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β-Thujaplicinol inhibits hepatitis B virus replication by blocking the viral ribonuclease H activity

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ABSTRACT

Hepatitis B virus (HBV) is a hepatotropic DNA virus that replicates by reverse transcription. It chronically infects >350 million people and kills about 1 million patients annually. Therapy primarily employs nucleos(t)ide analogs that suppress viral DNA synthesis by the viral reverse transcriptase very well but that rarely cure the infection, so additional therapies are needed. Reverse transcription requires the viral ribonuclease H (RNAseH) to destroy the viral RNA after it has been copied into DNA. We recently produced active recombinant HBV RNAseH and demonstrated that Human Immunodeficiency Virus (HIV) RNAseH antagonists could inhibit the HBV enzyme at a high frequency. Here, we extended these results to β-thujaplicinol, a hydroxylated tropolone which inhibits the HIV RNAseH. β-Thujaplicinol inhibited RNAseHs from HBV genotype D and H in biochemical assays with IC_{50} values of 5.9 ± 0.7 and 2.3 ± 1.7 μM, respectively. It blocked replication of HBV genotypes A and D in culture by inhibiting the RNAseH activity with an estimated EC_{50} of ~5 μM and a CC_{50} of 10.1 ± 1.7 μM. Activity of β-thujaplicinol against RNAseH sequences from multiple HBV genotypes implies that if chemical derivatives of β-thujaplicinol with improved efficacy and reduced toxicity can be identified, they would have promise as anti-HBV agents.

1. Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus that replicates by reverse transcription (Seeger et al., 2007). It has 8 genotypes (A–H) that differ by >8% at the nucleotide level (Kramvis et al., 2005; Kurbanov et al., 2010). HBV chronically infects >350 million people world-wide and causes liver failure and hepatocellular carcinoma, resulting in about 1 million deaths per year (Ganem and Prince, 2004; Lavanchy, 2004; Shepard et al., 2006; Sorrell et al., 2009).

HBV therapy primarily employs nucleos(t)ide analog drugs that suppress DNA synthesis by the viral polymerase protein (Cox and Tillmann, 2011; Kwon and Lok, 2011) by 4–5 $\log_{10}$ in up to 70–90% patients, often to below the typical detection limit of ~200 copies/ml (Marcellin et al., 2008; van Bommel et al., 2010; Woo et al., 2010). Nucleos(t)ide therapy also reduces the nuclear episomal form of the HBV genome (covalently-closed circular DNA, cccDNA) in the liver by ~1 $\log_{10}$ after 1–2 years (Cheng et al., 2011; Werle-Lapostolle et al., 2004; Wong et al., 2006). However, the nucleos(t)ide analogs eradicate HBV in only 3–6% of patients, even after years of treatment (Marcellin et al., 2008; van Bommel et al., 2010; Woo et al., 2010; Wursthorn et al., 2010). Therefore, treatment is essentially life-long, with ongoing expenses of $400–600/month (Buti et al., 2009; Lui et al., 2010; Ruggeri et al., 2011) and the potential for long-term side effects.

HBV reverse transcription occurs within nascent viral capsid particles in the cytoplasm (Fig. 1). Newly synthesized HBV genomes can either be secreted as virions, or they can be transported into the nucleus to replenish the cccDNA (Levrero et al., 2009; Tuttleman et al., 1986; Zoulim, 2004). The cccDNA is key to HBV persistence because it is the template for all HBV RNAs (Levrero et al., 2009; Zoulim, 2004), and hence curing HBV means eliminating the cccDNA. The nucleos(t)ide analogs suppress viral DNA titres well in most patients but they rarely eradicate the virus, in part because residual viral reverse transcription is sufficient to

Abbreviations: HBV, hepatitis B virus; cccDNA, covalently-closed circular DNA; RC DNA, relaxed circular DNA; RNAseH, ribonuclease H; HIV, human immunodeficiency virus; βTJ, β-thujaplicinol; HRHPL, recombinant wild-type HBV RNAseH; HRHPL-D702A, active site mutant of recombinant HBV RNAseH; HBc, HBV core protein; HBs, HBV surface proteins; Huh7, a human hepatoma cell line; HepG2, a hepatoblastoma cell line.

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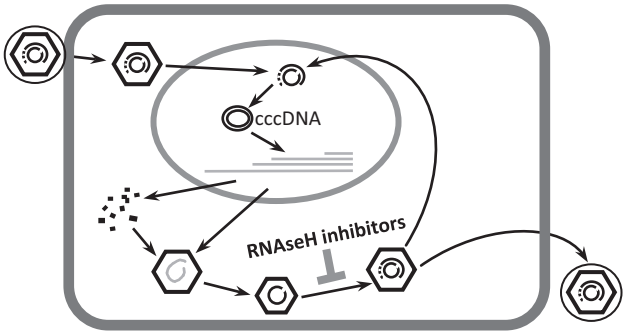

Fig. 1. Role of RNAseH inhibitors in blocking HBV replication and cccDNA accumulation. HBV reverse transcription occurs in cytoplasmic capsid particles. The viral RNA is encapsidated by the viral capsid protein HBc, copied by the viral polymerase to minus-polarity DNA, and then plus-polarity DNA strand is made. Mature capsid particles can either be transported to the nucleus to replenish the cccDNA, or they can be secreted as virions. RNAseH inhibitors would block plus-polarity DNA synthesis and consequently would suppress both secretion of functional virions and cccDNA replenishment. The hepatocyte is represented as a rectangle, the nucleus as an oval, HBV capsid particles as hexagons, and enveloped HBV virions as hexagons within a circle. RNAs are grey and DNAs are black. The figure is modified from Fig. 1 in Tavis et al. (Tavis et al., 2013) under the creative commons license.

support some cccDNA replenishment even in the absence of clinically detectable viremia. Residual replication during antiviral therapy is demonstrated by sequential accumulation of resistance mutations in the reverse transcriptase (Coffin et al., 2011; Ghany and Liang, 2007; Lee et al., 2013; Monto et al., 2010; Zoulim and Locarnini, 2009). Therefore, more patients may be cured if HBV replication could be suppressed far enough to suppress cccDNA replenishment rather than just blocking virion secretion, as is the usual outcome of nucleos(t)ide analog therapy.

HBV reverse transcription requires destruction of the viral genomic RNA after it has been copied into minus-polarity DNA to permit synthesis of the plus-polarity DNA strand. This process is catalyzed by the ribonuclease H (RNAseH) activity encoded at the C-terminus of the multifunctional viral polymerase protein. RNAseH enzymes are members of the nucleotidyl transferase superfamily, which also includes the retroviral integrases (Nowotny, 2009; Yang and Steitz, 1995). The canonical RNAseH structure is comprised of about 100 aa and the active site contains four conserved carboxylates (the "DEDD" motif) that coordinate two essential divalent cations, usually Mg^{+2} (Nowotny et al., 2005). The enzymatic mechanism is believed to involve both divalent cations (Klumpp et al., 2003; Yang and Steitz, 1995), and mutating HBV DEDD residues ablates RNAseH activity (Tavis et al., 2013). The similarity among the RNAseH and integrase enzymes is high enough that some inhibitors of the Human Immunodeficiency Virus (HIV) integrase can inhibit the HIV RNAseH and vice versa (Billamboz et al., 2008, 2011; Klarmann et al., 2002; Shaw-Reid et al., 2003; Williams et al., 2010).

Ablating the HBV RNAseH activity results in incomplete HBV genomes. This causes secretion of HBV particles with defective genomes (Gerelsaikhan et al., 1996; Wei et al., 1996), and would either block transport of the newly-synthesized genomes into the nucleus or prevent repair of the incomplete viral DNA into its biologically active cccDNA form (Fig. 1). Consequently, RNAseH inhibitors could augment the existing nucleos(t)ide analog drugs to improve suppression of the cccDNA. We hypothesized that the similarities between the HBV RNAseH and the HIV RNAseH and integrase enzymes would lead anti-HIV RNAseH and integrase compounds to inhibit the HBV enzyme at a high frequency. To test this hypothesis, we recently produced active recombinant HBV RNAseH and screened 21 compounds predicted to be RNAseH inhibitors based on previous work with HIV (Tavis et al., 2013). Twelve compounds inhibited the HBV RNAseH at $\leqslant$10 μM in biochemical assays, and one inhibited HBV replication in cell culture at low micromolar concentrations by blocking RNAseH activity.

β-Thujaplicinol (βTJ) is a hydroxylated tropolone isolated from the heartwood of Western Red Cedar trees that inhibits the HIV-1 RNAseH with a 50% inhibitory activity (IC_{50}) of 0.2–0.3 μM (Beilhartz et al., 2009; Budihas et al., 2005; Farias et al., 2011). It binds to the HIV-1 RNAseH active site and inhibits the enzyme by chelating the Mg^{+2} ions (Beilhartz et al., 2009; Farias et al., 2011; Himmel et al., 2009). Therefore, we extended our search for inhibitors of the HBV RNAseH by asking whether βTJ could inhibit the HBV RNAseH.

2. Materials and methods

2.1. Plasmids and viral strains employed

Codon-optimized coding sequences for recombinant genotypes D and H HBV RNAseH (HRHPL) with a C-terminal hexahistidine tag were cloned by gene synthesis in pTrcHis2B (Invitrogen). Genotype D HRHPL encodes HBV polymerase residues 684–845 from GenBank V01460; the genotype H clone encodes the homologous amino acid sequences from GenBank AB298362. The human RNAseH1 gene (NP_002927.2) was cloned with an N-terminal hexahistidine-tag in pRsetB (Invitrogen) by gene synthesis. pCMV-HBV-LE- is an HBV over-length genomic expression vector with mutations that block HBV surface protein expression for biosafety reasons. It contains 1.2 copies of the HBV(adw2, genotype A) genome (GenBank X02763.1) downstream of the CMV promoter inpBS (Promega).pCMV-HBV(gtD) is an analogous HBV genomic expression construct for genotype D that expresses wild-type surface antigen proteins.

2.2. Compound acquisition

βTJ was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. It was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at −80 °C.

2.3. RNAseH expression and enrichment

HRHPL and human RNAseH1 were expressed in *Escherichia coli* and enriched by nickel-affinity chromatography as described (Tavis et al., 2013). The enriched extracts were dialyzed into 50 mM HEPES pH 7.3, 300 mM NaCl, 20% glycerol, and 5 mM DTT, and stored in liquid nitrogen.

2.4. Biochemical RNAseH assays

Oligonucleotide-directed RNAseH cleavage assays (Gong et al., 2001) were performed as described (Tavis et al., 2013). Briefly, HBV RNAseH extracts were mixed with a 264 nt-long internally ^{32}P-labeled RNA plus an excess of a complementary DNA oligonucleotide or its inverse-complement as a negative control in a final concentration of 65 mM Tris pH 8.0, 190 mM NaCl, 5 mM $MgCl_2$, 5 mM dTT, 0.05% NP40, and 6% glycerol. βTJ was dissolved in DMSO and added at the indicated concentrations; the final concentration of DMSO in all reactions was 1%. The reactions were incubated at 42 °C for 90 min and terminated by addition of Laemmli protein electrophoresis buffer. The samples were resolved by SDS–PAGE, labeled RNA was detected by autoradiography, the autoradiograms were scanned, and the RNA cleavage products were quantified with ImageJ (National Institutes of Health). IC_{50} values were calculated by non-linear regression using GraphPad Prism (GraphPad Software, Inc.).

2.5. HBV replication inhibition assays

Inhibition of HBV replication by βTJ was measured as recently described (Tavis et al., 2013). Briefly, Huh7 cells were transfected with HBV genomic expression vectors using TransIT-LT1 (Mirus, Inc.), βTJ was added 12–16 h post-transfection at the indicated concentrations, and fresh medium containing the compounds was provided two days later. HBV core particles were isolated four days post-transfection by detergent lysis of the cells and sedimentation through a sucrose cushion as described (Tavis et al., 1998). Viral DNAs were isolated from cytoplasmic core particle preparations by proteinase K digestion followed by phenol/chloroform extraction as described (Gong et al., 2001). Duplicate aliquots of each nucleic acid preparation were treated with 2 U *E. coli* DNAse-free RNAseH (Invitrogen) at 37 °C for 30 min or were mock treated. The nucleic acids were resolved by electrophoresis on 1.2% agarose gels and HBV DNAs were detected by Southern blotting with ^{32}P-labeled double-stranded HBV DNA as a probe to detect both the plus- and minus-polarity HBV DNA strands. HBV core protein (HBc) and β-actin in the cytoplasmic lysates were monitored by western analysis using an anti-HBc antibody (HBP-023-9, Austral Biologicals) and a mouse anti-β-actin monoclonal antibody from ZSGB-BIO Co., Ltd., respectively. HBV surface antigen (HBs) proteins were quantified by ELISA (Shanghai Kehua Biotech).

2.6. Real-time PCR quantification of HBV DNA

Plus-polarity preferential quantitative TaqMan PCR for the HBV DNA was conducted employing forward primer GGAGGCTGTAGGCATAAATTGG, reverse primer AGATGATTAGGCAGAGGTGAAAAAG, and probe 5′-6Fam-CTGCGCACC-Zen-AGCACCATGCA-IabkFQ-3′ (Integrated DNA Technologies). PCR was conducted on purified HBV capsid-derived nucleic acids for 40 cycles of 95 °C for 15 s and 65 °C for 1 min employing the TaqMan universal PCR master mix (Applied Biosystems). Results were standardized against serial dilutions of cloned HBV DNA. The EC_{50} was estimated by linear regression of the real-time PCR data.

2.7. Cytotoxicity

Toxicity of βTJ in Huh7 cells was determined with3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) assays that measures mitochondrial dysfunction employing the Cell Titre 96 aqueous nonradioactive cell proliferation assay (Promega), and with the CytoTox-Glo assay (Promega) that reflects cellular integrity. Huh7 and HepG2 cells (1×10^4) were plated in 96-well tissue culture plates, medium containing βTJ at the indicated concentrations was added the next day, the medium was replaced with fresh medium containing βTJ after two days, and after four days the MTS or CytoTox-Glo assays were conducted according to the manufacturer's instructions. The 50% cytotoxicity concentrations (CC_{50}) were calculated by non-linear regression with GraphPad Prism.

3. Results

3.1. Inhibition of recombinant HBV RNAseH by βTJ

We recently expressed recombinant HBV genotype D and H RNAseH in *E. coli* and enriched the enzymes by nickel-affinity chromatography (Tavis et al., 2013). The HBV RNAseH was a minor component in these enriched extracts. It was undetectable by Coomassie staining but was detectable by western analysis with the anti-HBV RNAseH domain monoclonal antibody 9F9 (Fig. 2A). The concentration of full-length HRHPL was estimated to be 0.5 ng/μl in our extracts by comparison in western blots to full-length HBV polymerase at a known concentration. The RNAseH activity of this enzyme was readily detected using an oligonucleotide-directed RNA cleavage assay, and the HBV enzyme was proven to be the source of this activity by mutating two key active site "DEDD" residues (Fig. 2B and C). In this assay, a DNA oligonucleotide is annealed to a uniformly-labeled RNA to create an RNA:DNA heteroduplex and cleavage of the RNA in the heteroduplex yields two RNA fragments. The RNAs are resolved by electrophoresis, detected by autoradiography, and the cleavage products are quantified by densitometry. Control experiments that varied the reaction time from 20 to 90 min and the amount of RNAseH from 1 to 3 ng demonstrated that the assay is linear with respect to both time and enzyme concentration (Fig. 2D). Addition of βTJ to the RNAseH assays at concentrations ranging from 0.02 to 100 μM revealed that βTJ inhibited the RNAseH, with 50% inhibitory concentrations (IC_{50}) of 5.9 ± 0.7 μM for the genotype D RNAseH and 2.3 ± 1.7 μM for the genotype H enzyme (Fig. 3).

3.2. Effect of βTJ on human RNAseH1

RNAseH1 is responsible for about 80% of the RNAseH activity in human cells (Lima et al., 2001, 2007), and hence inhibition of it would be a possible contributor to cellular toxicity from anti-HBV RNAseH drugs. Therefore, we expressed the human RNAseH1 in *E. coli*, enriched it by nickel affinity chromatography as described (Tavis et al., 2013), and tested the effects of βTJ on the recombinant human enzyme in oligonucleotide-directed RNAseH assays. Inclusion of βTJ from 0.02 to 100 μM had no effect on RNAseH1 activity in this assay (Fig. 4).

3.3. Inhibition of HBV replication by βTJ

βTJ was tested for its ability to suppress HBV replication in culture. Huh7 human hepatoma cells were transfected with HBV genomic expression vectors to initiate HBV replication, medium containing vehicle or βTJ at 2.5, 10, 15, and 20 μM was added the following morning, and the βTJ-containing medium was refreshed two days later. After four days, the cells were lysed, HBV capsid particles were isolated by sucrose sedimentation, and nucleic acids were purified. Replicate nucleic acid aliquots were mock treated or treated with DNAse-free *E. coli* RNAseH to destroy RNA:DNA heteroduplexes, and then HBV DNAs were detected by Southern blotting employing a HBV DNA probe that detects both strands of the viral DNA.

RNAseH deficiency blocks synthesis of HBV plus-polarity DNA and consequently blocks production of the slowest-migrating, most-mature relaxed circular form of the viral DNA (RC DNA). It also causes accumulation of RNA:DNA heteroduplexes in which the DNA migrates as double-stranded species in the absence of exogenous RNAseH treatment, but as faster-migrating single-stranded species upon degradation of the RNA strand. Therefore, inhibiting the RNAseH activity in this experiment would have two effects. First, the amount of the RC DNA in the mock-treated sample would be reduced. Second, DNAs that appeared as double-stranded forms in the mock-treated aliquot would migrate faster in the RNAseH-treated aliquot of the same nucleic acid preparation.

DNAs produced by the wild-type genome contained the mature RC DNA, and mobility of the spectrum of double-stranded species was unaffected by RNAseH treatment (Fig. 5A, compare the mock-treated sample in lane 1 to the RNAseH-treated sample in lane 2). Cells transfected with an RNAseH-deficient genome carrying a mutation in an essential active site DEDD residue (D702A) did not produce mature RC DNA (Fig. 5A lane 3); rather, the viral DNAs were found in duplexes that collapsed to single-stranded forms in

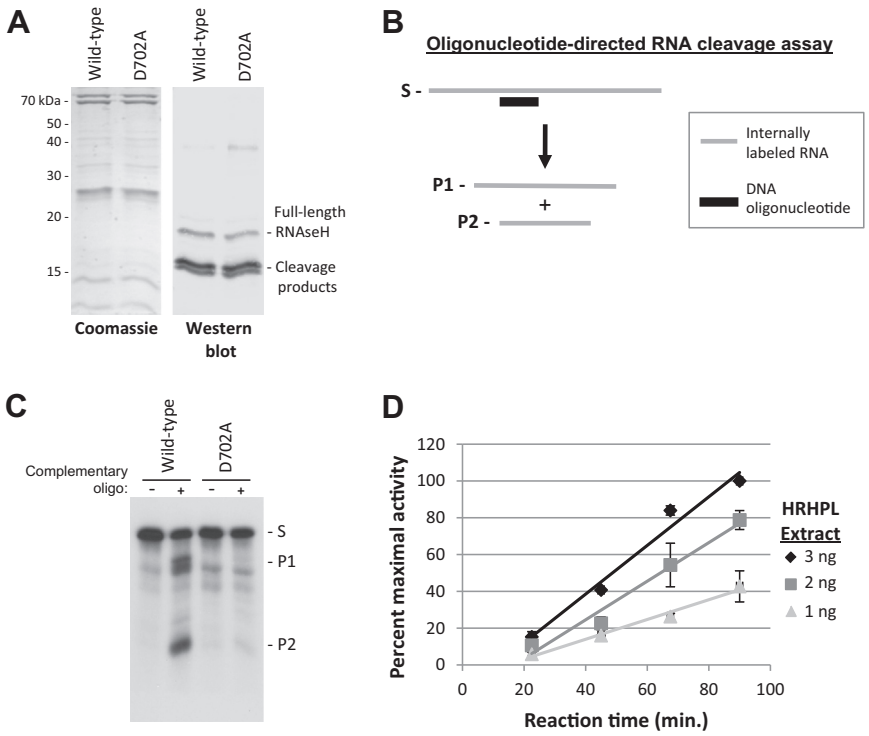

Fig. 2. RNAseH expression and activity. (A) Recombinant HBV RNAseH. Proteins in enriched extracts following nickel-affinity chromatography were detected by Coomassie blue staining or western analysis with monoclonal antibody 9F9 specific for the HBV RNAseH domain. D702A, RNAseH-deficient active-site mutant. (B) Oligonucleotide-directed RNAseH assay. Uniformly ^{32}P-labeled RNA substrate (S) is annealed to a complementary DNA oligonucleotide. RNAseH activity cleaves the RNA in the heteroduplex formed where the oligonucleotide anneals to the RNA and yields two products (P1 and P2). (C) RNAseH activity of enriched recombinant HBV RNAseH. An oligonucleotide-directed RNA cleavage assay was conducted with wild-type and RNAseH-deficient (D702) HBV RNAseH. +, complementary DNA oligonucleotide employed; –, non-complementary oligonucleotide employed. (D) Time- and enzyme-concentration dependence of the RNAseH reaction. Reactions containing 3, 2, or 1 ng RNAseH were incubated for 20–90 min and fit to linear relationships ($R^2 > 0.97$ for each fit). Error bars are ± 1 standard deviation from three experiments.

the RNAseH-treated aliquot (Fig. 5A compare lanes 3 and 4). The heterogeneity in length of the minus-polarity DNA strands revealed following treatment with *E. coli* RNAseH was due to stalling of minus-polarity DNA synthesis in the absence of removal of the RNA strand during reverse transcription that we previously reported (Gerelsaikhan et al., 1996).

Treating cells replicating a wild-type genotype A HBV isolate with βTJ suppressed accumulation of the RC DNA and led to accumulation of RNA:DNA heteroduplexes whose mobility increased upon removal of the RNA strand (Fig. 5B, compare lanes 1 and 2 to the pairs of βTJ-treated samples, for example, lanes 5 and 6). The proportion of HBV DNAs found in heteroduplexes (i.e., DNA strands whose mobility increased following treatment with exogenous RNAseH) increased at higher βTJ concentrations, and the total amount of viral DNA detected was suppressed at 15 and 20 μM. Similar results were observed when cells replicating a HBV genotype D isolate were evaluated (Fig. 5C).

The HBV plus-polarity DNA strand was measured to quantify the effects of βTJ on HBV replication because plus-polarity DNA cannot be made without RNAseH activity. Real-time PCR primers flanking the gap in the HBV minus-polarity DNA were designed that preferentially measure plus-polarity DNA because amplification across the gap in the minus-polarity DNA is inefficient. Plus-polarity preferential PCR revealed that βTJ suppressed HBV genotype A replication to 79 ± 20% relative the DMSO-treated control at 2.5 μM (Fig. 5D). Above 10 μM, βTJ suppressed HBV plus-polarity DNA levels to below the assay background of approximately 14% that was determined with the D702A RNAseH-deficient genome. This implies a 50% effective concentration (EC_{50}) for βTJ against this genotype A isolate in Huh7 cells of approximately 5 μM.

Three control experiments were conducted to determine whether βTJ treatment affected viral or cellular protein accumulation during this four day assay. First, levels of the HBV core protein (HBc) in cytoplasmic extracts were measured by western analysis. Although some sample-to-sample variation was observed in western analysis of HBc in the cellular extracts, HBc levels at the higher βTJ concentrations were similar to those in the DMSO vehicle control (Fig. 5B and C). Second, β-actin was detected in extracts from βTJ-treated cells by western blots as a marker for cellular protein accumulation. Actin levels were unchanged at day four post-transfection (Fig. 5B and C). Finally, levels of the viral surface proteins (HBs) in the medium were analyzed by ELISA to evaluate whether βTJ altered viral protein secretion. HBs expression and secretion by the genotype D isolate was unaffected by βTJ at the end of the experiment even at 20 μM (Fig. 5E). Therefore, βTJ did not substantially affect viral protein expression or overall cellular protein levels over the course of this experiment.

3.4. Cytotoxicity

Toxicity of βTJ in Huh7 cells was assessed by measuring mitochondrial function with an MTS assay, and also by measuring membrane integrity as reflected by release of cytoplasmic proteases into the culture medium employing the CytoTox-Glo assay (Promega). In both assays, cells were plated at the same density employed in the HBV replication assays, medium containing various concentrations of βTJ was added, the βTJ-containing medium was refreshed on day 2, and the toxicity assays were conducted after four days. βTJ was moderately toxic by the MTS assay, with a 50% cytotoxicity concentration (CC_{50}) value of 10.1 ± 1.7 μM. However, βTJ was much less toxic by the membrane integrity

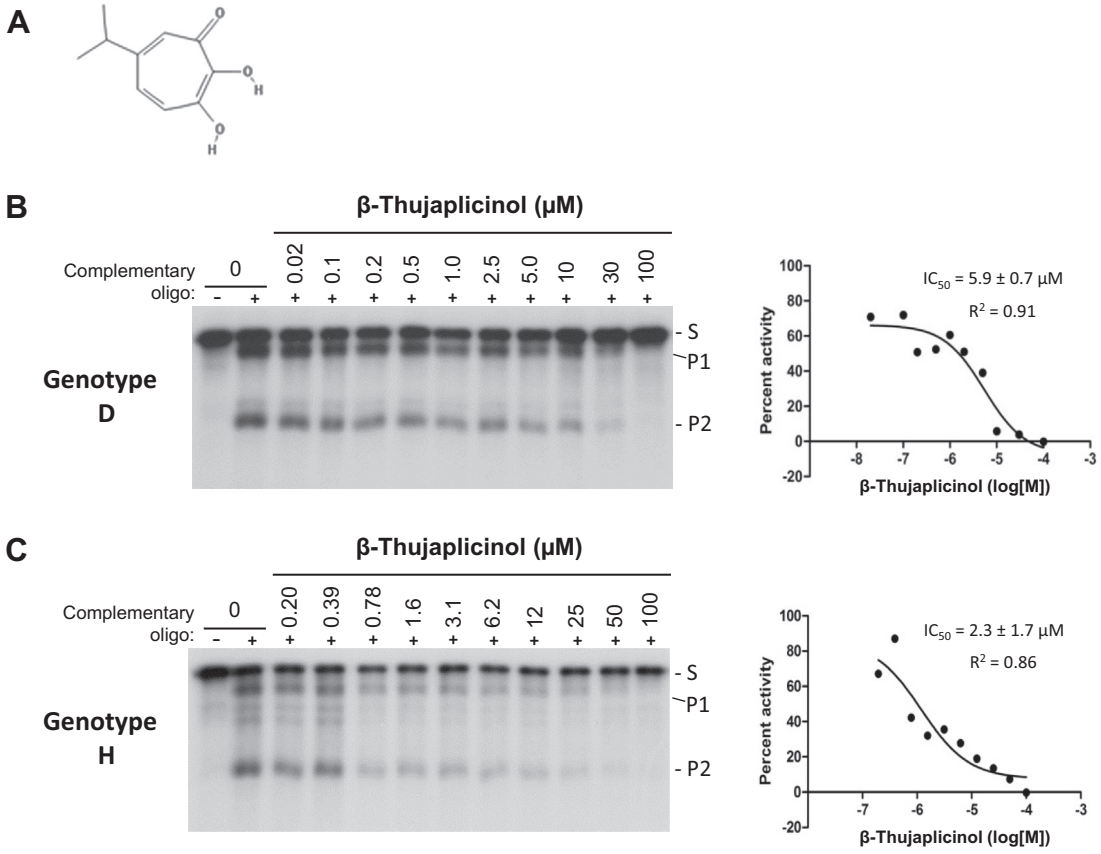

Fig. 3. Inhibition of the HBV RNAseH by βTJ. (A) Structure of β-Thujaplicinol. (B, C) Inhibition of the HBV RNAseH by βTJ was measured with oligonucleotide-directed RNAseH assays and dose–response curves were plotted. S, substrate; P1, larger RNA cleavage product; P2, smaller RNA cleavage product. (B) Genotype D RNAseH. (C) Genotype H RNAseH. The curves are from representative experiments and the IC_{50} values are the average ± 1 standard deviation from three to four experiments.

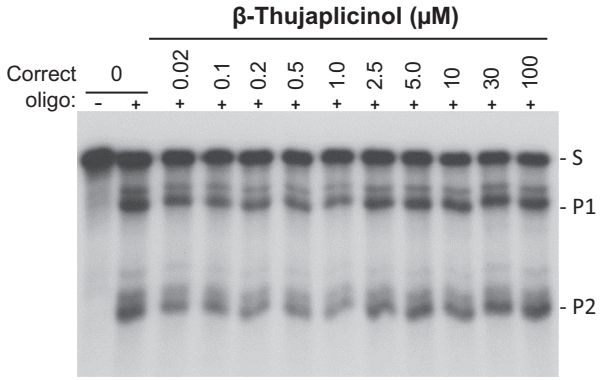

Fig. 4. βTJ does not inhibit recombinant human RNAseH1 in the oligonucleotide-directed RNAseH assay. Oligonucleotide-directed RNAseH assay employing recombinant human RNAseH1. S, substrate; P1, larger RNA cleavage product; P2, smaller RNA cleavage product.

assay, with a CC_{50} > 150 μM (Fig. 6A). The low toxicity in the membrane integrity assay was consistent with the unchanged levels of HBc, HBs, and β-actin detected at the end of the assay (Fig. 5). Therefore, βTJ has a significant impact on mitochondrial function in Huh7 cells, but this effect was not enough to kill the cells during the four day HBV replication assay.

Toxicity of βTJ was also assessed in HepG2 cells because these cells are another human hepatocyte-derived cell line that supports HBV reverse transcription. The CC_{50} of βTJ in HepG2 cells was 16.7 ± 3.7 μM by the MTS assay, similar to its effect on Huh7 cells (Fig. 6B).

4. Discussion

We hypothesized that βTJ may inhibit the HBV RNAseH because it is active against the HIV RNAseH (Beilhartz et al., 2009; Budihas et al., 2005; Farias et al., 2011). As predicted, βTJ inhibited recombinant HBV RNAseH with low micromolar IC_{50} values in biochemical assays, and it blocked HBV replication in cell culture by inhibiting the RNAseH in its native context within the full-length HBV polymerase. βTJ was effective against HBV genotype D and H isolates in biochemical assays and against genotype A and D isolates in the replication assays, indicating that its efficacy against HBV is not genotype-specific.

βTJ has an IC_{50} value of 0.2–0.3 μM against the HIV-1 RNAseH (Beilhartz et al., 2009; Budihas et al., 2005; Farias et al., 2011), but its IC_{50} against the HBV RNAseH was 5.9 and 2.3 μM for the genotype D and H enzymes, respectively. This ~10-fold difference was not surprising because the HBV enzyme is genetically distant from the HIV RNAseH, sharing only about 23% amino acid identity in the core RNAseH domain with its HIV counterpart.

βTJ can efficiently inhibit the HIV RNAseH in biochemical assays, but it is inactive against HIV replication in cells (Chung et al., 2011). In contrast, βTJ inhibited HBV replication in Huh7 cells by targeting the viral RNAseH activity (Fig. 5). βTJ is only the second compound demonstrated to inhibit HBV replication by blocking the viral RNAseH activity, and this is the first time that

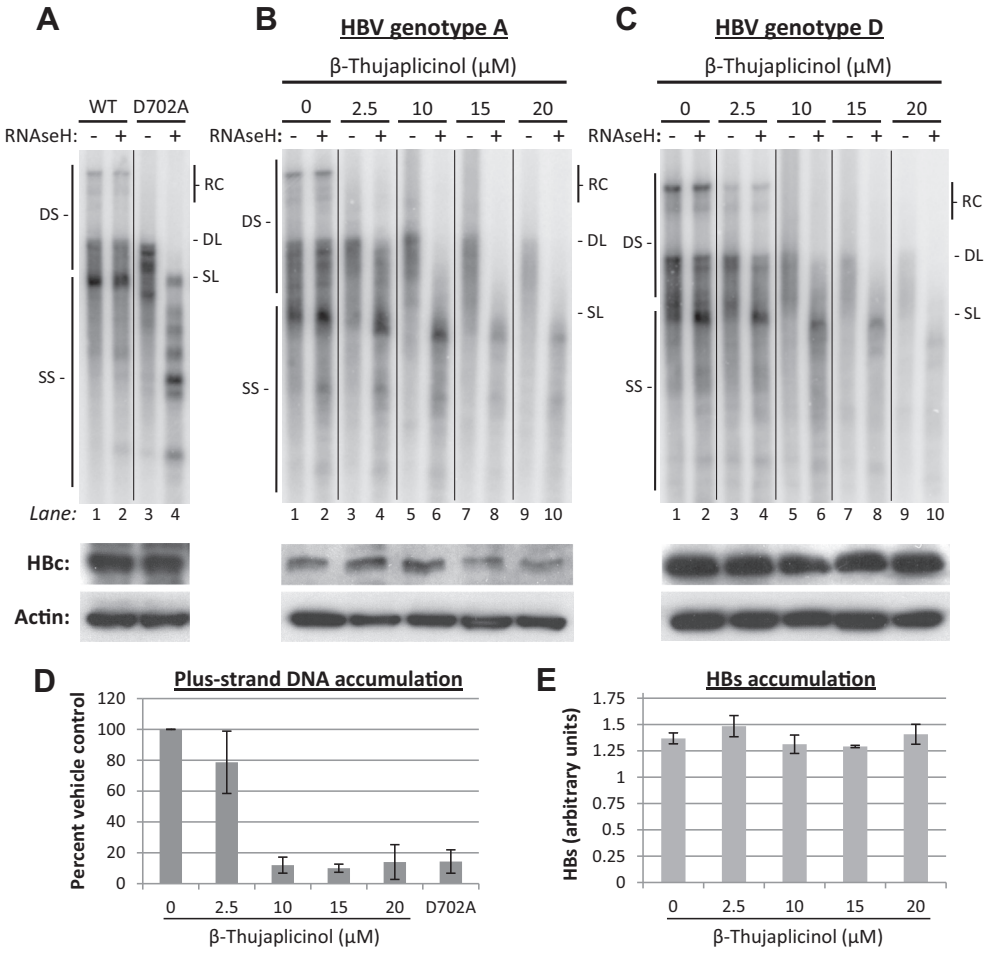

Fig. 5. βTJ inhibits HBV replication by blocking the RNAseH activity. (A) Control assay employing wild-type or D702A RNAseH-deficient HBV genotype A. (B) Effect of βTJ on replication of the wild-type HBV genotype A isolate. (C) Effect of βTJ on replication of a wild-type HBV genotype D isolate. The top images in panels A–C show Southern analyses of HBV capsid DNAs preparations from Huh7 cells replicating HBV. Replicate nucleic acid aliquots were either mock treated or treated with *E. coli* RNAseH to destroy RNA:DNA heteroduplexes. The positions of the mature relaxed-circular (RC), duplex linear (DL), and full-length single-stranded linear (SL) DNAs are shown. DS indicates the spectrum of double-stranded nucleic acids produced by reverse transcription, and SS indicates the spectrum of single-stranded nucleic acids. 0 μM indicates the DMSO vehicle control. The center and bottom images of panels A–C show western analyses of the HBV core protein (HBc) or β-actin in the cytoplasmic lysates. (D) Suppression of HBV plus-polarity DNA strand accumulation. Quantitative PCR preferential for the plus-polarity HBV DNA strand was performed on cytoplasmic capsid particle-derived nucleic acids isolated from Huh7 cells replicating genotype A HBV in the presence of βTJ. The results were normalized to the DMSO vehicle control. Error bars represent ± 1 standard deviation from three independent experiments. (E) HBs accumulation in the medium. Medium was collected after four days incubation in the presence of the indicated concentrations of βTJ for the HBV genotype D isolate and HBs was quantified by ELISA. Error bars are ± 1 standard deviation from three experiments.

a tropolone has been shown to inhibit the HBV RNAseH. We previously reported that napthyridinone inhibited the HBV RNAseH with an IC_{50} of 2.5 μM and that it blocked viral replication in Huh7 cells by ~93% at 10 μM (Tavis et al., 2013). Like βTJ, napthyridinone was moderately toxic, with an estimated CC_{50} in the low μM range in Huh7 cells by the MTS assay. Our observation with βTJ confirms that HBV replication can be pharmacologically inhibited in cells by targeting the RNAseH. Because βTJ was selected for analysis due to its ability to inhibit the HIV RNAseH, these data emphasize the high potential for success from screening inhibitors of the HIV RNAseH for ability to inhibit the HBV enzyme.

βTJ induced substantial toxicity during the four day HBV replication assays in Huh7 cells, with CC_{50} of 10.1 μM by the MTS assay (Fig. 6A). This is similar to the CC_{50} of 2.3 μM that has been reported for βTJ in CEM-SS cells (Chung et al., 2011) and the value of 16.7 μM in HepG2 cells (Fig. 6B). This toxicity appears to be due to mitochondrial dysfunction because the MTS assay measures mitochondrial function. Furthermore, mitochondrial toxicity has been reported for the related compounds β-thujaplicin, tropolone, and tropone in rat hepatocytes (Nakagawa and Tayama, 1998). An alternative mechanism of toxicity due to inhibition of the human RNAseH 1 appears unlikely because βTJ failed to inhibit recombinant human RNAseH1 in our RNAseH assay even when a wide range of enzyme and inhibitor concentrations were employed (Fig. 4 and data not shown). However, βTJ has been reported to have an IC_{50} of 3.5 μM against a similar recombinant N-terminally hexahistidine-tagged human RNAseH1 (Budihas et al., 2005). Our RNAseH1 preparation can be inhibited to varying degrees by other RNAseH antagonists (including napthyridinone) (Tavis et al., 2013), so the reason(s) for this discrepancy is unknown. The much lower toxicity of βTJ in Huh7 cells as measured by the membrane integrity assay (CC_{50} > 150 μM) in this short-term assay is consistent with the MTS assay reflecting a direct action of the inhibitor on the cell. In this context, the lower toxicity measured by the membrane integrity assay would reflect the time needed for mitochondrially-mediated toxicity to cause cellular lysis.

Direct inhibition of HBV replication by βTJ that was independent of its negative impact on cells could be discerned for three reasons. First, HBV capsid protein (HBc) accumulation in the cells (Fig. 5B and C, bottom panels) and secretion of the viral surface proteins (HBs) into the supernatant (Fig. 5E) was unaffected even at 20 μM βTJ. The HBs data are particularly important because this

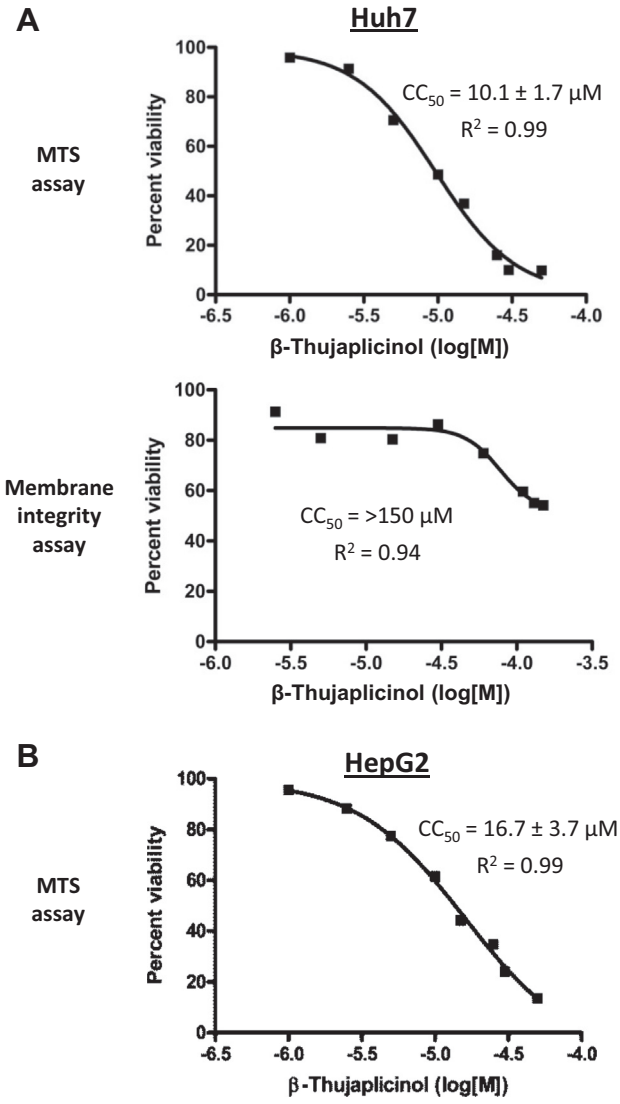

Fig. 6. Cytotoxicity of βTJ. Cells were incubated with the indicated concentrations of βTJ and cellular viability was measured with MTS or membrane integrity assays at day four post-transfection. The CC_{50} value is the average ± 1 standard deviation from three experiments. (A) Huh7 cells. (B) HepG2 cells.

assay measures secretion of HBs between the last medium change and harvesting of the cells (days 3 and 4 post-transfection), eliminating the possibility that we were detecting residual viral proteins that had been produced early in the experiment. Together, the HBc and HBs accumulation data demonstrate that viral protein production was unaffected despite the declining health of the cells at the higher βTJ doses late in the experiment. Second, toxicity in βTJ-treated cells was much lower when cell death was measured by detecting rupture of the plasma membrane (Fig. 6A). This indicates that the detrimental effects of βTJ on cellular metabolism had not progressed to the point where cells were dying and detaching from the plate at the end of the replication assay. Third, the key feature of our Southern blot assay is that it specifically measures the effect of RNAseH activity during viral replication by detecting RNA:DNA heteroduplexes. Comparing the patterns of HBV DNAs in mock-treated and RNAseH-treated aliquots of each HBV nucleic acid preparation reveals the presence of heteroduplexes regardless of the total yield of viral nucleic acids.

The HBV replication assays in Fig. 5 also measured the total yield of viral DNAs in the cultures, as reflected by the net intensity of the Southern blot signal. Part of the reduction in yield of HBV DNAs at high βTJ concentrations may have been due to impaired function of the cells from βTJ toxicity, but part of the loss was also due to inefficient elongation of the minus-polarity DNA strand and lack of synthesis of the plus-polarity DNA strand in the absence of RNAseH activity [Fig. 5A and D D702A samples, and (Chen and Marion, 1996; Chen et al., 1994; Gerelsaikhan et al., 1996)].

The EC_{50} of βTJ against HBV replication (~5 μM) and its CC_{50} by the MTS assay (10.1 μM) were similar in Huh7 cells. This precludes use of βTJ in humans, especially in a long-term treatment regimen such as would be envisioned for anti-HBV therapy. Therefore, chemical optimization will be needed if tropolone compounds are to be developed into anti-HBV drugs. Seven variants of the core hydroxylated tropolone moiety in βTJ have been tested against the HIV RNAseH (Budihas et al., 2005). Two compounds in addition to βTJ inhibited the RNAseH (α-thujaplicin and manicol) with IC_{50} values of 50 and 60 μM, respectively; the remaining compounds had IC_{50} values > 100 μM. Subsequent derivatization of manicol identified14 α-tropolones with IC_{50} values against HIV < 2.0 μM (Chung et al., 2011). Importantly, all of these compounds were less cytotoxic than either βTJ or manicol, and 12 of them could inhibit HIV replication in culture. These precedents with tropolone compounds imply that it may be possible to improve efficacy of βTJ against HBV and reduce its cytotoxicity through chemical derivatization.

The anti-HBV nucleos(t)ide analog drugs are extremely effective at suppressing viremia, and it is very unlikely that a derivative of βTJ would exceed their efficacy as a monotherapy. The clinical potential of βTJ derivatives therefore depends on their ability to work either additively or synergistically with drugs that act through other mechanisms. βTJ presumably inhibits the HBV RNAseH by binding to the enzyme's active site, as has been demonstrated for its effect against the HIV RNAseH (Beilhartz et al., 2009; Farias et al., 2011; Himmel et al., 2009). Binding of the nucleos(t)ide analogs and RNAseH inhibitors to different domains of the HBV polymerase indicates that additive or synergistic action with the nucleos(t)ide analogs against HBV replication is plausible. A precedent with HIV for simultaneous action by βTJ and an inhibitor of the DNA polymerase activity exists because βTJ acts synergistically with calanolide A, a non-nucleoside inhibitor of DNA synthesis by the HIV reverse transcriptase (Budihas et al., 2005).

Employing RNAseH inhibitors in combination with the nucleos(t)ide analogs would have two clinical benefits if they work additively or synergistically with the existing drugs. First, combining nucleos(t)ide analog and anti-RNAseH drugs may suppress HBV replication enough to eliminate HBV from more patients than is currently possible. Second, if HBV RNAseH inhibitors can be produced inexpensively, they could be combined with the inexpensive anti-HBV drug lamivudine. Long-term efficacy of lamivudine treatment is limited because resistance mutations appear in ~20% of patients in the first year and in ~80% after five years (Shaw et al., 2006; Zoulim and Locarnini, 2009). However, lamivudine monotherapy is still widely used in many parts of the world because it is the only drug many patients can afford. Combining lamivudine plus an RNAseH inhibitor would be predicted more effectively suppress viral replication than either drug could achieve alone, and this would slow development of resistance against both lamivudine and the RNAseH inhibitor. Slowing evolution of resistant HBV variants would prolong window of efficacy for these low-cost therapies and have a major impact on hepatitis B in resource-limiting settings.

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References

Beilhartz, G.L., Wendeler, M., Baichoo, N., Rausch, J., Le Grice, S., Gotte, M., 2009. HIV-1 reverse transcriptase can simultaneously engage its DNA/RNA substrate at both DNA polymerase and RNase H active sites: implications for RNase H inhibition. J. Mol. Biol. 388, 462–474.

Billamboz, M., Bailly, F., Barreca, M.L., De, L.L., Mouscadet, J.F., Calmels, C., Andreola, M.L., Witvrouw, M., Christ, F., Debyser, Z., Cotelle, P., 2008. Design, synthesis, and biological evaluation of a series of 2-hydroxyisoquinoline-1,3(2H,4H)-diones as dual inhibitors of human immunodeficiency virus type 1 integrase and the reverse transcriptase RNase H domain. J. Med. Chem. 51, 7717–7730.

Billamboz, M., Bailly, F., Lion, C., Touati, N., Vezin, H., Calmels, C., Andreola, M.L., Christ, F., Debyser, Z., Cotelle, P., 2011. Magnesium chelating 2-hydroxyisoquinoline-1,3(2H,4H)-diones, as inhibitors of HIV-1 integrase and/or the HIV-1 reverse transcriptase ribonuclease H domain: discovery of a novel selective inhibitor of the ribonuclease H function. J. Med. Chem. 54, 1812–1824.

Budihas, S.R., Gorshkova, I., Gaidamakov, S., Wamiru, A., Bona, M.K., Parniak, M.A., Crouch, R.J., McMahon, J.B., Beutler, J.A., Le Grice, S.F., 2005. Selective inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones. Nucleic Acids Res. 33, 1249–1256.

Buti, M., Brosa, M., Casado, M.A., Rueda, M., Esteban, R., 2009. Modeling the cost-effectiveness of different oral antiviral therapies in patients with chronic hepatitis B. J. Hepatol. 51, 640–646.

Chen, Y., Marion, P.L., 1996. Amino Acids Essential for RNAse H activity of hepadnaviruses are also required for efficient elongation of minus-strand DNA. J. Virol. 70, 6151–6156.

Chen, Y., Robinson, W.S., Marion, P.L., 1994. Selected mutations of the duck hepatitis B virus P gene RNase H domain affect both RNA packaging and priming of minus-strand DNA synthesis. J. Virol. 68, 5232–5238.

Cheng, P.N., Liu, W.C., Tsai, H.W., Wu, I.C., Chang, T.T., Young, K.C., 2011. Association of intrahepatic cccDNA reduction with the improvement of liver histology in chronic hepatitis B patients receiving oral antiviral agents. J. Med. Virol. 83, 602–607.

Chung, S., Himmel, D.M., Jiang, J.K., Wojtak, K., Bauman, J.D., Rausch, J.W., Wilson, J.A., Beutler, J.A., Thomas, C.J., Arnold, E., Le Grice, S.F., 2011. Synthesis, activity, and structural analysis of novel alpha-hydroxytropolone inhibitors of human immunodeficiency virus reverse transcriptase-associated ribonuclease H. J. Med. Chem. 54, 4462–4473.

Coffin, C.S., Mulrooney-Cousins, P.M., Peters, M.G., van, M.G., Roberts, J.P., Michalak, T.I., Terrault, N.A., 2011. Molecular characterization of intrahepatic and extrahepatic hepatitis B virus (HBV) reservoirs in patients on suppressive antiviral therapy. J. Viral Hepat. 18, 415–423.

Cox, N., Tillmann, H., 2011. Emerging pipeline drugs for hepatitis B infection. Expert Opin. Emerg. Drugs 16, 713–729.

Farias, R.V., Vargas, D.A., Castillo, A.E., Valenzuela, B., Cote, M.L., Roth, M.J., Leon, O., 2011. Expression of an Mg2+-dependent HIV-1 RNase H construct for drug screening. Antimicrob. Agents Chemother. 55, 4735–4741.

Ganem, D., Prince, A.M., 2004. Hepatitis B virus infection – natural history and clinical consequences. N. Engl. J. Med. 350, 1118–1129.

Gerelsaikhan, T., Tavis, J.E., Bruss, V., 1996. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. J. Virol. 70, 4269–4274.

Ghany, M., Liang, T.J., 2007. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology 132, 1574–1585.

Gong, Y., Yao, E., Tavis, J.E., 2001. Evidence that the RNAseH activity of the duck hepatitis B virus is unable to act on exogenous substrates. BMC Microbiol. 1, 12.

Himmel, D.M., Maegley, K.A., Pauly, T.A., Bauman, J.D., Das, K., Dharia, C., Clark Jr., A.D., Ryan, K., Hickey, M.J., Love, R.A., Hughes, S.H., Bergqvist, S., Arnold, E., 2009. Structure of HIV-1 reverse transcriptase with the inhibitor beta-thujaplicinol bound at the RNase H active site. Structure 17, 1625–1635.

Klarmann, G.J., Hawkins, M.E., Le Grice, S.F., 2002. Uncovering the complexities of retroviral ribonuclease H reveals its potential as a therapeutic target. AIDS Rev. 4, 183–194.

Klumpp, K., Hang, J.Q., Rajendran, S., Yang, Y., Derosier, A., Wong, K.I., Overton, H., Parkes, K.E., Cammack, N., Martin, J.A., 2003. Two-metal ion mechanism of RNA cleavage by HIV RNase H and mechanism-based design of selective HIV RNase H inhibitors. Nucleic Acids Res. 31, 6852–6859.

Kramvis, A., Kew, M., Francois, G., 2005. Hepatitis B virus genotypes. Vaccine 23, 2409–2423.

Kurbanov, F., Tanaka, Y., Mizokami, M., 2010. Geographical and genetic diversity of the human hepatitis B virus. Hepatol. Res. 40, 14–30.

Kwon, H., Lok, A.S., 2011. Hepatitis B therapy. Nat. Rev. Gastroenterol. Hepatol. 8, 275–284.

Lavanchy, D., 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J. Viral Hepat. 11, 97–107.

Lee, G.H., Inoue, M., Toh, J.K., Chong, R.H., Aung, M.O., Koay, E.S., Lim, S.G., 2013. Two-step evolution of the hepatitis B drug-resistant mutations in a patient who developed primary entecavir resistance. Liver Int. 33, 642–646.

Levrero, M., Pollicino, T., Petersen, J., Belloni, L., Raimondo, G., Dandri, M., 2009. Control of cccDNA function in hepatitis B virus infection. J. Hepatol. 51, 581–592.

Lima, W.F., Rose, J.B., Nichols, J.G., Wu, H., Migawa, M.T., Wyrzykiewicz, T.K., Siwkowski, A.M., Crooke, S.T., 2007. Human RNase H1 discriminates between subtle variations in the structure of the heteroduplex substrate. Mol. Pharmacol. 71, 83–91.

Lima, W.F., Wu, H., Crooke, S.T., 2001. Human RNases H. Methods Enzymol. 341, 430–440.

Lui, Y.Y., Tsoi, K.K., Wong, V.W., Kao, J.H., Hou, J.L., Teo, E.K., Mohamed, R., Piratvisuth, T., Han, K.H., Mihm, U., Wong, G.L., Chan, H.L., 2010. Cost-effectiveness analysis of roadmap models in chronic hepatitis B using tenofovir as the rescue therapy. Antivir. Ther. 15, 145–155.

Marcellin, P., Heathcote, E.J., Buti, M., Gane, E., De Man, R.A., Krastev, Z., Germanidis, G., Lee, S.S., Flisiak, R., Kaita, K., Manns, M., Kotzev, I., Tchernev, K., Buggisch, P., Weilert, F., Kurdas, O.O., Shiffman, M.L., Trinh, H., Washington, M.K., Sorbel, J., Anderson, J., Snow-Lampart, A., Mondou, E., Quinn, J., Rousseau, F., 2008. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N. Engl. J. Med. 359, 2442–2455.

Monto, A., Schooley, R.T., Lai, J.C., Sulkowski, M.S., Chung, R.T., Pawlotsky, J.M., McHutchison, J.G., Jacobson, I.M., 2010. Lessons from HIV therapy applied to viral hepatitis therapy: summary of a workshop. Am. J. Gastroenterol. 105, 989–1004.

Nakagawa, Y., Tayama, K., 1998. Mechanism of mitochondrial dysfunction and cytotoxicity induced by tropolones in isolated rat hepatocytes. Chem. Biol. Interact. 116, 45–60.

Nowotny, M., 2009. Retroviral integrase superfamily: the structural perspective. EMBO Rep. 10, 144–151.

Nowotny, M., Gaidamakov, S.A., Crouch, R.J., Yang, W., 2005. Crystal structures of RNase H bound to an RNA/DNA hybrid: substrate specificity and metal-dependent catalysis. Cell 121, 1005–1016.

Ruggeri, M., Cicchetti, A., Gasbarrini, A., 2011. The cost-effectiveness of alternative strategies against HBV in Italy. Health Policy 102, 72–80.

Seeger, C., Zoulim, F., Mason, W.S., 2007. Hepadnaviruses. In: Knipe, D.M., Howley, P., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 2977–3029.

Shaw-Reid, C.A., Munshi, V., Graham, P., Wolfe, A., Witmer, M., Danzeisen, R., Olsen, D.B., Carroll, S.S., Embrey, M., Wai, J.S., Miller, M.D., Cole, J.L., Hazuda, D.J., 2003. Inhibition of HIV-1 ribonuclease H by a novel diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid. J. Biol. Chem. 278, 2777–2780.

Shaw, T., Bartholomeusz, A., Locarnini, S., 2006. HBV drug resistance: mechanisms, detection and interpretation. J. Hepatol. 44, 593–606.

Shepard, C.W., Simard, E.P., Finelli, L., Fiore, A.E., Bell, B.P., 2006. Hepatitis B virus infection: epidemiology and vaccination. Epidemiol. Rev. 28, 112–125.

Sorrell, M.F., Belongia, E.A., Costa, J., Gareen, I.F., Grem, J.L., Inadomi, J.M., Kern, E.R., McHugh, J.A., Petersen, G.M., Rein, M.F., Strader, D.B., Trotter, H.T., 2009. National institutes of health consensus development conference statement: management of hepatitis B. Ann. Intern. Med. 150, 104–110.

Tavis, J.E., Cheng, X., Hu, Y., Totten, M., Cao, F., Michailidis, E., Aurora, R., Meyers, M.J., Jacobsen, E.J., Parniak, M.A., Sarafianos, S.G., 2013. The hepatitis B virus ribonuclease h is sensitive to inhibitors of the human immunodeficiency virus ribonuclease h and integrase enzymes. PLoS Pathog. 9, e1003125.

Tavis, J.E., Massey, B., Gong, Y., 1998. The duck hepatitis B virus polymerase is activated by Its RNA packaging signal, Epsilon. J. Virol. 72, 5789–5796.

Tuttleman, J.S., Pourcel, C., Summers, J., 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 47, 451–460.

van Bommel, F., De Man, R.A., Wedemeyer, H., Deterding, K., Petersen, J., Buggisch, P., Erhardt, A., Huppe, D., Stein, K., Trojan, J., Sarrazin, C., Bocher, W.O., Spengler, U., Wasmuth, H.E., Reinders, J.G., Moller, B., Rhode, P., Feucht, H.H., Wiedenmann, B., Berg, T., 2010. Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. Hepatology 51, 73–80.

Wei, Y., Tavis, J.E., Ganem, D., 1996. Relationship between Viral DNA synthesis and virion envelopment in hepatitis B viruses. J. Virol. 70, 6455–6458.

Werle-Lapostolle, B., Bowden, S., Locarnini, S., Wursthorn, K., Petersen, J., Lau, G., Trepo, C., Marcellin, P., Goodman, Z., Delaney, W.E., Xiong, S., Brosgart, C.L.,

Chen, Gibbs, C.S., Zoulim, F., . Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 126, 1750–1758.

Williams, P.D., Staas, D.D., Venkatraman, S., Loughran, H.M., Ruzek, R.D., Booth, T.M., Lyle, T.A., Wai, J.S., Vacca, J.P., Feuston, B.P., Ecto, L.T., Flynn, J.A., DiStefano, D.J., Hazuda, D.J., Bahnck, C.M., Himmelberger, A.L., Dornadula, G., Hrin, R.C., Stillmock, K.A., Witmer, M.V., Miller, M.D., Grobler, J.A., 2010. Potent and selective HIV-1 ribonuclease H inhibitors based on a 1-hydroxy-1,8-naphthyridin-2(1H)-one scaffold. Bioorg. Med. Chem. Lett. 20, 6754–6757.

Wong, D.K., Yuen, M.F., Ngai, V.W., Fung, J., Lai, C.L., 2006. One-year entecavir or lamivudine therapy results in reduction of hepatitis B virus intrahepatic covalently closed circular DNA levels. Antivir. Ther. 11, 909–916.

Woo, G., Tomlinson, G., Nishikawa, Y., Kowgier, M., Sherman, M., Wong, D.K., Pham, B., Ungar, W.J., Einarson, T.R., Heathcote, E.J., Krahn, M., 2010. Tenofovir and entecavir are the most effective antiviral agents for chronic hepatitis B: a systematic review and Bayesian meta-analyses. Gastroenterology 139, 1218–1229.

Wursthorn, K., Jung, M., Riva, A., Goodman, Z.D., Lopez, P., Bao, W., Manns, M.P., Wedemeyer, H., Naoumov, N.V., 2010. Kinetics of hepatitis B surface antigen decline during 3 years of telbivudine treatment in hepatitis B e antigen-positive patients. Hepatology 52, 1611–1620.

Yang, W., Steitz, T.A., 1995. Recombining the structures of HIV integrase, RuvC and RNase H. Structure 3, 131–134.

Zoulim, F., 2004. Antiviral therapy of chronic hepatitis B: can we clear the virus and prevent drug resistance? Antivir. Chem. Chemother. 15, 299–305.

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