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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 nucelos(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 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.

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

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 acids; 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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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; Gerelsaikhan 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^{++} (Nowotny et al., 2005). The RNase cleavage mechanism requires both cations to promote a hydroxyl-mediated nucleophilic scission reaction (Klumpp 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; Osiowy 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; Osiowy 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 (Osiowy 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., 2008, 2010). All compounds were ≥95% pure. They were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at −80 °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 L 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 $MgCl_2$, 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 h onto a 5 ml His-Trap column (GE Healthcare) equilibrated with