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The Hepatitis B Virus Ribonuclease H as a Drug Target

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Abstract

Chronic hepatitis B virus (HBV) infection is a leading cause of hepatitis, liver failure, and hepatocellular carcinoma. An outstanding vaccine is available; however the number of infections remains high. Current anti-HBV treatments with interferon α and nucleos(t)ide analogs clear the infection in only a small minority of patients, and either induce serious side-effects or are of very long duration. HBV is a small, enveloped DNA virus that replicates by reverse transcription via an RNA intermediate. The HBV ribonuclease H (RNaseH) is essential for viral replication, but it has not been exploited as a drug target. Recent low-throughput screening of compound classes with anti-Human Immunodeficiency Virus RNaseH activity led to identification of HBV RNaseH inhibitors in three different chemical families that block HBV replication. These inhibitors are promising candidates for development into new anti-HBV drugs. The RNaseH inhibitors may help improve treatment efficacy enough to clear the virus from the liver when used in combination with existing anti-HBV drugs and/or with other novel inhibitors under development. This article forms part of a symposium in *Antiviral Research* on "An unfinished story: from the discovery of the Australia antigen to the development of new curative therapies for hepatitis B."

Keywords

Hepatitis B Virus; Ribonuclease H; Reverse transcription; Inhibitors

1. HBV: Disease and genomic replication

Hepatitis B Virus (HBV) is an enveloped DNA virus that replicates in the liver. It is a member of the *Hepadnaviridae* family that includes the animal viruses Duck Hepatitis B Virus (DHBV) and Woodchuck Hepatitis Virus (WHV) (Dandri et al., 2005). HBV chronically infects up to 350 million people world-wide (Seeger et al., 2013). The infection causes hepatitis, fibrosis, cirrhosis, liver failure and over half of all cases of hepatocellular carcinoma (Lavanchy, 2005). Together, this leads to an annual death toll of over 500,000 (Sorrell et al., 2009).

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HBV replicates its genome by reverse transcription of a viral pregenomic RNA within cytoplasmic capsid particles (Seeger et al., 2007; Summers and Mason, 1982; Tavis and Badtke, 2009). Reverse transcription is catalyzed by two enzymatic activities located on different domains of the viral polymerase protein (Chang et al., 1990; Radziwill et al., 1990). The reverse transcriptase copies the pregenomic RNA into minus-polarity DNA, and the ribonuclease H (RNaseH) destroys the viral RNA after it has been copied so that the plus-polarity DNA strand can be made. The direct product of HBV replication is a partially double-stranded DNA molecule within cytoplasmic capsid particles. These capsids may be enveloped and secreted from the cell as mature virions, or they may be transported to the nucleus where the DNA is converted to an episomal covalently-closed circular molecule (cccDNA) (Fig. 1). The cccDNA is key to HBV biology because it is the transcriptional template for all HBV RNAs (it is functionally equivalent to an integrated retroviral provirus).

2. Limitations to current anti-HBV therapy

The nucleos(t)ide analog drugs that dominate HBV therapy have transformed management of HBV chronic infections. The best drugs, tenofovir and entecavir, suppress HBV replication by 4–5 $\log_{10}$ or more in up to 70–90% of patients, often to below the common detection limit of ~200–400 copies/ml (Cox and Tillmann, 2011; Kwon and Lok, 2011; van Bommel et al., 2010; Woo et al., 2010) with little to no drug resistance even after prolonged treatment (Zoulim, 2011). This remarkable success for a monotherapy has made HBV infection controllable for those able to afford its high costs (Block et al., 2013; Lui et al., 2010), with major health benefits for the treated individuals (Dienstag, 2009; Liaw, 2013; Marcellin and Asselah, 2014).

Despite the profound suppression of HBV titers induced by nucleos(t)ide analogs, treatment reduces cccDNA levels by only about 1 $\log_{10}$ even after years of continuous drug exposure (Cheng et al., 2011; Werle-Lapostolle et al., 2004; Wong et al., 2006). Consequently, HBV infections are cleared in only 2–8% of patients after many years of treatment (Liaw, 2013). This persistence of the cccDNA causes viral titers to resurge if the nucleos(t)ide analogs are withdrawn, and hence treatment is essentially life-long. cccDNA persistence is in part due to its long apparent halflife, which is estimated to be 10 days in non-dividing tissue culture cells (Cai et al., 2012) and may be up to 30–60 days in the liver based on inhibitor studies in animal models (Addison et al., 2002; Moraleda et al., 1997; Zhu et al., 2001). However, maintenance of the cccDNA is also due to ongoing viral HBV replication during nucleos(t)ide analog therapy. This is revealed by sequential accumulation of resistance mutations to the nuceos(t)ide analogs (Ghany and Liang, 2007; Monto et al., 2010; Zoulim and Locarnini, 2009), and is confirmed by genetic analyses of viral DNA in the liver during therapy that explicitly demonstrate replenishment of the cccDNA even in the absence of clinically detectable viremia (Coffin et al., 2011). This opens an opportunity for improving antiviral therapy by suppressing HBV replication further than is currently possible.

3. RNaseH enzymes

3.1. General features of RNaseH enzymes

RNaseHs (Hostomsky et al., 1993) cleave RNA when the RNA is bound to DNA in a heteroduplex. They help destroy failed transcription products, remove RNA primers during DNA synthesis, and contribute to reverse transcription of viral and retrotransposon genomes. They belong to the nucleotidyl transferase enzyme superfamily whose members share a similar protein fold and similar enzymatic mechanisms (Nowotny, 2009; Yang and Steitz, 1995). This family includes *E. coli* RNaseH I and II (Katayanagi et al., 1990; Lai et al., 2000), human RNaseH 1 and 2 (Lima et al., 2001), retroviral RNaseH and integrases (Dyda et al., 1994), and many other nucleases and transposases. The canonical RNaseH structure contains about 100 amino acids with four conserved carboxylates (the “DEDD” motif) that coordinate two divalent cations (Nowotny et al., 2005). Integrases have a similar protein fold, but they employ only three carboxylates to coordinate the essential divalent cations (the “DDE” motif) (Nowotny, 2009). The nucleic acid cleavage mechanism is believed to require both divalent cations to promote a hydroxyl-mediated nucleophilic scission reaction (Klumpp et al., 2003; Nowotny and Yang, 2006; Yang and Steitz, 1995).

3.2. The HBV RNaseH

The HBV RNaseH was originally identified by sequence homology to known RNaseH enzymes (Khudyakov and Makhov, 1989; Schodel et al., 1988), and its existence was confirmed by evaluating the effects on viral replication of mutating the predicted RNaseH motifs (Chang et al., 1990; Chen and Marion, 1996; Gerelsaikhan et al., 1996). Ablating HBV RNaseH activity causes accumulation of long RNA:DNA heteroduplexes, truncates most minus-polarity DNA strands, and blocks production of the plus-polarity DNA strand. Virions can still be secreted when the RNaseH activity is blocked, but they contain defective RNA:DNA heteroduplex genomes (Gerelsaikhan et al., 1996; Wei et al., 1996). These genomes are biologically inert due to the severe damage to the viral DNA, especially the suppression of the second and third strand transfer reactions needed to produce mature genomes. Consequently, inhibiting the RNaseH would block both release of infectious virions and replenishment of the nuclear cccDNA (Fig. 1). The RNaseH has not been analyzed biochemically because the full-length polymerase protein is unable to accept exogenous RNaseH substrates (Gong et al., 2001), and because production of active recombinant HBV RNaseH has been a significant challenge.

The HBV and Human Immunodeficiency Virus (HIV) RNaseHs are only distantly related to one another. The genetic distance between the HBV and HIV RNaseHs is so large that the HBV enzyme is genetically about equally related to the HIV RNaseH and integrase, sharing 23% and 19% identity, respectively, in the core catalytic domains (Tavis et al., 2013). Despite this distance, the HBV and HIV RNaseHs perform the equivalent biological roles during viral replication (Freed and Martin, 2007; Tavis and Badtke, 2009).

3.3. HIV RNaseH and integrase as drug targets

The HIV RNaseH has attracted much attention as a drug target, with over 100 compounds based on a variety of chemical scaffolds being reported to have anti-HIV RNaseH activity

(Cao et al., 2014; Chung et al., 2011; Esposito and Tramontano, 2014; Klumpp and Mirzadegan, 2006; Le Grice, 2012). Most of these inhibitors act by binding to the RNaseH active site, in part through direct interactions of three adjacent Lewis basic moieties on the inhibitors with the divalent cations in the active site (Billamboz et al., 2011; Chung et al., 2011; Kirschberg et al., 2009; Su et al., 2010). IC_{50} values for the majority of the inhibitors are in the low micromolar or high nanomolar range. This limited efficacy appears to be primarily due to the shallow, open shape of the HIV RNaseH catalytic groove which provides few contact sites for the inhibitors other than interactions with the two metal ions (Billamboz et al., 2011; Chung et al., 2011; Kirschberg et al., 2009; Su et al., 2010). The inhibitors usually induce modest cytotoxicity, leading to therapeutic index (TI) values that are often <10. Despite these limitations, compounds with efficacy and TI values appropriate for a drug have been reported (Himmel et al., 2006; Williams et al., 2010).

HIV integrase inhibitors are based on multiple chemical scaffolds, especially the diketo- and β-keto acids (Li et al., 2014). Three drugs have been approved: raltegravir, elvitegravir, and dolutegravir (Mesplede et al., 2014). Like the RNaseH inhibitors, the integrase inhibitors usually function by binding to and chelating the divalent cations in the active site (Agrawal et al., 2012; Espeseth et al., 2000; Hare et al., 2010; Hazuda et al., 2009), although binding of the inhibitors is strengthened in many cases by interactions with both the enzyme and the DNA substrate.

As predicted from their common membership in the nucleotidyl transferase superfamily (Nowotny, 2009; Yang and Steitz, 1995) and similar orientation of the two cations in their active sites (Hare et al., 2012), some anti-HIV RNaseH compounds inhibit the integrase, and some anti-HIV integrase compounds inhibit the RNaseH (Billamboz et al., 2008; Klarmann et al., 2002; Williams et al., 2010). This ability of some compounds to cross-inhibit distal members of the nucleotidyl transferase superfamily prompted us to develop the means to screen HIV RNaseH and integrase inhibitors against the HBV RNaseH.

4. Development of a low throughput anti-HBV RNaseH screening pipeline

4.1. Production of active recombinant HBV RNaseH

Although a few reports of active recombinant HBV RNaseH were published prior to our efforts (Choi et al., 2002; Lee et al., 1997; Potenza et al., 2007; Wei and Peterson, 1996), these systems were not developed far enough to be employed for antiviral drug discovery. Consequently, no anti-HBV RNaseH drug discovery efforts have been reported by groups other than our own, although unpublished efforts may have been conducted by the pharmaceutical industry.

We expressed the HBV RNaseH in *E. coli* as a C-terminally hexahistidine-tagged protein (Tavis et al., 2013). The RNaseH was recovered following nickel-affinity purification at very low levels that were detectable only by western analysis. RNaseH activity was evaluated using an oligonucleotide-directed RNA cleavage assay. In this assay, a DNA oligonucleotide is annealed to a uniformly ^{32}P-labeled RNA to create an RNA:DNA heteroduplex. Cleavage of the RNA in the heteroduplex yields two RNA fragments that are resolved by electrophoresis Fig. 2A. Wild-type HBV RNaseH could cleave the RNA,

cleavage required a complementary oligonucleotide, and the size of the RNA products varied as predicted when the oligonucleotide annealing site on the RNA was altered, formally proving RNaseH activity. Most of this activity was due to the HBV enzyme because mutating essential DEDD residues in the active site sharply reduced RNaseH activity. The enzyme had the predicted Mg^{++} dependence and could tolerate up to 2% DMSO (Tavis et al., 2013). We recently produced Coomassie-stainable amounts of the RNaseH as a maltose-binding protein fusion with a hexahistidine tag at its C-terminus (unpublished). HBV is a genetically diverse virus with eight or nine genotypes (A – I) that differ from each other by ≥8% at the nucleotide level (Schaefer, 2007). The RNaseH sequences differ between genotypes by ~6% at the amino acid level (Hayer et al., 2014). Currently, active HBV RNaseH can be produced from HBV genotypes B, C, D, and H.

This recombinant HBV RNaseH has been employed successfully in low throughput drug screening efforts (Cai et al., 2014; Hu et al., 2013; Lu et al., 2015; Tavis et al., 2013), but it remains far from ideal. Although we can produce large amounts of protein at relatively high purity, its specific activity is very low, and its specificty for RNA:DNA heteroduplexes is reduced by oxidizing conditions, divalent cations other than Mg^{++}, and low ionic strength. Furthermore, the yield of RNAseH activity is roughly proportional to the size of the bacterial culture from which the protein is derived, not yield of the RNaseH protein. This implies that an unidentified bacterial component is limiting in formation of active enzyme. High throughput screening against the HBV RNaseH will not be feasible until these challenges are surmouonted. Ongoing efforts to solve these problems include optimization of the expression and purification protocols, truncating the enzyme's C-terminus which aligns poorly with other RNaseHs and is predicted to be intrinsically disordered, and identifying the limiting component in the bacteria.

4.2. Cell-based HBV RNaseH inhibition assays

Inhibition of HBV replication by interfering with the RNaseH activity is best assessed by measuring preferential inhibition of HBV plus-polarity DNA strand synthesis. This is because the plus-polarity DNA cannot be made if the RNaseH has not removed the viral RNA from the newly synthesized minus-polarity DNA strand. In contrast, production of the 5′ half of the minus-polarity DNA strand is largely unaffected (Cai et al., 2014; Gerelsaikhan et al., 1996; Hu et al., 2013). Therefore, we developed a strand-preferential quantitative PCR assay [Fig. 2B; (Cai et al., 2014)]. Replication inhibition is usually measured in HepDES19 cells, which contain a tetracycline-repressible expression cassette for a replication-competent HBV genotype D genome (Guo et al., 2007). Antiviral activity of the RNaseH inhibitors is tested by treating cells replicating HBV with compounds for three days, followed by quantitative PCR detection of the HBV plus- and minus-polarity DNA strands within viral capsid particles (Cai et al., 2014; Lu et al., 2015).

4.3. Low-throughput screening pipeline for HBV RNaseH inhibitors

These assays have been integrated into a low-throughput drug screening pipeline (Fig. 3). Compounds are initially evaluated in a semi-quantitative biochemical assay against HBV RNaseH from a genotype D isolate using the oligonucleotide-directed cleavage assay. Primary hits are confirmed by screening against enzyme from HBV genotype B or C isolates

to eliminate hits specific for only one of HBV's genotypes. Confirmed hits are counter-screened against recombinant human RNaseH1 to identify compounds with a high probability of having unacceptable toxicity. IC_{50} values are then measured for compounds active against RNaseHs from two or more genotypes but have minimal activity against the human enzyme. HBV inhibitors with IC_{50} values <30 μM are selected for evaluation against HBV replication in cell culture. Compounds showing selective suppression of the HBV plus-polarity DNA strand in cells using a semi-quantitative form of the strand-preferential quantitative PCR assay are then evaluated for cytotoxicity, and EC_{50} values are determined for compounds with low toxicity at the effective concentrations. Compounds with favorable therapeutic indexes then undergo *in vitro* and *in vivo* absorption, distribution, metabolism, and excretion studies, plus basic pharmacokinetic and toxicity analyses in mice. Finally, promising compounds undergo efficacy studies in HBV transgenic mice that produce authentic HBV in their livers (Dandri et al., 2006), using the strand-preferential quantitative PCR assay as a readout. The leading anti-RNaseH compounds are just beginning the *in vivo* analyses.

5. α-Hydroxytropolones as example HBV RNaseH inhibitors

5.1. Discovery of α-hydroxytropolone inhibitors of HBV RNaseH

α-hydroxytropolones (αHT) have a seven-membered aromatic ring with three adjacent hydroxyl and carbonyl moieties oriented so that they can chelate the divalent cations of nucleotidyl transferase superfamily enzymes. Consequently, αHT compounds can suppress replication of HIV, the Herpes Simplex Viruses, many bacteria, and some tumors (Meck et al., 2014; Tavis et al., 2014; Zhao, 2007). Fifty-one αHT compounds or related troponoids were purchased, obtained from the NCI Developmental Therapeutics compound repository, provided by Dr. Stuart Le Grice at the National Cancer Institute, or synthesized by Dr. Ryan Murelli at the City University of New York (Chung et al., 2011; Hirsch et al., 2014; Meck et al., 2014; Meck et al., 2012; Williams et al., 2013). Key αHT compounds are in Fig. 4. Thirteen αHTs inhibited the HBV RNaseH at ≤60 μM, with five inhibiting at ≤20 μM in the primary screening assays (compounds #46, 106, 107, 110, and 113) (Lu et al., 2015). The best IC_{50} against the genotype D enzyme was 5.9 μM for compound #46 (β-thujaplicinol) (Table 1). Eleven αHTs were tested against HBV replication in cell culture, and seven suppressed HBV replication, six with EC_{50} values below 10 μM. The best EC_{50} values for αHTs against viral replication were 0.34 for #110 and 1.0 μM for #46. CC_{50} values by MTT assays ranged from 25 to 79 μM, leading to TI values from 3.8 up to a high of 94 for compound #110 (Lu et al., 2015).

5.2. Preliminary structure-activity relationship αHTs against the HBV RNaseH

Four constraints on the structure-activity relationship for the αHTs are apparent. First, the intact α-tropolone moiety is needed because deleting one of the three oxygens on the tropolone ring ablates inhibition (Lu et al., 2015). This implies that αHTs inhibit the HBV RNaseH by the metal-chelating mechanism employed against the HIV RNaseH (Budihas et al., 2005; Chung et al., 2011; Didierjean et al., 2005). Second, at least one appendage must exist at positions R^1, R^2, or R^3 on the αHT scaffold for activity [unsubstituted α-hydroxytropolone was inactive but 13 substituted derivatives were active (Lu et al., 2015);

the positions of the R groups are indicated in Fig. 4]. Third, a wide variety of polar, aliphatic, or aromatic groups are permitted in the appendages. Finally, the appendages at R^1, R^2, and R^3 had to be less than about four atoms in length for substantial activity (Lu et al., 2015). This size limitation implies that the HBV RNaseH active site is probably narrower than the HIV RNaseH active site. The probability of a narrow active site is reinforced by our studies on the N-hydroxyisoquinolinediones, where only the smallest compound tested worked against the HBV enzyme (compound #1) despite many of the larger molecules being active against the HIV RNaseH (Cai et al., 2014). A narrower active site would imply that designing compounds with adequate specificity for the RNaseH will be easier for HBV than HIV. Unfortunately, the breadth of the HBV RNaseH active site cannot be experimentally confirmed because no crystal structure exists.

6. Known anti-HBV RNaseH compounds

Over 190 compounds in the αHT, N-hydroxyisoquinolinedione, napthyridinone, dioxobutanoic acid, hydroxychromenone, Elvitegravir, Raltegravir, aminocyanothiophene, hydroxyxanthenone, cyanopyran, and thienopyrimidinone chemical families have been screened against the HBV RNaseH. Nineteen compounds inhibited the HBV RNaseH at ≤20 μM, with the best hits having low micromolar IC_{50} values [(Cai et al., 2014; Hu et al., 2013; Lu et al., 2015; Tavis et al., 2013) and unpublished]. Strong hits were observed among the αHTs, N-hydroxyisoquinolinediones, napthyridinones, and hydroxychomenones. In all cases, the compounds had similar activities against HBV genotypes C, D, and/or H. The inhibitors appear to act by chelating the divalent cations in the RNaseH active site because removing one of the three Lewis basic moieties or altering their angles relative to each other ablates inhibition [(Cai et al., 2014; Lu et al., 2015) and unpublished]. Counter-screening against human RNaseH1 revealed that most of these screening hits were also active against the human enzyme, but differences in the sensitivity of the two enzymes were apparent (Lu et al., 2015). This indicates that increasing selectivity for the HBV enzyme must be a major focus during chemical optimization.

Thirty-seven compounds have been tested for ability to suppress HBV replication in culture; 10 inhibited HBV replication at ≤20 μM (Table 1). Preferential suppression of the viral plus-polarity DNA strand confirmed that inhibition was due to blocking the viral RNaseH activity for all compounds except #56. Moderate cytotoxicity was observed for all active compounds, with CC_{50} values ranging from 6.1 to 79 μM. Therefore, it will be essential to minimize cytotoxicity during development of RNaseH inhibitors. The EC_{50} values for most of these inhibitors was substantially lower than their IC_{50} values. The cause for this quantitative discrepancy is unknown, but it could be due to accumulation of the compounds in cells, metabolism of the inhibitors to more active forms, or most likely, that the recombinant RNaseH does not recapitulate all aspects of the enzyme in its natural context as part of the full-length HBV polymerase protein. Nevertheless, strong inhibition of the recombinant enzyme is an excellent predictor of a compound's potential to inhibit HBV replication in cells.

7. Outlook

The HBV RNaseH is a promising but unexploited target for antiviral drug development. Anti-RNaseH drugs are envisioned to be used in combination with the existing nucleos(t)ide analogs to suppress HBV replication much further than is currently possible. The goal will be to suppress viral replication enough to block replenishment of the cccDNA in the liver, permitting turnover of the cccDNA and elimination of infected cells by the immune system to clear the infection. The long apparent halflife of the cccDNA implies that therapy will be prolonged and may well benefit from co-administration with novel immune-stimulating therapies that are under development (Bertoletti and Gehring, 2013; Gehring et al., 2014). Therefore, minimizing toxicity and optimizing specificity and pharmacokinetic properties of potential RNaseH drugs will be especially important.

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HIGHLIGHTS

- The HBV ribonuclease H is a promising but unexploited drug target.
- Recent technical advances permit low-throughput screening for ribonuclease H inhibitors.
- Some of the known HIV ribonuclease H inhibitors also inhibit HBV.
- Combining ribonuclease H inhibitors with existing or novel drugs may significantly improve treatment efficacy for HBV.

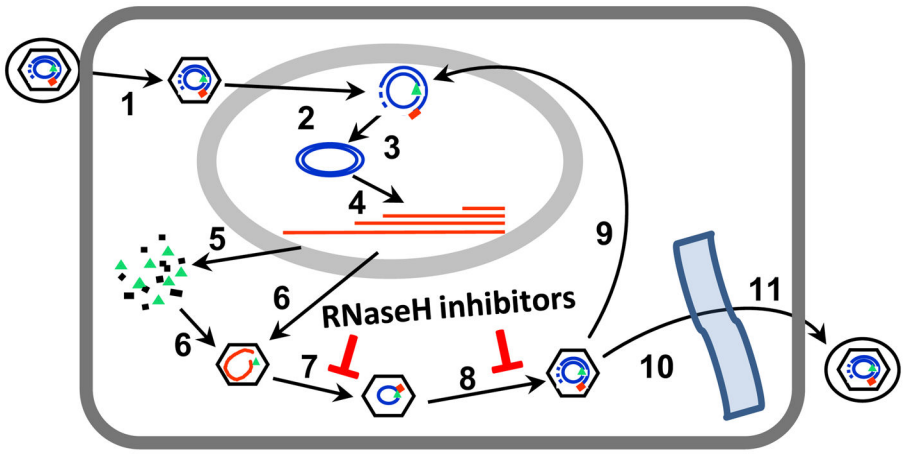

Fig. 1. HBV replication cycle

Binding of HBV virions to hepatocytes followed by fusion of the viral envelope with the plasma membrane releases core particles into the cytoplasm **(1)**. Core particles are transported to the nucleus, where they release the partially double-stranded viral DNA **(2)**, and the DNA is converted into cccDNA inside the nucleus **(3)**. Viral RNAs are transcribed **(4)** and translated to produce the viral proteins **(5)**. The viral pregenomic RNA is encapsidated into core particles as a complex with viral polymerase protein **(6)**. The minus-polarity DNA strand is synthesized by the reverse transcriptase activity of the polymerase with concomitant degradation of the pregenomic RNA by the RNaseH activity **(7)**. The plus-polarity DNA strand is synthesized by the reverse transcriptase **(8)**. Mature core particles are then either transported back into the nucleus to maintain the cccDNA pool **(9)** or are enveloped by budding into the endoplasmic reticulum **(10)** and are non-cytolytically secreted as mature virions **(11)**. RNaseH inhibitors block steps 7 and 8. Modified from (Hu et al., 2013).

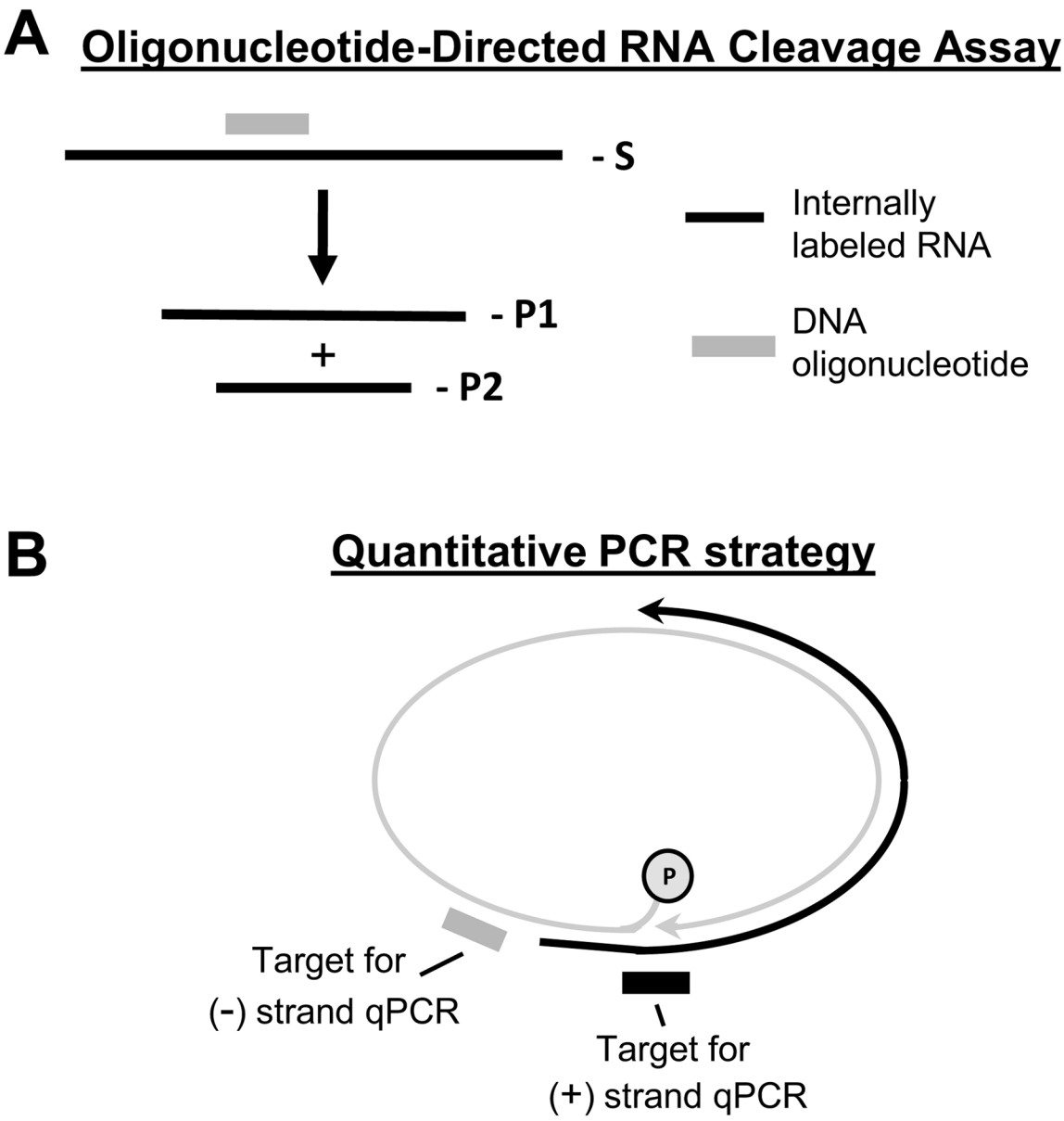

Fig. 2. Principles of the assays to study RNaseH inhibitors

A. Oligonucleotide-directed RNA cleavage assay to measure RNaseH activity. The substrate (S) is ^{32}P-labeled RNA (black line) annealed to a complimentary DNA oligo (grey line). The RNaseH cleaves the RNA within the RNA/DNA heteroduplex and forms two products (P1 and P2). The reaction products are resolved by denaturing PAGE and detected by autoradiography. **B.** Basis of the strand-preferential quantitative PCR assay used to measure effects of RNaseH inhibitors on viral replication. The minus-polarity DNA strand (grey line) is detected by amplifying the sequence close to its 5′ end. The plus-polarity DNA strand (black line) is detected by amplifying across the gap in the minus-polarity DNA strand. Modified from (Cai et al., 2014).

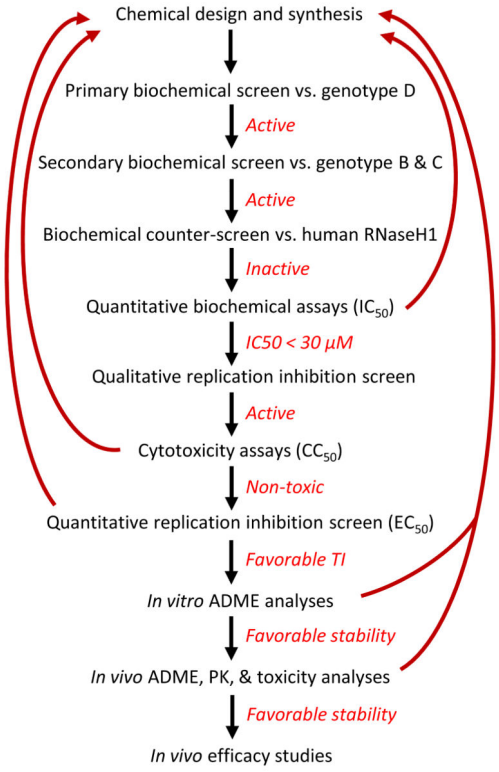

Fig. 3.
Low-throughput screening pipeline for HBV RNaseH inhibitors.

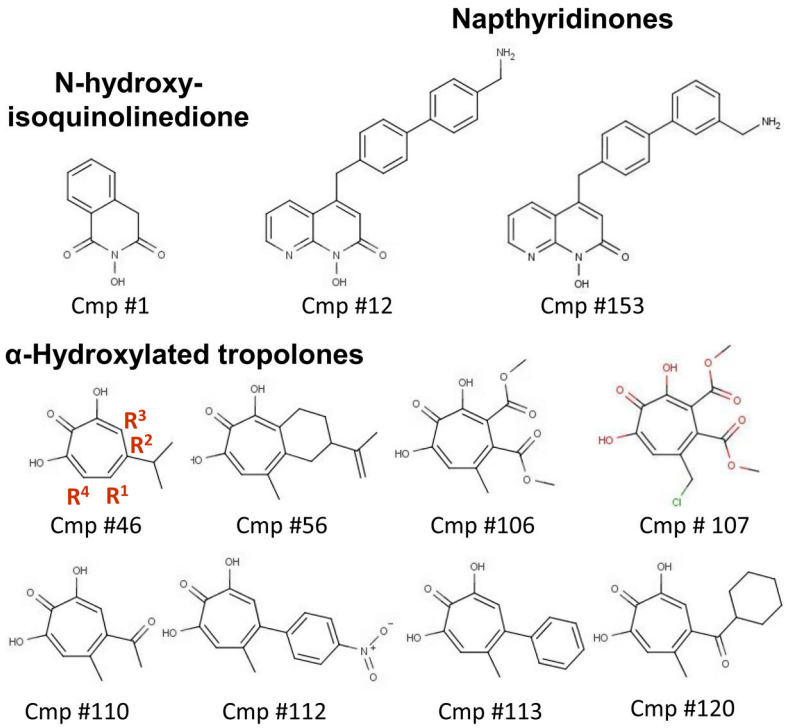

Fig. 4. Select HBV RNaseH inhibitors

Derivatization sites on the tropolone ring are numbered in red for compound #46.

Table 1

Inhibitors of the HBV RNaseH. Data for compound #1 are in (Cai et al. 2014); qualitative data for #12 are in (Tavis et al., 2013); data for #46 – 120 are in (Hu et al. 2013) and (Lu et al. 2015). Unpublished data are the quantitative values for #12, all values for #153, and the cell-based results for #120. Quantitative data are not available for all compounds. −, inactive; +, active at 60 μM; ++, active at 5 μM; t, toxic at 20 μM. gtD, genotype D.

Compound number	Name	Chemical class	IC_{50}, gtD (μM)	EC_{50} (μM)	CC_{50} (μM)	TI
1	2-Hydroxyisoquinoline-1,3(2H,4H)-dione	N-hydroxyisoquinolinedione	28	4.2	75	18
12	CWHM-000618	Napthyridinone	5.7	3.4	7.1	2.1
153	CWHM-000613		4.1	4.4	6.1	1.4
46	beta-thujaplicinol	αHydroxytropolone	5.9	1	25	25
56	Manicol			9.1	35	3.8
106	CM1012-6a		29.6	2.7	38	14
110	CM1012-6e		34.6	0.34	32	94
112	CM1012-6i		>100	2.5	79	32
113	RM-YM-1-0613		>100	4.2	66	16
120	RM-MD-1-0713		+	++	t	