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Chemical Approaches to Inhibiting the Hepatitis B Virus Ribonuclease H

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Abstract

Hepatitis B virus (HBV) chronically infects >250 million people and kills nearly a million annually, and current antivirals cannot clear the infection or adequately suppress disease. The virus replicates by reverse transcription, and the dominant antiviral drugs are nucleos(t)ide analogs that target the viral reverse transcriptase. We are developing antivirals targeting the other essential viral enzymatic activity, the ribonuclease H (RNaseH). HBV RNaseH inhibitors with efficacies in the low micromolar to nanomolar range against viral replication in culture have been identified in the α-hydroxytropolone and hydroxyimide chemotypes. Here, we review the promise of RNaseH inhibitors, their current structure–activity relationships, and challenges to optimizing the inhibitors into leads for clinical assessment.

■ HEPATITIS B VIRUS, DISEASE, AND TREATMENT

Hepatitis B virus (HBV) is a hepatotropic DNA virus that replicates by reverse transcription and chronically infects >250 million people worldwide.[1] Viral replication induces hepatic inflammation that leads to a decades-long disease progression from asymptomatic infection to chronic hepatitis, hepatic fibrosis, and cirrhosis. Infection often terminates in death from liver failure or hepatocellular carcinoma, and HBV kills >880 000 people annually.[2]

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Treatment for HBV infection is dominated by monotherapy with a nucleos(t)ide analogs (lamivudine, adefovir, telbivudine, entecavir, or tenofovir) that target the reverse transcriptase of the multifunctional HBV polymerase protein (Figure 1A). These drugs suppress HBV replication by 4–5 $\log_{10}$ in most patients, often to below the limit of detection. Therapy can also suppress the nuclear form of the HBV genome, the covalently closed circular DNA (cccDNA) that templates all HBV RNAs (Figure 1B), by ~1 $\log_{10}$ after 1–2 years.[3] However, HBV is cleared in only 3–6% of patients even after years of treatment, and treatment reduces chances of liver failure or hepatocellular carcinoma by only 2- to 4-fold after 10 years.[4] The costs of this partial suppression of disease progression are indefinite drug administration and potential side effects from decades of drug exposure.

The failure of monotherapy with very potent nucleos(t)ide analogs to clear HBV implies that eliminating HBV will require combination therapy with compounds that act by different mechanisms, analogous to treatment for human immunodeficiency virus (HIV) infection. Many approaches to identifying drugs that act by novel mechanisms are being explored, including efforts targeting the HBV ribonuclease H (RNaseH).

■ HBV RIBONUCLEASE H

RNaseHs cleave RNA in a RNA/DNA heteroduplex, and the role of the HBV RNaseH is to destroy the HBV RNA after it has been copied into DNA by the reverse transcriptase[1] (Figure 1B). RNaseHs belong to the nucleotidyl transferase superfamily that contains host and retroviral RNaseHs, including the HBV and HIV RNaseHs and human RNaseH 1 and 2. Hydrolysis of RNA by RNaseHs requires two Mg^{2+} ions in the enzyme active site that are bound to a “DEDD” motif.

Difficulties in expressing recombinant HBV RNaseH have severely restricted study of the enzyme and hampered anti-RNaseH drug screening. Therefore, work with the HBV RNaseH has been based on studies with the more tractable HIV RNaseH. Unfortunately, the HBV RNaseH and the HIV enzyme share only 23% amino acid identity, and the HBV RNaseH acts within a polymerase monomer compared to the HIV enzyme being part of a heterodimer. The HIV RNaseH structure is known, but no structural information exists for the HBV enzyme, and the HBV structure cannot be confidently modeled on other RNaseHs due to limited homologies. Therefore, the degree to which the HIV enzyme can serve as a model for the HBV RNaseH is limited.

■ HBV RNaseH AS A DRUG TARGET

HBV reverse transcription is catalyzed by coordinated function of the reverse transcriptase and RNaseH activities of the HBV polymerase protein. Inhibiting the RNaseH causes premature truncation of minus-polarity DNA strands, accumulation of RNA/DNA heteroduplexes within viral capsids, and failure to synthesize the viral plus-polarity DNA strand. This lethally damages the genome, rendering it unable to function in virions or be converted to cccDNA. Therefore, monotherapy with RNaseH inhibitors could be as effective as inhibiting the reverse transcriptase with nucleos(t)ide analogs. As novel inhibitors targeting a distinct essential enzymatic activity of the virus, RNaseH inhibitors would be

good candidates for use in combination therapies with nucleos(t)ide analog and other HBV drugs against new targets that are under development.

■ IDENTIFICATION OF RNaseH INHIBITORS

We screened >500 compounds, primarily based on their similarity to inhibitors of the HIV RNaseH, in biochemical HBV RNaseH assays and/or cell-based HBV replication inhibition assays. Over 100 inhibitors that suppress HBV replication in culture with 50% effective concentration (EC_{50}) values from ~100 nM to the low micromolar range were identified. 50% cytotoxic concentrations (CC_{50}) range from 3 to >100 μM by MTS assays, leading to therapeutic indexes (TIs, CC_{50}/EC_{50}) of up to 700. These inhibitors are found mostly in two chemotypes, the α-hydroxytropolones (αHTs; Figure 2B) and *N*-hydroxyimides (Figure 2C).[5–9] The *N*-hydroxyimides can be subdivided into three classes we have studied: *N*-hydroxyisoquinolinediones (HIDs), *N*-hydroxypyridinediones (HPyDs), and *N*-hydroxynapthyridinones (HNOs). In addition, Huber et al. recently screened 52 structurally related *N*-hydroxypyrimidinediones and found two hits, the most potent of which suppressed HBV replication in cells with an EC_{50} of 5.5 μM and a CC_{50} > 100 μM[10] (17 in Huber et al., Figure 2C).

Most HIV RNaseH inhibitors coordinate the two catalytic Mg^{2+} ions in the RNaseH active site, usually through a trident array of Lewis basic atoms or electron donors. Likewise, all HBV RNaseH inhibitors identified to date contain a trident of oxygen or nitrogen atoms. Disrupting the trident ablates inhibition, implying a similar inhibition mechanism between HBV and HIV RNaseH inhibitors. Furthermore, hydroxytropolones and *N*-hydroxyimides are relatively acidic, which may allow them to chelate to the metals in an ionic or even dianioinic state (Figure 2A).

The compounds inhibit HBV RNaseHs from three genotypes, indicating that HBV's high genetic diversity probably will not hinder drug development. The RNaseH inhibitors are synergistic with lamivudine and additive with the HBV capsid protein assembly modifier Hap12 without exacerbating cytotoxicity.[11] Two RNaseH inhibitors, an HPyD (**208**) and an αHT (**110**), can significantly suppress HBV replication in chimeric mice carrying humanized livers.[12] These data validate the RNaseH as a drug target.

■ α-HYDROXYTROPOLONE INHIBITORS

αHTs are highly oxygenated troponoids that can inhibit dinuclear metalloenzymes (Figure 2B),[13] including viral nucleases such as the HIV, HBV, and xenotropic murine leukemia-related virus RNaseHs. Antiviral activity is believed to depend on their ionic character at physiological pH due to the acidity of both hydroxyls and the Lewis basic nature of the tropolone carbonyl that promotes coordination of the active site Mg^{2+} ions.

Most of what is known about the biological activity of αHTs has been driven by studies of natural product troponoids. The first αHT found to inhibit the HBV RNaseH was the natural product β-thujaplicinol (**46**; Figure 2B), which has an EC_{50} of 1.0 μM and a CC_{50} of 25 μM. There are only a few sources of natural αHTs, and until recently, there have been few

strategies available to make synthetic αHTs. As a result, development of therapeutic αHTs has been stifled beyond the discovery phase.

The Murelli group pioneered new approaches for αHT synthesis, focusing on an oxidopyrylium cycloaddition/ring-opening strategy.[14] 165 structurally diverse αHTs and related troponoids have been assessed against HBV, and some αHTs have been synthesized on multigram scales. Eighty αHTs are active against HBV replication at ≤20 μM, with EC_{50}'s as low as 0.11 μM. These studies have led to structurally novel synthetic αHTs such as **110** that have improved potency, TI values, and pharmacokinetic properties. While a clear structure—activity relationship (SAR) is still being established, it appears that small electron-withdrawing appendages are beneficial, and cytotoxicity increases with lipophilicity and the number of aromatic rings attached to the αHT pharmacophore.[8]

■ *N*-HYDROXYIMIDE AND CLOSELY RELATED INHIBITORS: *N*-HYDROXYISOQUINOLINEDIONES, *N*-HYDROXYPYRIDINEDIONES, AND *N*-HYDROXYNAPTHYRIDINONES

Three chemotypes containing a hydroxyimide or *N*-hydroxyimide-like moiety can inhibit the HBV RNaseH: the HIDs, HPyDs, and HNOs (Figure 2C).[7,9] Like the αHTs, both the HPyD and HID scaffolds have an oxygen trident that is essential for activity. The HNO scaffold, exemplified by **12**, lacks this trident because one of the oxygens is replaced with an aromatic nitrogen that also has a lone pair of electrons available to coordinate a Mg^{2+} cation. The major stabilizing interactions of the HBV RNaseH—inhibitor complexes are probably electrostatic, and they appear to be formed primarily between the chelating moiety of the ligands (*N*-hydroxyimide group) and the polar catalytic center of the enzyme. In each case, strong ionic interactions along with charge-assisted hydrogen bonds presumably anchor the chelating moiety to the Mg^{2+} ions.

The various *N*-hydroxyimide cores provide different directionality for branching, and empirically developed preliminary SARs for these appendages to some of the cores have been generated. For example, placing an alkyl groups at C4 on the HID scaffold is not tolerated, but including a carbonyl group as either an ester or amide at C4 (e.g., **86**) is acceptable. This is believed to be due to the increased acidity of the α-hydrogen and therefore the ability to coordinate the Mg^{2+} ions in the ionic form. As is also illustrated in **86**, extension of large groups in the 7-position is also tolerated. In contrast, adding a third ring that condenses positions 6 and 7 and a second nitrogen on the HID scaffold to create indole-flutimide analogues is not tolerated.[9] Molecules generated from the HNO class focus mostly on the C4 position, which for this molecule is *para* or 180° to the *N*-hydroxyimide. The top molecule of this class to date, **12**, has a methylenebiarylmethyl-amine appendage, although this activity is only slightly better than truncated versions possessing an aminobenzyl group. At present, the HPyDs appear to be the most promising *N*-hydroxyimides for further development.

SCREENING FOR NOVEL INHIBITORY CHEMOTYPES

The existing HBV RNaseH inhibitors were discovered by screening compounds similar to HIV RNaseH inhibitors. Expanding this repertoire will require exploring novel chemical space. Unfortunately, high-throughput biochemical screening is currently not feasible due to limitations of the recombinant HBV RNaseH. Although the enzyme can be used for mechanistic confirmation of RNaseH inhibition, it underestimates efficacy compared to replication inhibition assays by 50- to 100-fold and has an unacceptably high false-negative rate. Phenotypic screens measuring suppression of viral replication are feasible, but they must measure reduction of the viral plus-polarity DNA strand because the truncated minus-polarity DNA strands produced in the absence of RNaseH activity make standard PCR detection of RNaseH inhibitors very insensitive. Therefore, a strand-preferential screening protocol was developed[8,9] that is amenable to a 96-well format, but its low throughput and high cost limit use to midthroughput screening at present. This continues to necessitate hypothesis-based screening rather than unbiased sampling of chemical diversity.

OUTLOOK FOR RNaseH INHIBITORS

Drugs to improve control and hopefully eliminate HBV will have to address the unique features of this infection. Patients typically begin treatment after decades of chronic infection, when serious liver damage has usually already occurred. In addition, treatment will take many months to years to clear or stably control HBV due to the unusual stability of the cccDNA that is the key molecule to be eradicated or inactivated to achieve a cure. As the cccDNA, like virions, is produced by reverse transcription[1] (Figure 1B), control of HBV replication will need to be continuous and profound to prevent replenishment of the cccDNA. Therefore, new drugs against HBV will have to have very low toxicity, act by a variety of mechanisms, have outstanding pharmacokinetic properties to ensure long-lasting efficacy, be active against a wide range of HBV's diverse genotypes, and be compatible with many other drugs. It is unlikely that any one drug will fully meet these demands, emphasizing the need for a broad approach for developing new anti-HBV drugs.

In this light, RNaseH inhibitors are promising candidates for further development given their relative insensitivity to HBV's genetic diversity and ability to synergize with drugs from other classes.[11] Key challenges to be addressed for RNaseH inhibitors to achieve their promise include developing high-throughput screening assays to access more chemical space, advancing synthetic methods to more rapidly derivatize the pharmacophores already identified, and exhaustively assessing the pharmacological and toxicological profiles of the inhibitors. If successful, RNaseH inhibitors may one day take their place in the cocktail(s) of drugs that will be deployed against HBV.

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■ ABBREVIATIONS

αHT	α-hydroxytropolone
CC_{50}	50% cytotoxic concentration
cccDNA	covalently closed circular DNA
EC_{50}	50% effective concentration
HBV	hepatitis B virus
HID	*N*-hydroxyisoquinolinedione
HIV	human immunodeficiency virus
HNO	*N*-hydroxynapthyridinone
HPyD	*N*-hydroxypyridinedione
RNaseH	ribonuclease H
SAR	structure—activity relationship
TI	therapeutic index

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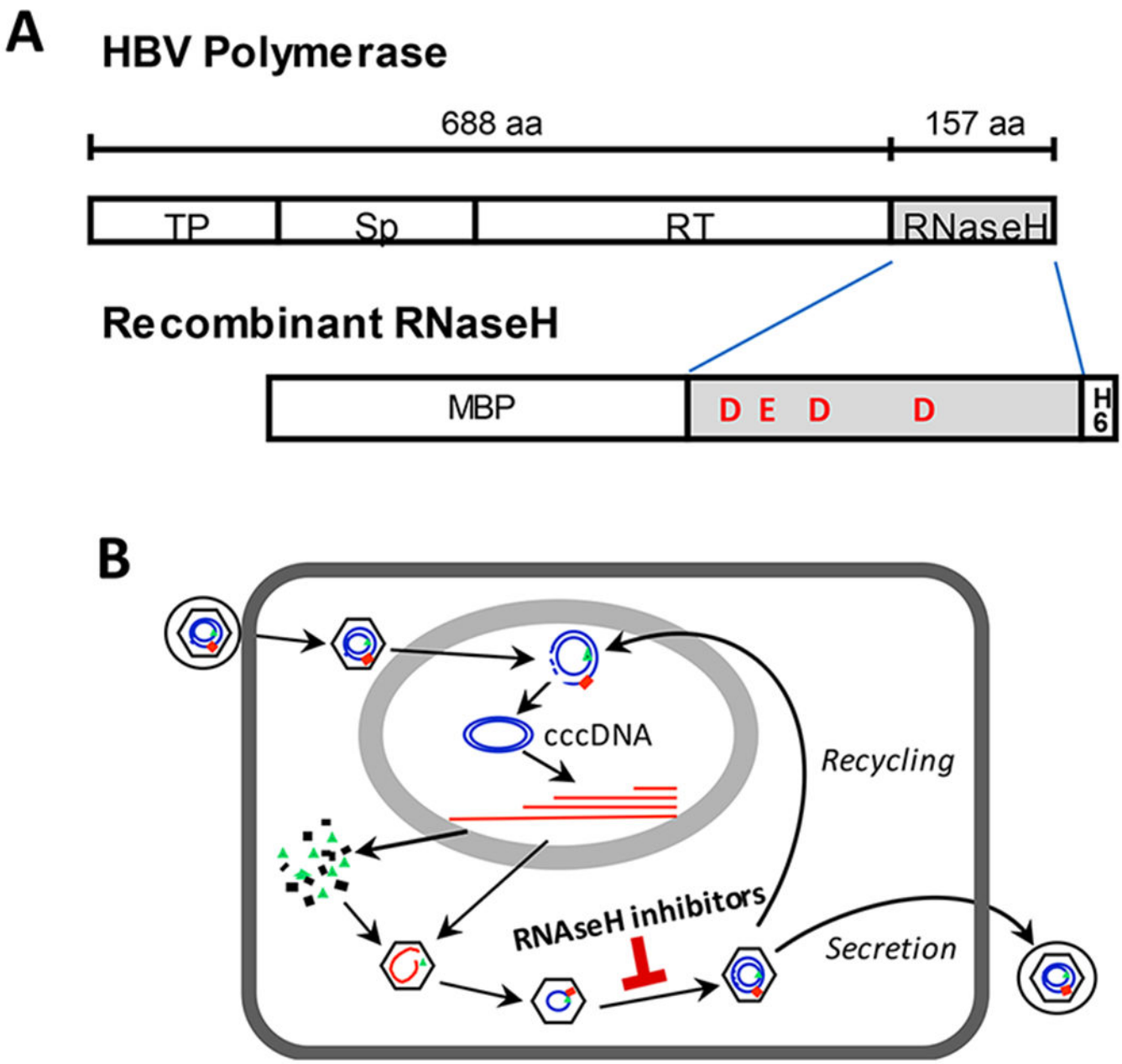

Figure 1.

The HBV RNaseH. (A) The RNaseH is the C-terminal domain of the multifunctional HBV polymerase protein. The RNaseH can be expressed as a functional recombinant protein with N-terminal maltose-binding protein (MBP) and C-terminal hexahistidine (H6) tags. TP, terminal protein domain that primes DNA synthesis; Sp, spacer domain; RT, reverse transcriptase domain; RNaseH, RNase H domain. The relative locations of the carboxylic amino acids (D and E) that presumably coordinate the catalytic Mg^{2+} ions are shown for the recombinant RNaseH. (B) HBV replication cycle. Newly synthesized genomes can be secreted as mature virions or converted via “recycling” to the nuclear cccDNA. DNA is in blue and RNA is in red. The stage at which RNaseH inhibitors act is indicated. Panel B

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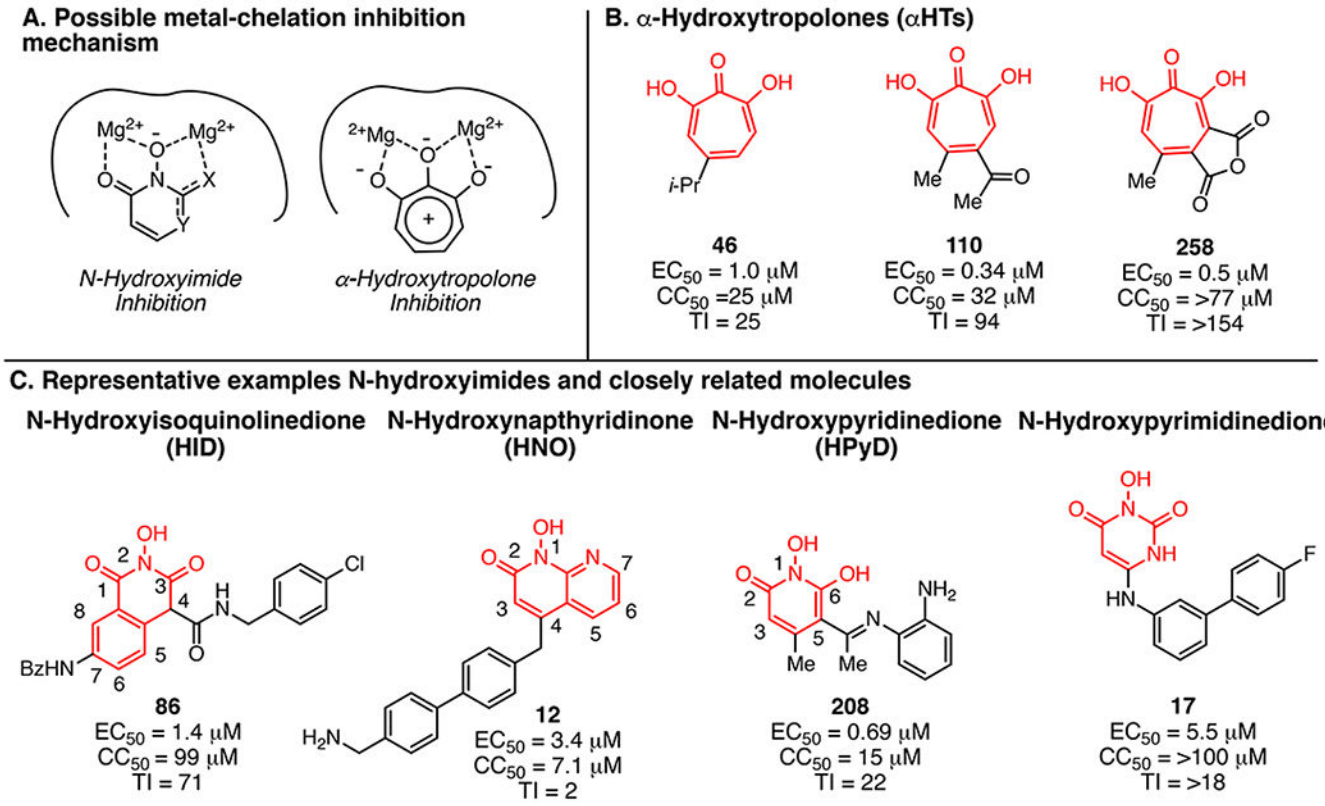

Figure 2.

Presumed mechanism of RNaseH inhibition and exemplary compounds. (A) Coordination of two Mg^{2+} ions in the geometry in which they are found in RNaseH active sites by the *N*-hydroxyimide and α-hydroxytropolone pharmacophores. (B) Example α-hydroxytropolone RNaseH inhibitors. (C) Example *N*-hydroxyimide and closely related classes of inhibitors.