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Hydroxylated Tropolones Inhibit Hepatitis B Virus Replication by Blocking Viral Ribonuclease H Activity

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Hepatitis B virus (HBV) remains a major human pathogen despite the development of both antiviral drugs and a vaccine, in part because the current therapies do not suppress HBV replication far enough to eradicate the virus. Here, we screened 51 troponoid compounds for their ability to suppress HBV RNaseH activity and HBV replication based on the activities of α-hydroxytropolones against HIV RNaseH, with the goal of determining whether the tropolone pharmacophore may be a promising scaffold for anti-HBV drug development. Thirteen compounds inhibited HBV RNaseH, with the best 50% inhibitory concentration (IC_{50}) being 2.3 μM. Similar inhibition patterns were observed against HBV genotype D and C RNaseHs, implying limited genotype specificity. Six of 10 compounds tested against HBV replication in culture suppressed replication via blocking of viral RNaseH activity, with the best 50% effective concentration (EC_{50}) being 0.34 μM. Eighteen compounds inhibited recombinant human RNaseH1, and moderate cytotoxicity was observed for all compounds (50% cytotoxic concentration [CC_{50}] = 25 to 79 μM). Therapeutic indexes ranged from 3.8 to 94. Efficient inhibition required an intact α-hydroxytropolone moiety plus one or more short appendages on the tropolone ring, but a wide variety of constituents were permissible. These data indicate that troponoids and specifically α-hydroxytropolones are promising lead candidates for development as anti-HBV drugs, providing that toxicity can be minimized. Potential anti-RNaseH drugs are envisioned to be employed in combination with the existing nucleos(t)ide analogs to suppress HBV replication far enough to block genomic maintenance, with the goal of eradicating infection.

More than 2 billion people have been infected with hepatitis B virus (HBV) at some time in their lives and up to 350 million remain chronically infected as carriers of HBV (1, 2). Approximately 20% of chronic hepatitis B patients develop liver cirrhosis, leading to hepatic insufficiency and portal hypertension (3). Furthermore, there is a 100-fold higher risk of development of hepatocellular carcinoma in chronic HBV patients than in noncarriers (4). Every year, HBV infection kills more than 500,000 people from cirrhosis, liver failure, and hepatocellular carcinoma (5).

The global level of chronic HBV infection still mandates development of new drugs despite the development of excellent vaccines and drugs against the virus. Seven drugs have been approved by the U.S. Food and Drug Administration for treating HBV infection. Interferon alpha and pegylated interferon alpha are immunomodulatory agents. However, the need for subcutaneous administration, the poor long-term responses, the very low cure rates, and the high frequency of adverse side effects make interferon far from an ideal drug (6). The nucleos(t)ide analog drugs lamivudine, adefovir, entecavir, telbivudine, and tenofovir are phosphorylated to their triphosphate derivatives by cellular enzymes and become chain-terminating substrates of the HBV reverse transcriptase (7). While these drugs profoundly suppress HBV replication in most patients, often to below the clinical limit of detection (8, 9), they have a number of limitations. The emergence of resistant HBV variants with mutations in the reverse transcriptase is a major problem for the nucleos(t)ide analogs except entecavir and tenofovir, and multidrug resistance has increased the risk of exacerbation of liver disease (5, 10). Furthermore, viral replication almost always resurges following termination of therapy. Thus, although viral replication can be controlled, the drugs must be given essentially indefinitely. This therapy is very expensive and may cause unpredictable adverse effects after long-term drug administration (11, 12).

HBV encodes two enzymes essential for its replication that are attractive drug targets (13). The reverse transcriptase domain of the viral polymerase protein contains the DNA polymerase activity that synthesizes new DNA. The RNaseH domain is encoded at the carboxy terminus of the viral polymerase and degrades the

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viral RNA template after it is copied into minus-polarity DNA, allowing the subsequent synthesis of the plus-polarity DNA strand. No drugs against HBV RNaseH are available, primarily due to difficulties in establishing screening assays. We recently developed a low-throughput screening pipeline for HBV RNaseH inhibitors (14–16), opening the door to anti-RNaseH drug discovery.

Both the HBV and HIV RNaseH enzymes belong to the nucleotidyl transferase superfamily (17, 18). We previously demonstrated that many compounds selected for the ability to suppress HIV RNaseH activity also inhibit HBV RNaseH, and some can also block HBV replication in cell cultures via suppression of the viral RNaseH activity (15, 16). One of these compounds was the natural product β-thujaplicinol (14). β-Thujaplicinol is a member of the most widely studied class of troponoids, the α-hydroxytropolones, that have been identified as anticancer agents (19, 20) as well as lead therapeutic agents for a number of infections, including HIV (21–25), herpes simplex virus (26), malaria (27), and many bacteria (28). This antimicrobial activity is often attributed to the ability of the compounds to sequester the divalent cations in the active sites of dinuclear metalloenzymes, a feature arising from the three contiguous oxygen atoms on the troponoid ring (29–31). Here, 51 troponoids, including 36 α-hydroxytropolones, were screened for their ability to inhibit HBV RNaseH activity and viral replication to assess whether tropolones and specifically α-hydroxytropolones may be attractive candidates for development as anti-HBV drugs.

MATERIALS AND METHODS

Compound acquisition and synthesis. The compounds employed are listed in Table 1, and their structures are in Fig. S1 in the supplemental material. Compounds 46 to 57 were acquired from the National Cancer Institute (NCI) Developmental Therapeutics Program, and compounds 59 to 63 were purchased. α-Hydroxytropolone (compound 172) was synthesized in 3 steps from tropolone based on the procedure of Takeshita et al. (32). Compounds 92 to 105 were synthesized from manicol as previously described (21), and compounds 106 to 120 and 143 to 147 were synthesized in 5 to 7 steps from kojic acid as previously described (33–35). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C.

RNaseH expression and purification. Recombinant HBV RNaseH and human RNaseH1 were expressed in *Escherichia coli* and partially purified by nickel-affinity chromatography as previously described (15). The enriched extracts were dialyzed into 50 mM HEPES (pH 7.3), 300 mM NaCl, 20% glycerol, and 5 mM dithiothreitol (DTT) and stored in liquid nitrogen.

Oligonucleotide-directed RNA cleavage assay. The RNaseH activity was measured using an oligonucleotide-directed RNA cleavage assay as previously described (14–16). Briefly, 6 μl protein extract was mixed on ice with an internally ^{32}P-labeled 264-nucleotide (nt) RNA derived from the duck hepatitis B virus genome (DRF+ RNA) plus 3 μg oligonucleotide D2507− or its inverse-complement oligonucleotide D2526+ as a negative control. This mixture was incubated with test compounds in 50 mM Tris (pH 8.0), 190 mM NaCl, 5 mM $MgCl_2$, 3.5 mM DTT, 0.05% NP-40, 6% glycerol, and 1% DMSO at 42°C for 90 min. The cleavage products were resolved by denaturing polyacrylamide gel electrophoresis, detected by autoradiography, and quantified using ImageJ. The nonspecific background values were determined from the incorrect oligonucleotide negative-control lane and subtracted from all experimental values. The 50% inhibitory concentrations (IC_{50}s) were then calculated with GraphPad Prism using three-parameter log(inhibitor) versus response nonlinear curve fitting with the curve minimum set to zero to reflect background subtraction.

TABLE 1 Anti-RNaseH profiles of hydroxytropolones and related compounds

Compound no.	Qualitative biochemical screening results for[a]:			Mean IC_{50} ± SD (μM) for:		
	HBV gtD	HBV gtC	Human RNaseH1	HBV gtD	HBV gtC	Human RNaseH1
46	+++	+++	++	5.9 ± 0.7	2.3 ± 1.7	58 ± 14.4
47	−	−	−			
48	−	−	−			
49	−	−	−			
50	−	−	−			
51	+	−	−			
52	−		−			
53	−	−	−			
54	−		−			
55	+	−	+			
56	+/−	+/−	−			
57	−		+			
59	−		+			
60	−		+			
61	−		++			
62	+	−	−			
63	−		+			
92	−	−	−			
93	−	−	−			
94	−		−			
95	−		−			
96	−		+			
97	−		−			
98	−	−	+			
99	−	−	−			
100	−	−				
101	−	−				
102	−					
103	−					
104	−					
105	−					
106	+++	+++	+++	29.6 ± 1.4	29.0 ± 0.4	47.8 ± 3.5
107	++	+	++	40 ± 18	65 ± 38	ica[b]
108	+	+	++			
109	+	+	++			
110	++	++	+++	34.6 ± 25	22.9 ± 2	29.3 ± 3.3
111	−					
112	+	+	+++	>100	>100	91.2 ± 23
113	++	+	++	>100	>100	27.4 ± 8.9
114	−					
115	−					
117	−					
118	−					
119	−					
120	+	+	+++			
143	+	+	+			
144	−					
145	−					
146	−					
147	−					
172	−	−	−			

[a] +++, inhibition at 10 μM; ++, inhibition at 20 μM; +, detectable inhibition at 60 μM; +/−, moderate inhibition in some but not all assays; −, no inhibition. gtD, genotype D; gtC, genotype C.
[b] ica, insufficient compound available.

HBV replication assay. Inhibition of the HBV replication was measured in HepDES19 cells as previously described (16). Cells were seeded in 6-well plates and incubated in Dulbecco's modified Eagle's medium (DMEM)/F12, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (P/S) with 1 μg/ml tetracycline. The tetracycline was withdrawn after 24 h. The test compound was applied to duplicate wells 48 h later in medium containing a final DMSO concentration of 1%, and medium containing the compound was refreshed daily for the following 2 days. Cells were harvested, and nonencapsidated nucleic acids were digested with micrococcal nuclease (New England BioLabs). HBV DNA was purified from capsids using a QIAamp cador pathogen minikit (Qiagen) with proteinase K incubation overnight at 37°C. TaqMan PCR was performed for 40 cycles at an annealing temperature of 60°C. The primers and probe (IDT Inc.) for the plus-polarity strand were 5′CATGAACAAGAGATGATTAGGCAGAG3′, 5′GGAGGCTGTAGGCATAAATTGG3′, and 5′/56-FAM/CTGCGCACC/ZEN/AGCACCATGCA/3IABkFQ. The primers and probe for the minus-polarity strand were 5′GCAGATGAGAAGGCACAGA3′, 5′CTTCTCCGTCTGCCGTT3′, and 5′/56-FAM/AGTCCGCGT/ZEN/AAAGAGAGGTGCG/3IABkFQ.

MTT cytotoxicity assays. HepDES19 cells (1.0×10^4 cells per well) were seeded in 96-well plates and incubated in DMEM with 10% FBS plus 1% P/S, 1% nonessential amino acids, and 1% glutamine. The compounds were diluted in the medium to the indicated concentrations plus 1% DMSO and added to the cells 24 h after plating, with each concentration tested in triplicate. The medium containing the compound was refreshed daily for the next 2 days. Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) was added to 0.25 mg/ml, the cultures were incubated for 60 min, the metabolites were solubilized in acidic isopropanol, and the absorbance was read at 570 nm.

RESULTS

Biochemical screening against HBV RNaseH. Fifty-one tropolones (Table 1; see also Fig. S1 in the supplemental material) were screened for anti-HBV RNaseH activity. The screening employed an oligonucleotide-directed RNA cleavage assay (Fig. 1A) in which an internally ^{32}P-labeled RNA is annealed to a cDNA oligonucleotide, and the cleavage at the heteroduplex at the annealing site is detected by resolving the RNA fragments by denaturing polyacrylamide gel electrophoresis followed by autoradiography. A qualitative primary screening assay was initially performed with compounds at concentrations of 60, 20, and 10 μM. The screening was first performed against a recombinant genotype D HBV RNaseH isolate and subsequently against a genotype C HBV RNaseH isolate to ensure that screening hits were not specific for only one of HBV's 8 major genotypes. Finally, IC_{50}s were determined for select compounds against the genotype D and C enzymes.

Eight compounds had activity at only 60 μM against the genotype D and/or C enzymes (Table 1 and Fig. 1B). Three additional compounds inhibited the genotype D and/or C enzymes at 20 μM, and the two most active compounds (compounds 46 and 106) inhibited RNaseHs from both genotypes at 10 μM. The results with compound 46 (β-thujaplicinol) were consistent with those from our previous studies (14). Compound 56 gave inconsistent results in these assays. In some assays, it inhibited RNaseH at 20 μM, but it was inactive in the large majority of assays. The IC_{50}s were determined for the six most active compounds against both genotype D and C enzymes. Compounds 46, 106, 107, and 110 had IC_{50}s of ≤40 μM against both the genotype D and/or C enzymes (Fig. 2 and Table 1).

Counterscreening against human RNaseH1. Thirty-five of the 51 compounds were counterscreened against recombinant human RNaseH1 in an initial effort to evaluate the specificity of the inhibition. Four compounds were active against the human enzyme at 10 μM, and 14 had activity at 20 μM or 60 μM. The inhibition patterns differed between HBV and human RNaseH1, with compounds generally having greater efficacy against the human enzyme than against HBV RNaseH. However, three compounds, compounds 46, 51, and 62, were more active against the HBV enzyme in the primary screening assays (Table 1). IC_{50}s were determined against human RNaseH1 for the five compounds selected for full replication analyses (Table 1). These data confirmed the different patterns of the inhibition between the HBV and hu-

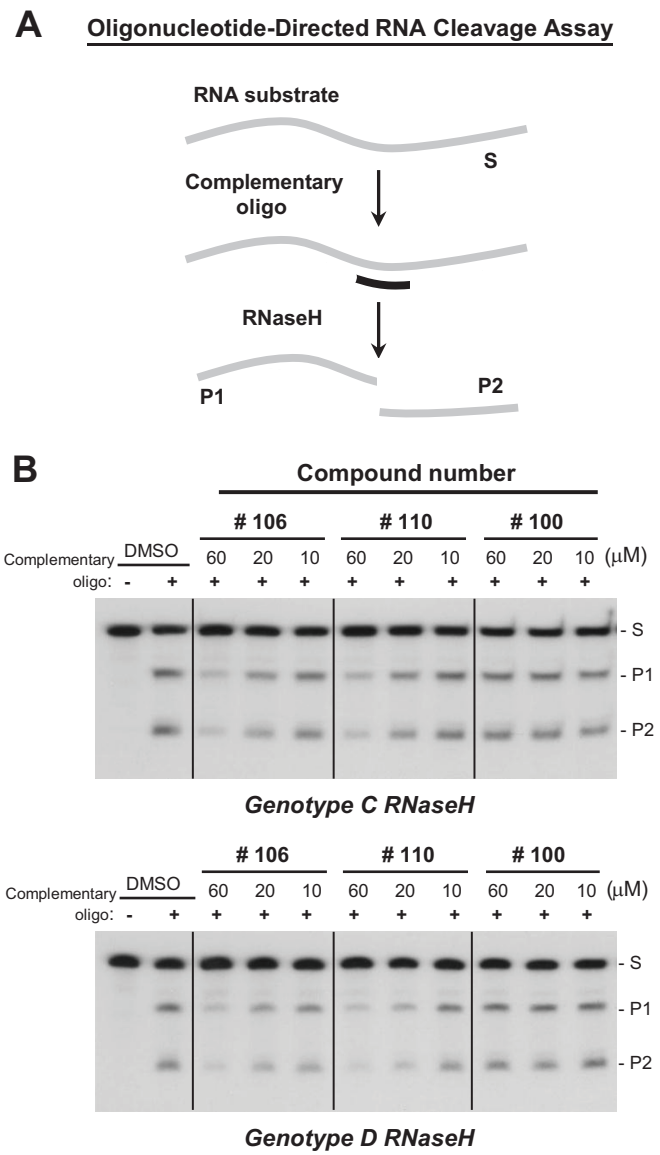


FIG 1 Primary screening of hydroxytropolones against HBV RNaseH. (A) Oligonucleotide-directed RNA cleavage assay. Reaction products were resolved by denaturing polyacrylamide gel electrophoresis and detected by autoradiography. Gray line, ^{32}P-labeled RNA; black line, DNA oligonucleotide; S, substrate; P1, product 1; P2, product 2. (B) Examples of primary screening assays against HBV genotype C and D RNaseH enzymes. DMSO, vehicle control; +, complementary oligo, −, inclusion of a non-complementary DNA oligonucleotide as a specificity control.

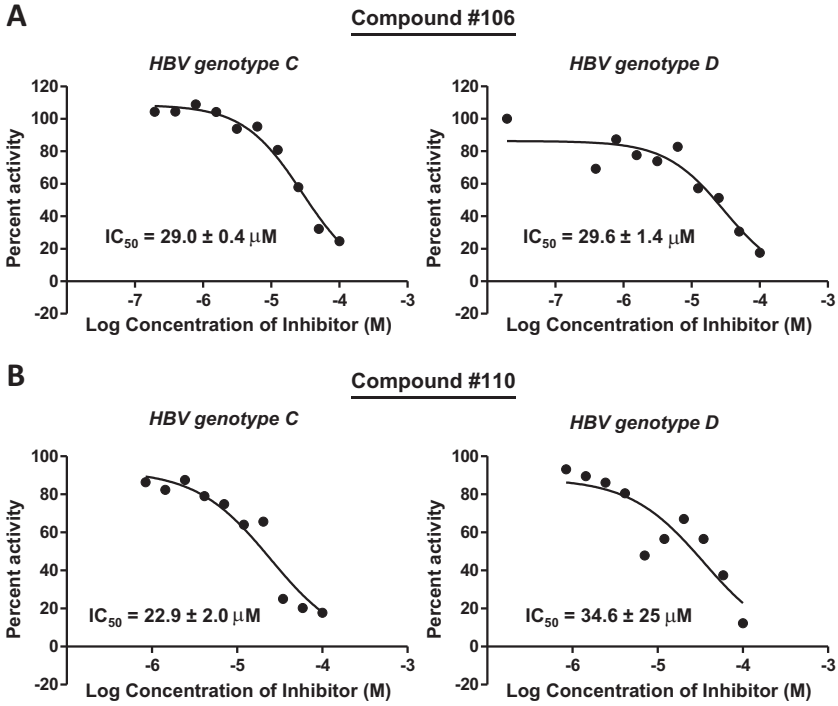


FIG 2 IC_{50}s for hydroxylated tropolone inhibitors of HBV RNaseH. IC_{50}s were measured using the oligonucleotide-directed RNA cleavage assay. (A) IC_{50} curves for compound 106 against genotype C and D HBV RNaseHs. (B) IC_{50} curves for compound 110 against genotype C and D HBV RNaseHs. The IC_{50} curves are from representative assays, and the IC_{50}s are the averages ± 1 standard deviation from two or three assays.

man enzymes, with compound 46 being 10- to 20-fold more active against the HBV enzyme, compound 106 being almost 2-fold more active against the HBV enzyme, compounds 110 and 112 having similar IC_{50}s, and compound 113 being about 4-fold more active against human RNaseH1.

HBV replication inhibition and cytotoxicity. The inhibition of viral replication was measured in HepDES19 cells (HepG2 cells stably transfected with an HBV genotype D genome under the control of a tetracycline-repressible promoter [36]) using a protocol we recently developed (16). Tetracycline was withdrawn from the medium, cells were incubated with the compound for 3 days with daily replenishment of the medium and compound, and cytoplasmic lysates containing viral capsid particles were prepared. The lysates were treated with micrococcal nuclease to destroy contaminating chromosomal DNA, and the capsids were treated with proteinase K to release the viral DNAs. Synthesis of the HBV plus-polarity DNA strand is suppressed by RNaseH inhibitors, and many minus-polarity DNA strands are truncated prematurely (14–16, 37). Therefore, we employed a strand-preferential quantitative PCR to measure accumulation of the amount of plus- and minus-polarity HBV DNAs recovered from the capsids (Fig. 3A).

Ten compounds were tested in the initial semiquantitative HBV replication inhibition assays at 60, 20, or 6.7 μM (Table 2) based on their efficacy in biochemical screening. Five compounds that inhibited RNaseH (compounds 46, 106, 110, 112, and 113) were selected; compound 107 was not tested because insufficient compound was available. We also selected five compounds that lacked activity against HBV RNaseH (compounds 47, 48, 50, 53, and 56) as specificity controls to evaluate the accuracy of our biochemical assay in identifying the inhibitors of viral replication. All five RNaseH inhibitors selectively inhibited accumulation of plus-polarity HBV DNA at 6.7 μM. Three of the five compounds that were negative in the biochemical assays were also negative in the replication inhibition assays (compounds 47, 48, and 53). Compound 50 was negative in the biochemical assays but suppressed accumulation of both DNA strands at 60 μM, consistent with inhibition being due to cytotoxicity and/or other mechanisms not involving RNaseH. Compound 56 occasionally had low activity in the biochemical assays. It suppressed accumulation of both DNA strands at 60 μM but had a weak preferential effect against the plus-polarity DNA at 20 μM. The cytotoxicity of the six compounds that selectively suppressed the plus-polarity DNA strand was assessed in HepDES19 cells using an MTT assay under the culture conditions employed for the replication inhibition assays. Moderate cytotoxicity was observed for all compounds, with 50% cytotoxic concentration (CC_{50}) values ranging from 25 to 79 μM (Table 2 and Fig. 4).

The 50% effective concentrations (EC_{50}s) were then determined for the six compounds which preferentially suppressed the plus-polarity DNA strand (compounds 46, 56, 106, 110, 112, and 113) (Table 2 and Fig. 3). All EC_{50}s were <10 μM, with the best value being 0.34 μM for compound 110. The minus-polarity DNA strand in the same samples was either not suppressed by treatment with the compounds or was suppressed at much higher compound concentrations for all compounds except compound 56, confirming that synthesis of mature HBV genomes was suppressed by inhibition of the viral RNaseH activity. This led to therapeutic indexes (TI) (CC_{50}/EC_{50}) from 3.8 for compound 56 up to 94 for compound 110. For compound 56, minus-polarity DNA accumulation was suppressed in parallel with plus-polarity DNA (data not shown) over a concentration range in which sig-

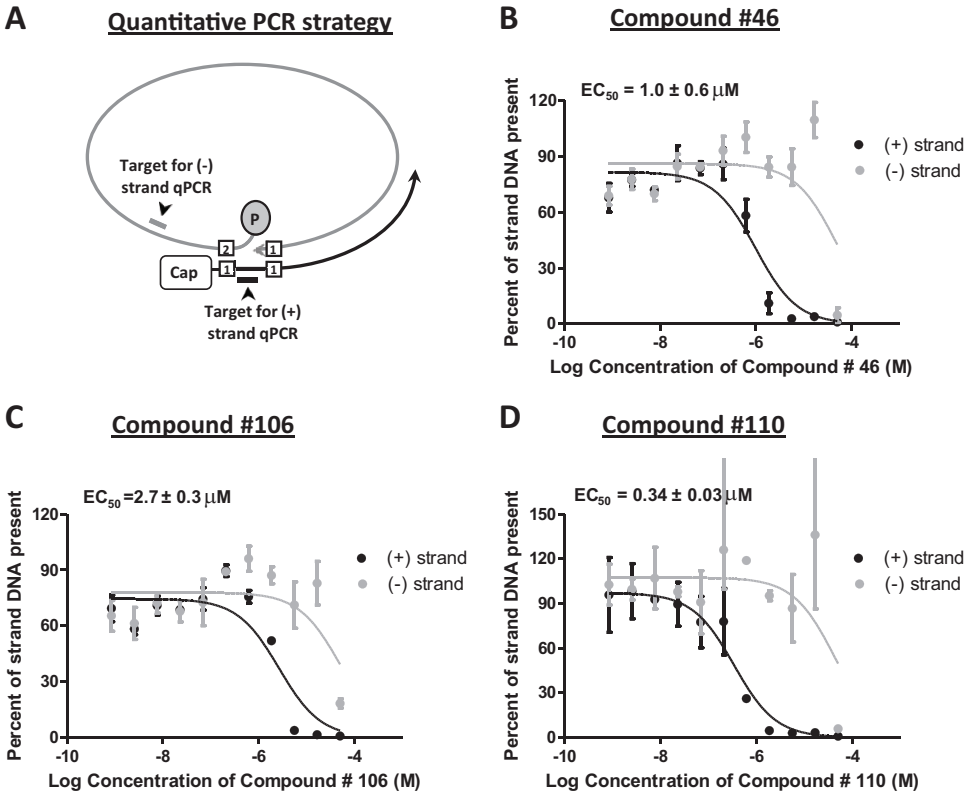


FIG 3 Inhibition of HBV replication by hydroxylated tropolones. The inhibition of replication by hydroxylated tropolones was measured against an HBV genotype D isolate in HepDES19 cells. (A) Principle underlying the strand-preferential quantitative PCR (qPCR) assay. Plus-polarity DNA is measured by amplifying HBV DNA across the gap in the minus-polarity DNA strand. Minus-polarity DNA strands are measured by amplifying sequences downstream of the 3′ end of the vast majority of plus-polarity DNA strands in viral capsids. (B to D) Select EC_{50} curves. EC_{50}s were calculated based on the decline of the plus-polarity DNA strand. The curves and the EC_{50}s are the means ± 1 standard deviation from two or three independent experiments, each conducted in duplicate.

nificant cytotoxicity was evident in the MTT assays. This implies that compound 56 inhibited HBV replication in large part by inducing cytotoxicity.

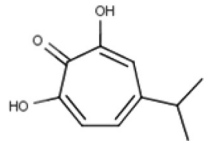
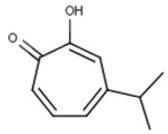
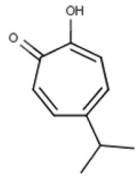
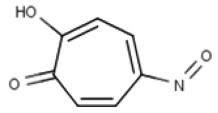
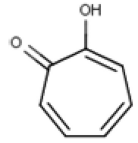
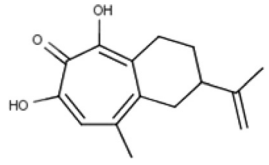
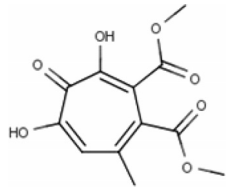
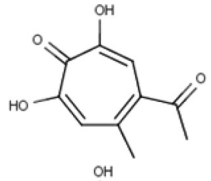
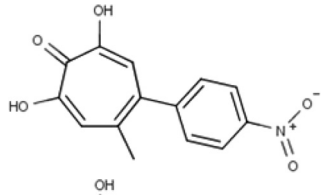
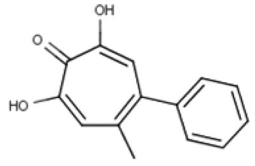
DISCUSSION

The HBV and HIV RNaseH enzymes both belong to the nucleotidyl transferase superfamily (17, 18), and they share about 23% identity and 33% similarity in the core catalytic domain (15, 38). Previously, we identified the α-hydroxytropolone natural product β-thujaplicinol (compound 46) as an inhibitor of HBV RNaseH (14) based on its efficacy against HIV RNaseH (21–25). Here, 50 additional troponoids, including 36 α-hydroxytropolones, were screened against HBV RNaseH based on our previous results and the ability of this compound class to inhibit the HIV enzyme and to cross-inhibit other viruses such as the herpes simplex viruses (26) and foamy virus (39). Our goal was to determine whether tropolones are candidates for development as anti-HBV drugs and, if so, to derive initial structure-activity relationships (SAR) that could help guide their chemical optimization. Screening against recombinant HBV RNaseH identified five compounds that were active at ≤20 μM. These compounds inhibited HBV DNA synthesis with EC_{50}s of <5 μM, and their selective suppression of the HBV plus-polarity DNA strand accumulation confirmed that inhibition of viral replication was due to blocking of the RNaseH activity.

The compounds were screened against HBV RNaseH enzymes from both genotype C and D isolates to maximize the chances of finding hits with efficacy against genetically divergent HBV isolates. Ten of the 13 compounds active against the genotype D RNaseH at ≤60 μM were also active against the genotype C enzyme (Table 1), indicating that hydroxylated tropolones can inhibit the RNaseH enzymes from multiple HBV genotypes. Five of these compounds had somewhat different efficacies against the two genotypes in primary screening, suggesting a potential for the HBV genotype to affect compound efficacy. However, the small magnitude of these differences implies that any potential genotype specificity is unlikely to present an insurmountable hurdle during drug development.

Two major observations regarding the SAR for the tropolones against HBV RNaseH can be drawn from the biochemical studies (Fig. 5). First, the α-hydroxytropolone moiety is needed for strong inhibition (compare compound 46 to compounds 47 and 48). The four most active inhibitors (compounds 46, 106, 107, and 110) and five of the six less efficient inhibitors (compounds 108, 109, 112, 113, and 120) are all α-hydroxytropolones. Almost every other free tropolone tested, including the close analogs of compound 46 (compounds 47 and 48), was inactive. Interestingly, two of the four benzoylated tropolones, compounds 62 and 51, showed minor activity, and broader follow-up of this class of molecules may be warranted. This need for three adjacent Lewis basic

TABLE 2 Inhibition of HBV replication by tropolones

Compound no.	Structure	Results for qualitative replication inhibition[a]	Mean EC_{50} ± SD (μM)	Mean CC_{50} ± SD (μM)	TI
46		+++	1.0 ± 0.6	25 ± 20	25
47		−			
48		−			
50		+			
53		−			
56		++	9.1 ± 0.4	35	3.8
106		+++	2.7 ± 0.3	38 ± 2	14
110		+++	0.34 ± 0.03	32 ± 5	94
112		+++	2.5 ± 1.3	79 ± 15	32
113		+++	4.2 ± 0.8	66 ± 10	16

[a] +++, inhibition at 6.7 μM; ++, inhibition at 20 μM; +, detectable inhibition at 60 μM; −, no inhibition.

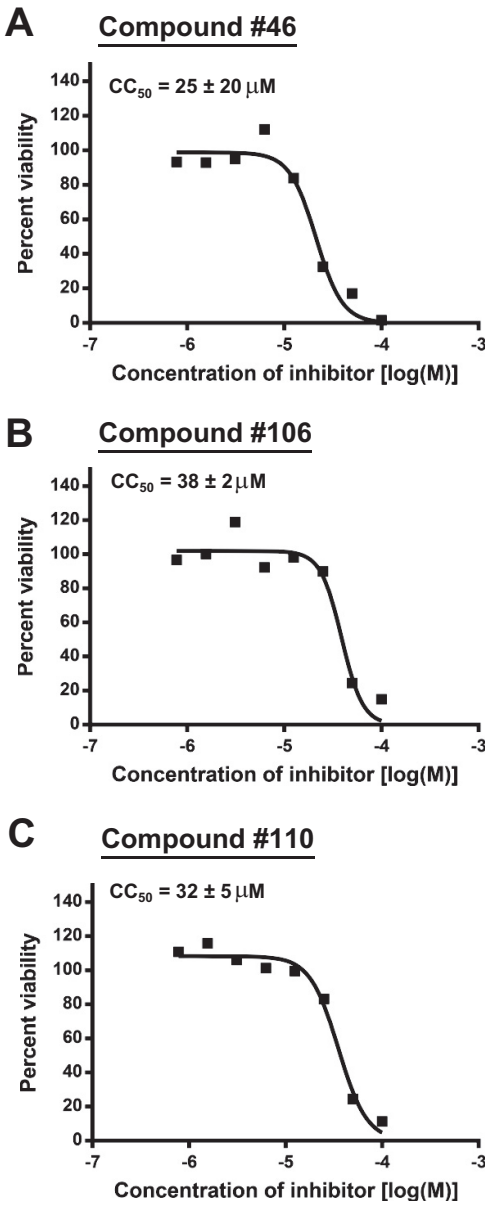


FIG 4 Representative cytotoxicity measurements. Representative MTT cytotoxicity assay results are shown. The curves are from representative experiments conducted in triplicate, and the CC_{50} values are the averages ± 1 standard deviation from two or three independent experiments, each conducted in triplicate.

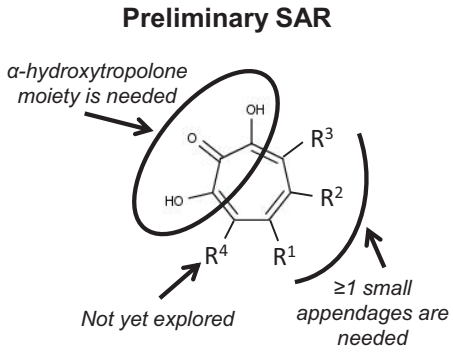


FIG 5 Preliminary SAR for hydroxylated tropolones against HBV RNaseH.

moieties on the tropolone ring for efficient inhibition of RNaseH activity is consistent with mechanistic studies indicating that the α-hydroxytropolones bind to the active site and sequester the divalent cations via interactions with the Lewis basic moieties (21–25). Most reported HIV RNaseH inhibitors from other chemical classes also act by the same mechanism (40–43), but compounds that inhibit HIV RNaseH by altering its conformation or its interaction with nucleic acids have also been reported (44, 45).

Second, there appears to be a size limit for substitutions on the hydroxytropolone ring above which the compounds fail to inhibit RNaseH, although the lack of activity of the parent α-hydroxytropolone (compound 172) demonstrates that some substitution is necessary. The most active α-hydroxytropolone was the least-substituted molecule, compound 46, which has a single isopropyl group, and the other most active inhibitors (compounds 106, 107, and 110) all have similarly small appendages on the hydroxytropolone ring (methyl ester, methyl ketone, methyl, and chloromethyl). As these appendages lengthen, however, the activity diminishes. For example, compound 108 differs from compounds 106 and 107 only at R^1, where it has a larger methoxymethylene group, and this change makes it inactive. The trend continues with compounds 109, 143, 120, 111, and 118, which differ from compound 110 only at R^2, where they have an ethyl ester, an isopropyl ketone, a cyclohexyl ketone, a phenyl ketone, and a biphenyl ketone, respectively. An ethyl ester and an isopropyl ketone are only moderately larger than the methyl ketone, and in this case the activities of compounds 109 and 143 decrease but are not ablated. On the other hand, the biphenyl ketone is the longest of the series, and compound 118 is inactive. The phenyl ketone and cyclohexyl ketone are similar in size and are each considerably longer then the methyl ketone. Interestingly, while the phenyl ketone-containing compound 111 is inactive, the cyclohexyl ketone-containing compound 120 retains minor activity. This could be due to the relative flexibility of the cyclohexane ring to adopt chair-like conformations that would shorten the side chain. Similarly, other larger molecules, such as manicol (compound 56) and its derivatives (compounds 92 to 105), lacked activity. This is in contrast to their enzymatic inhibition of HIV RNaseH, where both compounds 46 and 56 are highly active (21). This size limitation suggests that the binding site of HBV RNaseH may be less open than that of HIV RNaseH, providing an opportunity for exploitation during inhibitor development.

Several compounds that differ from compound 110 with an aryl substitution at R^2 were also tested. The smallest of these, compound 113, has a phenyl group at R^2 and retained some activity. However, even a minor change to this structure, such as introducing a halogen (compounds 114, 119, and 144), resulted in no inhibition (see also compound 115 and compounds 145 to 147). The only close derivative of compound 113 with activity was compound 112, which has a nitroaryl at R^2. It is unclear why this substitution would give greater activity, but it contains the most electronically poor aryl group of the series. This suggests that the favorable interactions between the electronically poor aryl group of the ligand and the electronically rich aromatic residues in the binding cavity may offset any negative effects, as we have seen in other projects (46).

Overall, the relationship between the longer appendage length and lack of activity at R^1 and R^2 suggests unfavorable interactions due to steric strain either within the binding site or perhaps with the substrate. It is unclear whether similar effects would be seen with substitution at the R^3 position. However, the inactivity of compound 117, which differs from the moderately active compound 109 by only an added methyl group at R^3, implies that substantial changes in activity will be observed.

Eighteen of the 35 compounds that were counterscreened against human RNaseH1 inhibited the enzyme, including 11 of the 13 inhibitors of HBV RNaseH. Therefore, the potential exists for cross-inhibition of the human enzyme that should not be overlooked during drug development. However, differences were observed in the sensitivity profiles for HBV and human RNaseHs, implying that selectivity is achievable. For example, compound 46 was 10- to 20-fold more active against HBV RNaseH than against the human enzyme and compound 106 was almost 2-fold more active against the HBV enzyme (Table 1). Furthermore, the human enzyme was modestly sensitive to compounds with substituents attached to one of the two oxygens on the tropolone ring (compounds 59 and 61 to 63), whereas the HBV enzyme was not, and two manicol derivatives (compounds 96 and 98) which were inactive against HBV RNaseH had modest activity against human RNaseH1.

All five of the HBV RNaseH inhibitors that were active at ≤20 μM against HBV RNaseH in biochemical assays (compounds 46, 106, 110, 112, and 113) also preferentially suppressed accumulation of the viral plus-polarity DNA strand in the replication assays. Each compound had an EC_{50} of <5 μM, with compound 110 having an EC_{50} of 0.34 μM. The CC_{50} values for these compounds were 25 to 79 μM by MTT assays (Table 2), leading to TI values from 14 for compound 106 to 94 for 110. Together, these observations indicate that HBV replication was inhibited by these five compounds primarily via suppression of the viral RNaseH activity.

Some of the cytotoxicity (measured as a loss of mitochondrial function in the MTT assays) may be due to inhibition of human RNaseH1 because RNaseH contributes to mitochondrial DNA replication and pre-rRNA processing (47–49). However, the cytotoxicity did not correlate perfectly with inhibition of RNaseH1 (Tables 1 and 2). Compound 56 was inactive against human RNaseH1 yet had a CC_{50} of 35 μM, whereas compounds 106 and 110 had CC_{50} values similar to that of compound 56 but inhibited human RNaseH1 well at 10 μM. This discord implies that inhibition of human RNaseH1 is not the sole source of toxicity in our assays; this possibility is consistent with reports of direct toxicity against isolated mitochondria for some troponoids (50).

In all cases for which both EC_{50}s and IC_{50}s were determined, the EC_{50} was from ~6-fold (for compound 46) to 102-fold (compound 110) lower. This is reminiscent of the lower EC_{50} than IC_{50} that we recently observed with an *N*-hydroxyisoquinolinedione (4.2 versus 28 μM) (16). Some possible explanations for this difference include cellular retention of the compounds, enzymatic conversion to more active derivatives, or greater efficacy against RNaseH in the context of the native enzyme than the recombinant form used in the biochemical studies. Given that this greater efficacy in cells has been observed with seven compounds from two different chemical classes, we favor the possibility that this reflects differences between the native and recombinant forms of RNaseH. However, the good correlation between inhibition at 20 μM in the biochemical assays and activity against replication in culture (Tables 1 and 2) indicates that the biochemical screening assay provides a valuable screening tool with respect to viral replication in the context of simultaneous cytotoxicity testing.

Our studies indicate that α-hydroxytropolones are promising candidates for development as novel anti-HBV RNaseH drugs. They provide the initial guidance for chemical optimization, and they highlight the particular need for care to exploit differences between HBV RNaseH and human RNaseH1 and to address mitochondrial toxicity to avoid unacceptable side effects during the long treatment period envisioned for curative HBV therapy. RNaseH inhibitors may work additively or synergistically with the nucleos(t)ide analogs because the two classes of inhibitors target physically distinct enzyme active sites (51), but this remains to be determined. If so, RNaseH inhibitors would be good candidates for use in the multidrug regimens that will be needed to suppress viral replication far enough to block HBV genomic maintenance in chronically infected individuals (11, 52, 53).

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