This diversity is reflected in the viral RNaseH enzyme, raising the possibility that divergent HBV genotypes or isolates may have varying sensitivity to RNaseH inhibitors. To evaluate this possibility, we expressed and purified 18 patient-derived RNaseHs from genotypes B, C, and D. Basal RNaseH activity and sensitivity to three novel RNaseH inhibitors from three different chemotypes were assessed. We also evaluated four consensus HBV RNaseHs to determine if such sequences would be suitable for use in antiviral drug screening. The patient-derived enzymes varied by over 10-fold

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

JAV and GL designed and conducted most of the experiments, assisted with data interpretation, and wrote the manuscript. MJD conducted the genetic analyses and chose the variant RNaseHs for analysis. XC assisted with protein purification and data analyses. MJM and RH synthesized compound #12. JET conceived of the project, directed its execution, helped interpret all data, and edited the manuscript. All authors approved the final version.

SUPPLEMENTAL DATA

Supplemental Data File 1. RNaseH and RNA sequences employed.

Supplemental Table 1. Genbank accession numbers for the 300 HBV isolates employed to identify the variant RNaseHs for phenotypic evaluation.

Supplemental Figure 1. Alignment of the RNaseH sequences employed.

in their basal RNaseH activities, but they were equivalently sensitive to each of the three inhibitors. Similarly, all four consensus HBV RNaseH enzymes were active and were equally sensitive to an RNaseH inhibitor. These data indicate that a wide range of RNaseH sequences would be suitable for use in antiviral drug screening, and that genotype- or isolate-specific genetic variations are unlikely to present a barrier during antiviral drug development against the HBV RNaseH.

Keywords

Hepatitis B virus; DNA virus; genetic diversity; ribonuclease H

1. INTRODUCTION

Hepatitis type B is caused by hepatitis B virus (HBV), a member of the *Hepadnaviridae* family. HBV chronically infects up to 350 million people worldwide and kills over 600,000 patients annually, and it is the leading infectious cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma worldwide (Ganem and Prince, 2004; Lavanchy, 2004; Shepard et al., 2006; Sorrell et al., 2009). HBV is an enveloped virus that contains an icosahedral nucleocapsid core particle surrounding the viral DNA genome. The nucleocapsid also contains the viral polymerase protein (P). P is a reverse transcriptase that copies the RNA form of the viral genome into partially double-stranded DNA. The principal therapy for hepatitis B employs nucleos(t)ide analog drugs that inhibit DNA polymerization by P. This treatment suppresses HBV levels in serum to near or below the clinical limit of detection (Cox and Tillmann, 2011; Kwon and Lok, 2011), but viral replication is not completely eliminated (Coffin et al., 2011; Zoulim, 2004) and viremia rebounds in the vast majority of patients when the drugs are withdrawn. However, HBV infections are cleared in a few percent of HBV patients after years of nucleos(t)ide analog therapy (Marcellin et al., 2008; van Bommel et al., 2010; Woo et al., 2010; Wursthorn et al., 2010), implying that the infection could be cleared in more patients by suppressing HBV further. Greater suppression of HBV will require new drugs that will probably be used in combination with the nucleos(t)ide analogs treatments that already exist (Block et al., 2013; Tavis et al., 2013b).

HBV reverse transcription is catalyzed by two enzymatic activities that are both located on the viral P protein. The DNA polymerase activity synthesizes new DNA, and the ribonuclease H (RNaseH) destroys the RNA template after it has been copied into DNA (Seeger et al., 2013). Blocking either activity prevents synthesis of mature viral genomes, including both the cccDNA nuclear form of the genome that is the template for all viral RNAs and the partially double-stranded form found in infectious virions. Inhibiting the RNaseH causes viral genomic replication to stall, leading to an incomplete minus-polarity DNA strand and failure to synthesize the plus-polarity DNA strand (Chen and Marion, 1996; Chen et al., 1994; Gerelsaikhon et al., 1996). Drugs have not been developed against the HBV RNaseH despite it being a logical target, in large part due to technical difficulties in developing screening assays. We recently developed a low-throughput screening pipeline to identify inhibitors of the HBV RNaseH (Tavis et al., 2013a; Tavis and Lomonosova, 2015).

This pipeline has been used to identify over 60 compounds that block viral DNA replication by suppressing the RNaseH.

RNaseH enzymes cleave RNA within a DNA:RNA heteroduplex (Hostomsky et al., 1993). RNaseH enzymes belong to the nucleotidyl transferase superfamily, whose members share a similar protein fold and enzymatic mechanisms (Nowotny, 2009; Yang and Steitz, 1995). This large family of proteins includes the retroviral RNaseHs and integrases, including the HIV enzymes (Dyda et al., 1994). The RNaseH active site contains four conserved carboxylates (the "DEDD" motif) that coordinate two divalent cations, usually Mg^{2+} (Nowotny et al., 2005). The RNase cleavage mechanism requires both cations to promote a hydroxyl-mediated nucleophilic scission reaction (Klump et al., 2003; Nowotny and Yang, 2006; Yang and Steitz, 1995), and the HBV RNaseH inhibitors appear to function by binding to the Mg^{++} ions and impeding their function during catalysis (Tavis and Lomonosova, 2015).

HBV has 8 genotypes (A–H), plus provisional identification of genotypes I and J, that differ by >8% at the sequence level (Kramvis et al., 2005; Kurbanov et al., 2010b; Osioy et al., 2006). Most genotypes are divided into subtypes that differ by >4.5%. Distribution of the genotypes varies around the world, with genotypes B and C being dominant in southeast Asia and genotype A and D being more common in the USA and Europe. Genotypes C and D are somewhat more virulent than other genotypes (Li et al., 2015; Lu et al., 2013; Osioy et al., 2006). Despite the large genetic variation between and among the genotypes, there is little evidence that they differ significantly in their sensitivity to nucleos(t)ide analog drugs (Osioy et al., 2015).

Little is known regarding how HBV's genetic diversity affects its sensitivity to the newly discovered RNaseH inhibitors, although cross-genotypic inhibition is possible because we found that RNaseH inhibitors can block replication of both genotype A and D isolates in cell culture (Hu et al., 2013; Tavis et al., 2013a). Therefore, we evaluated RNaseH activity and sensitivity to RNaseH inhibitors for 18 patient-derived HBV RNaseHs from genotypes B, C, and D, plus four consensus RNaseH sequences derived from genotypes B, C, and D. These genotypes were selected because they are among the most common (Kramvis et al., 2005; Kurbanov et al., 2010a) and they account for most of HBV's pathology (Tanwar and Dusheiko, 2012). The RNaseH inhibitors were selected from three different chemical classes (Cai et al., 2014; Hu et al., 2013; Tavis et al., 2013a) to evaluate potential chemotype-specific effects of HBV variation on inhibitor efficacy.

2. MATERIALS AND METHODS

2.1 Compound acquisition and synthesis

The compounds employed are shown in Fig 3. Compound #46 (β -thujaplicinol) was acquired from the NCI Developmental Therapeutics Program, compound #1 was purchased from Toronto Research Chemicals, and compound #12 was synthesized at Saint Louis University following established protocols (Williams et al., 2010; Williams et al., 2008). All compounds were 95% pure. They were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at $-80^{\circ}C$ in small aliquots in opaque tubes.

2.2 HBV RNaseH genotype specific and consensus sequences

One hundred complete genomes of each HBV genotype (B, C and D) were downloaded from NCBI in April, 2014 (accession numbers in Supplemental Table 1). The reading frames were extracted using customized perl scripts and protein sequences were generated from each reading frame. The protein sequences corresponding to the RNaseH domain (amino acids 684–845 of the full-length polymerase protein; reference strain ADW2, genotype A) were extracted using a customized perl script. The protein sequences were aligned using MUSCLE (<http://www.ncbi.nlm.nih.gov/pubmed/15034147>) with default parameters. The aligned protein sequences were imported into Geneious (v 5.8, Biomatters Limited) and the consensus sequences were generated using a 50% threshold. The pan-genotype meta-consensus was generated from an alignment of the three consensus genotype sequences. Phylogenetic trees were generated employing the neighbor-joining algorithm with a WAG substitution model implemented in the Topali program (Milne et al., 2009). Six RNaseH sequences distributed throughout the genetic space occupied by each of the three genotypes were selected for activity analyses to ensure adequate sampling of genetic variation within the genotype. The RNaseH sequences employed are in Supplemental Data File 1 and an alignment of the sequences is in Supplemental Fig. 1.

2.3 Cloning variant RNaseH sequences

Variant HBV RNaseH sequences were cloned into the expression vector pMAL-c5xHis (Invitrogen) by gene synthesis to create pMal-HRHgtB#1-B#6, pMal-HRHgtC#1-C#6, pMal-HRHgtD#1-D#6, pMal-HRHConB, pMal-HRHConC, pMal-HRHConD, and pMal-HRHConBCD. Ala-Gly-Ala was inserted between the N-terminal maltose binding protein (MBP) tag and the HBV RNaseH sequences, and Gly-Ala-Gly was inserted between the RNaseH sequences and the C-terminal hexahistidine tag.

2.4 Expression and purification of the HBV RNaseH domain

The consensus sequences were purified from *E. coli* LOBSTR-BL21(DE3) (Andersen et al., 2013) cells harboring the RNaseH expression plasmids. Overnight cultures were diluted 20-fold in 1 liter of LB broth in the presence of 50 µg/ml of ampicillin and incubated at 37°C with shaking until $A_{600}=0.6$ was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25 mM. After 3 h of incubation at 16°C, cells were pelleted and frozen at –80°C. The pellet was suspended in 30 ml lysis buffer, (buffer A: 50 mM HEPES pH 8.0, 0.1 M NaCl, 2% Tween20, 30% glycerol, 25 mM imidazole) plus 1 mM protease inhibitor cocktail (Sigma), 5 mM ATP, 1 mM MgCl₂, 0.5 mM EDTA and the cells were disrupted by sonication. Cellular debris was eliminated by centrifugation at 54,000 g for 45 min. The supernatant was loaded for 1 hour onto a 5 ml His-Trap column (GE Healthcare) equilibrated with buffer A. The column was washed with 50 ml of buffer A for 2 hours. Bound proteins were eluted with a linear gradient of lysis buffer A: buffer B (buffer B: 50 mM HEPES pH 8.0, 0.1 M NaCl, 2% Tween20, 30% glycerol 0.5 M imidazole) in 25 column volumes. The products were evaluated by 10% SDS-PAGE electrophoresis and Coomassie brilliant blue staining. Samples were dialyzed into 50 mM HEPES pH 7.3, 300 mM NaCl, 20% glycerol, and 5 mM DTT, and stored in liquid nitrogen. The variant patient-derived RNaseH enzymes were purified in a simplified version of this

protocol employing 100 ml bacterial cultures and gravity-feed nickel-affinity columns. RNaseH concentrations were balanced between all samples evaluated in each experiment.

2.5 Oligonucleotide-directed RNA cleavage assay

DNA oligonucleotide-directed RNA cleavage assays were conducted as previously described (Hu et al., 2013; Tavis et al., 2013a) employing the DRF+ substrate RNA (a 264 nucleotide RNA derived from the duck hepatitis B virus genome, sequence in Supplemental Data File 1). Briefly, uniformly ^{32}P -labeled DRF+ RNA was combined with a complementary DNA oligonucleotide or a non-complementary control oligonucleotide. The complementary DNA oligonucleotide was D2526(-) CCACATAGGCTATGTGGAAC, and the non-complementary oligonucleotide was D2507(+) GTTCCACATAGCCTATGTGG. These substrates were incubated with the RNaseH at a final concentration of 50 mM Tris pH 8.0, 190 mM NaCl, 5 mM MgCl_2 , 3.5 mM DTT, 0.05% NP40, 6% glycerol, and 1% DMSO at 42 °C for 90 min. The products were resolved by 7% denaturing polyacrylamide gel electrophoresis, detected by autoradiography, and quantified using ImageJ. Non-specific background values were determined from the incorrect oligonucleotide negative control lane and subtracted from all experimental values.

3 RESULTS

3.1 Activity of variant RNaseHs

One hundred patient-derived HBV sequences for each genotypes B, C and D were obtained from Genbank (Supplemental Table 1) and the RNaseH sequences were deduced from the genomic sequences. The sequences were aligned at the protein level and phylogenetic trees were constructed. Six representative sequences distributed throughout the trees were selected for biochemical analysis for each of genotypes B, C, and D, yielding a total of eighteen variant patient-derived sequences (Table 1 and Fig. 1). These sequences were cloned, expressed in *E. coli* as dual fusion proteins with a maltose binding protein tag at the N-termini and a hexahistidine tag at their C-termini, and purified by nickel affinity chromatography.

Activity of the variant RNaseHs was measured using an DNA oligonucleotide (ODN)-directed RNaseH assay. In this assay, an RNA:DNA heteroduplex substrate is formed by annealing an ODN (20 mer) to the 264 nucleotide (nt) ^{32}P -labeled DRF+ RNA. Cleavage of the RNA by the RNaseHs within the heteroduplex yields two fragments of about 160 and 100 nts that are resolved by denaturing polyacrylamide gel electrophoresis and detected by autoradiography. Quantification of the activity of each protein was calculated as the average of three or more independent replicates. All data were normalized to the activity of a reference RNaseH genotype C isolate that was included in each experiment. All variant enzymes were purified at least twice to exclude variation stemming from purification differences.

A wide variation in basal RNaseH activity was observed for the variant enzymes, ranging from about 10–110% the activity of the reference genotype C RNaseH (Fig. 2). One-way ANOVA analyses indicated that these differences were statistically significant within each of

the three genotypes ($p < 0.01$). The differences between the three genotypes were also statistically significant ($p = 0.05$), with genotype C enzymes being somewhat more active on average. Therefore, the genetic variation in the HBV RNaseH is reflected in the variable enzymatic activity of patient-derived enzymes.

3.2 Biochemical sensitivity of variant RNaseHs to RNaseH inhibitors

Efficacy of HBV RNaseH inhibitors from three different chemical families that we previously identified was evaluated against variant genotype B, C, and D HBV RNaseHs. The compounds were an N-hydroxyisoquinolinedione (compound #1), a naphthyridinone (compound #12), and an α -hydroxytropolone (compound #46) (Fig. 3) (Cai et al., 2014; Hu et al., 2013; Lu et al., 2015; Tavis et al., 2013a). The inhibitors were employed in ODN-directed RNaseH assays at their respective IC_{50} values against the MBP-HRHgtB, C, and D variants: 30 μ M for compounds #1 and #46, and 15 μ M for compound #12. Note that these IC_{50} values are higher than we originally reported because although the MBP-tagged version of the HBV RNaseH is a more robust enzyme and is much easier to purify to homogeneity than our original version of the recombinant HBV RNaseH (Villa et al., 2016), it is 3- to 5-fold less sensitive to inhibition by these compounds. Four variants were selected for analysis from each genotype (variants with the highest and lowest activity plus two intermediates). Treatment of each of these 12 variant HBV RNaseH enzymes with each of the three inhibitors led to suppression of RNaseH activity by ~40–60% relative to their respective DMSO-treated vehicle controls (Fig. 4), and none of the efficacy differences among the variant enzymes within a genotype or between the genotypes was statistically significant. This degree of inhibition is consistent with the expected inhibition levels from use of the inhibitors at $1 \times IC_{50}$.

We extended these results by obtaining IC_{50} values for the six genotype C RNaseH variants against compound #12 using ODN-directed RNaseH assays. The IC_{50} s varied from 5.6 to 16.4 μ M, with an average IC_{50} for the set of enzymes being 11.2 μ M (Table 2). In all cases, RNaseH activity was suppressed to <20% of the activity in the DMSO vehicle control at 50 μ M of compound #12. The differences in the IC_{50} values were not statistically significant by the one-way ANOVA test ($p = 0.69$). Therefore, although minor differences may exist among the IC_{50} values for variant HBV RNaseHs against compound #12, the magnitude of the differences is likely to be too small to be clinically significant if #12 were a therapeutically useful RNaseH inhibitor.

3.3 Characterization of consensus sequences for genotype B, C and D

Employing a single RNaseH enzyme that is representative of HBV's genetic variation during high throughput screening for RNaseH inhibitors would maximize chances of identifying pan-genotypic inhibitors. Consensus sequences have the advantage of representing the center point of a virus' genetic diversity cloud and hence may be more representative than a given individual isolate. However, consensus sequences can also yield non-representative data due to their potential to be artificial sequences not found in nature and from disruption of natural covariances, epistatic interactions, etc. Therefore, to determine if consensus HBV RNaseH sequences may present advantages for antiviral drug screening, we evaluated the RNaseH activity and sensitivity to RNaseH inhibitors for consensus sequences representing

genotypes B, C, and D. The consensus sequences for HBV RNaseH genotype B, C and D were obtained by aligning one hundred sequences for each different genotype, and a genotype BCD metaconsensus (consensus among genotypes B, C, and D) was obtained by aligning the individual consensus sequences for genotypes B, C, and D (Fig. 1 and Supplemental Data File 1). The proteins encoded by these consensus sequences were expressed in *E. coli*, purified, and their activities were tested in an ODN-directed RNaseH assay. MBP-HRHConB, C, D and BCD were each active in the RNA cleavage assays, with no significant differences among them (Fig. 5). Treatment of these four consensus enzymes with $1 \times \text{IC}_{50}$ of compound #12 led to suppression of HBV RNaseH activity by approximately 60% in all cases (Fig. 5). The small differences in degree of inhibition among the four enzymes were not statistically significant. Therefore, the consensus RNaseHs are active enzymes that can be inhibited by RNaseH antagonists similarly to what is observed with the natural patient-derived RNaseH sequences. However, there appears to be no advantage to employing the consensus sequences over a natural isolate for inhibitor screening.

4. DISCUSSION

HBV is a very genetically diverse virus with at least eight genotypes that differ by >8% at the sequence level (Kramvis et al., 2005; Kurbanov et al., 2010a; Tanwar and Dusheiko, 2012). These differences are large enough to cause variable efficacy of direct-acting antiviral compounds that would be analogous to what was encountered with the earlier generations of protease inhibitors for Hepatitis C virus, which were largely specific for only genotype 1 (Murphy et al., 2014). Here, we evaluated the impact of HBV's genetic variation on its RNaseH activity and sensitivity to representative RNaseH inhibitors from three newly discovered chemotypes. We observed a roughly 10-fold variation in the basal RNaseH activity among a collection of 18 variant patient-derived RNaseHs from genotypes B, C, and D, with genotype C enzymes being somewhat more active on average than the genotype B and D enzymes (Fig. 2). Despite this large variation in overall RNaseH activity, there were no significant differences in sensitivity of the enzymes to inhibition by any of the three RNaseH inhibitors (Fig. 4 and Table 2). Similarly, consensus RNaseH enzymes derived from the genotype B, C, and D enzymes were enzymatically active and were inhibited with similar efficacy by an RNaseH inhibitor (Fig. 5).

The ~10-fold variance in basal RNaseH activity in Fig. 2 indicates that genetic variation in the RNaseH domain (Fig. 1 and Supplemental Fig. 1) affects enzymatic phenotype, but the data do not implicate a mechanism for the variation. Uneven quality of the viral purifications was excluded as a source of this enzymatic variation because each enzyme was purified at least twice and they maintained their phenotypes in the repeat preparations. Both the degree of genetic diversity and the magnitude of variability in the RNaseH activity were smaller than we previously observed among 31 variant HCV RNA-dependent RNA polymerases (Cao et al., 2011), in which a 300-fold variation in RNA polymerization efficiency was found. We cannot address whether the HBV RNaseH enzymatic variation correlates with replication fitness differences among the HBV isolates because the variant sequences were derived from Genbank and these entries are not annotated with fitness information. The lack of accumulation of RNA:DNA heteroduplexes within HBV capsids despite the large

variation in basal RNaseH activity among the variant isolates implies that basal RNaseH activity is unlikely to be rate-limiting for HBV genomic synthesis. This could be due to even the least active enzymes having a higher activity than would be minimally needed to keep up with DNA elongation, and/or to compensatory variation in basal activity of the other activities needed for replication (e.g., DNA priming, the three strand transfers, or DNA elongation).

The lack of marked sensitivity differences among the variant HBV RNaseHs to the inhibitors is similar to the modest differences found in sensitivity of HBV to the two classes of drugs used to treat HBV, the nucleos(t)ide analogs and (pegylated)interferon α . Although there are reports of discernable differences in responses to the established therapies among HBV's genotypes (Osiowy et al., 2015), they are not nearly as pronounced as what was seen with the first-generation Hepatitis C virus protease inhibitors (Murphy et al., 2014). The limited variation in HBV's sensitivity to interferon-based therapies is due to interferon acting through host-mediated mechanisms. The limited variability in efficacy of the nucleos(t)ide analogs appears to be due to their binding to the active site of the DNA polymerase active site on P, with the catalytic cleft having very low genetic diversity due to its essential nature during reverse transcription. There is no structural information available for the HBV RNaseH, so genetic variation within the RNaseH catalytic cleft cannot be formally evaluated. However, the ability to identify key catalytic motifs in the HBV RNaseH from sequence alignments implies a similar genetic constraint for the RNaseH active site (Wang et al., 2016). Furthermore, although HBV is genetically diverse, HCV is much more so, leading to greater mean genetic distances between the HCV genotypes compared to the HBV genotypes (8% for HBV, 15% for HCV) (Horner and Naggie, 2015; Kramvis et al., 2005).

The lack of sensitivity differences among the variant patient-derived and consensus RNaseHs to these three RNaseH inhibitors has two implications for anti-RNaseH drug development. First, the choice of which variant enzyme to employ during compound screening can be driven by practical considerations of the assays, such as having higher basal activity or ease of purification, rather than concerns about the applicability of the isolate to the infected patient population. More importantly, it implies that drugs targeting the HBV RNaseH will have broad efficacy among patients infected with a diverse range of HBV isolates from many genotypes. However, continued analysis of variant enzymes from additional HBV genotypes and against additional chemotypes of RNaseH inhibitors as they are identified is warranted because inhibitors that act by other mechanisms and/or that bind to the RNaseH active site in a different manner may be more affected by HBV's genetic variation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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JET is an inventor on USA Patent applications 14/647,331, 61/174,385, 62/174,350, and 62/309,332 that cover therapeutic use of RNaseH inhibitors against HBV.

Abbreviations

HBV	Hepatitis B virus
RNaseH	Ribonuclease H
MBP	Maltose binding protein
gt	Genotype
nt	Nucleotide
DEDD	Active center formed by aspartic, glutamic, aspartic and aspartic
IPTG	Isopropyl- β -D-thiogalactopyranoside
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
bp	Base pair
ODN	DNA oligonucleotide

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Highlights

- Genetic diversity within the HBV RNaseH domain causes variable enzymatic activity of the encoded RNaseH enzymes.
- RNaseH enzymes from genotype C isolates are typically more enzymatically active than isolates from genotypes B and D.
- RNaseHs from HBV genotypes B, C, and D are equivalently sensitive to inhibitors from three chemotypes.
- Consensus RNaseHs are active and their sensitivity to RNaseH inhibitors is similar to that of natural isolates.

Figure 1. Phylogenetic relationship of the variant HBV RNaseH enzymes

Phylogenetic trees were generated for the HBV RNaseH protein sequences employed in this study using the Neighbor-joining algorithm with a WAG substitution model.

Figure 2. Basal RNaseH activity of the variant patient-derived RNaseHs

RNaseH activity was determined employing ODN-directed RNaseH assays, with all values being normalized to activity of a genotype C reference RNaseH that was included in each assay (control,). Values are averages $\pm$ 1 standard deviation from at least three independent experiments. **, $p < 0.01$; * $p = 0.05$.

Compound #1

Compound #12

Compound #46

Figure 3. Compounds employed

Figure 4. Sensitivity of variant HBV RNaseHs to RNaseH inhibitors

Sensitivity of the variant patient-derived RNaseH enzymes to compounds #1, 12, and 46 at their respective IC_{50} values was determined with ODN-directed RNaseH assays, with all values being normalized to activity of a genotype C reference RNaseH that was included in each assay (control). Values are averages $\pm$ 1 standard deviation from at least three independent experiments.

Figure 5. RNaseH activity and inhibitor sensitivity of consensus HBV RNaseHs

A. Representative ODN-directed RNaseH assay with the consensus RNaseH enzymes. –, negative control reactions in which an ODN of the wrong polarity was included; +, DMSO vehicle-treated reactions containing the correct polarity ODN; #12, reactions containing the correct ODN plus 15 μ M compound #12. Lanes labeled “+” show the basal activity of the enzyme. S, substrate; P1, product 1; P2, product 2. **B.** Sensitivity of the consensus RNaseH enzymes to compound #12 at its IC_{50} value (15 μ M) was determined with ODN-directed RNaseH assays, with activity being compared to the activity of a genotype C reference

RNaseH. Only the P2 product band was quantified because the P1 band is subject to preferential degradation by the HBV RNaseH's 3'-5' exonuclease activity (Villa et al., 2016) and hence is present in sub-stoichiometric amounts. The Y axis is arbitrary absorption units measured by densitometry of the autoradiograms. Values are the average of three to four independent experiments $\pm$ 1 standard deviation.

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Table 1

Nomenclature for the HBV RNaseH sequences evaluated

Isolate or sequence ^I	Name
Consensus clones	
Genotype B, C, D metaconsensus	ConBCD
Genotype D consensus	ConD
Genotype C consensus	ConC
Genotype B consensus	ConB
Genotype B clones	
GU815549	gtB#1
AB493827	gtB#2
GU815590	gtB#3
GU815731	gtB#4
AB219430	gtB#5
AB073825	gtB#6
Genotype C clones	
FJ882614	gtC#1
GQ475346	gtC#2
DQ478899	gtC#3
AB042282	gtC#4
AB493838	gtC#5
AF223954	gtC#6
Genotype D clones	
EU594396	gtD#1
EU594382	gtD#2
AB090270	gtD#3
GQ205380	gtD#4
FJ904435	gtD#5
DQ486024	gtD#6

^I Genbank accession numbers or genotypes comprising the consensus sequences.

Table 2IC₅₀ values for the variant genotype C RNaseHs against compound #12

Isolate	IC ₅₀ (μM)
gtC#1	10.2 ± 2.1
gtC#2	12.3 ± 3.0
gtC#3	5.6 ± 0.7
gtC#4	13.3 ± 8.9
gtC#5	9.6 ± 6.2
gtC#6	16.4 ± 10