3-Hydroxypyrimidine-2,4-Diones as Novel Hepatitis B Virus Antivirals Targeting the Viral Ribonuclease H

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ABSTRACT Hepatitis B virus (HBV) RNase H (RNH) is an appealing therapeutic target due to its essential role in viral replication. RNH inhibitors (RNHIs) could help to more effectively control HBV infections. Here, we report 3-hydroxypyrimidine-2,4-diones as novel HBV RNHIs with antiviral activity. We synthesized and tested 52 analogs and found 4 that inhibit HBV RNH activity in infected cells. Importantly, 2 of these compounds inhibited HBV replication in the low micromolar range.

KEYWORDS RNase H, antiviral agents, hepatitis B virus

Approximately 350 to 400 million people worldwide are chronically infected with hepatitis B virus (HBV), a condition that can lead to cirrhosis, hepatocellular carcinoma, and death (1–5). Current HBV treatment options include only certain nucleoside reverse transcriptase inhibitors (NRTIs) and the innate immune modulator, interferon alpha (1–6). Interferon has only limited efficacy in patients and has many adverse side effects. NRTIs can effectively control virus production and disease progression, but they do not clear the infection. Accordingly, HBV-infected individuals currently require lifelong treatment that can be very costly and that has the potential for adverse side effects due to long-term administration of a therapeutic agent (1–6).

HBV replicates through a pregenomic RNA (pgRNA) intermediate that is converted to the circular, partially double-stranded DNA genome by the viral polymerase, which has priming, reverse transcriptase, and RNase H (RNH) activities. RNH activity degrades the pgRNA during production of the negative-sense DNA strand, allowing for synthesis of the positive-sense DNA strand. The abolishment of RNH activity results in replication-incompetent viruses (7–11), suggesting that HBV RNH represents a potential drug target. To date, β -thujaplicinol, naphthyridinones, α -hydroxylated tropolones, and N-hydroxy-isoquinolinediones have shown activity against HBV replication as RNH inhibitors (RNHIs) (7, 10–13). We report here that 3-hydroxypyrimidine-2,4-diones (HPDs) are novel HBV RNHIs with inhibitory activity against fully infectious HBV.

Previously reported HBV RNHIs were discovered by testing known inhibitors of HIV-1 RNH and integrase, which have similar enzymatic mechanisms. Such compounds act by chelating the two metal cofactors that are coordinated to the catalytic RNH active site

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FIG 1 Primary antiviral and cytotoxicity screening of compounds. (A) HepAD38 cells were treated with compounds (20 μ M) and assessed for HBV core-associated (+)-strand DNA by qPCR. (B) HepG2 cells were treated with compounds (20 μ M) and assessed for cell viability by XTT assay. Values reported are the means $\pm$ standard deviation from 2 independent experiments.

residues (14–18). The HPDs chosen for this study (see Table S1 in the supplemental material) were previously reported to potently inhibit HIV-1 RNH activity (18) and thus were appealing candidates for HBV RNH screening.

We conducted a cell-based screen of 52 compounds for anti-HBV activity in HepAD38 cells, a tetracycline (*tet*)-responsive stable cell line that produces fully infectious HBV upon removal of *tet* from the growth medium (19). HepAD38 cells were treated with compounds (20 μ M) every 2 days for 4 days, and HBV core-associated DNA was extracted as previously described (12, 13). Antiviral activity was assessed using a reported strand-specific quantitative PCR (qPCR) assay (12, 13) that measures inhibition of (+)-strand DNA synthesis due to inhibition of viral RNH activity (Fig. 1A). In order to ascertain that observed inhibition was due to antiviral activity, and not cytotoxicity, HepG2 cells were treated with compounds (20 μ M) every 2 days for 4 days and evaluated for viability by XTT assay (Roche) (Fig. 1B). Considerable toxicity was associated with many of the compounds, thus reducing the pool of suitable compounds. However, compounds 17–20 had a substantial antiviral effect ($P < 0.05$ for 17, 18, and 20 and $P = 0.13$ for 19) without exhibiting toxicity and were therefore chosen for further study. Furthermore, compounds 17–20 lack a methyl substituent at the N-1 position, a modification that is unique to only 5 of the 52 compounds tested (Fig. 2 and Table S1).

For assessment of potency, HepAD38 cells were treated with compounds 17–20 (0–100 μ M) as above and assayed for (+)-strand HBV DNA content by qPCR. Values were plotted in GraphPad Prism 5 and analyzed with the log (inhibitor) versus normalized

FIG 2 Antiviral potency and cytotoxicity of compounds 17 and 18. Center panels: HepAD38 cells were treated with compounds (0–100 μM) and assessed for HBV core-associated (+)-strand DNA by qPCR. Right panels: HepG2 cells were treated with compounds (0–100 μM) and assessed for cell viability by XTT assay. IC_{50} and CC_{50} values reported are means $\pm$ standard deviation from at least 2 independent experiments. IC_{50} , half-maximal inhibitory concentration; CC_{50} , cytotoxic concentration 50; DMSO dimethyl sulfoxide.

response–variable slope equation. Compounds 17 and 18 inhibited HBV (+)-strand DNA synthesis with half-maximal inhibitory concentrations (IC_{50} s) of 5.5 ± 0.6 and $8.0 \pm 0.5 \mu M$, respectively (Fig. 2). Although compounds 19 and 20 had slight antiviral activity, each had an IC_{50} greater than 30 μM . Compounds (0–100 μM) were further tested for cytotoxicity in HepG2 cells using the XTT assay and did not show toxicity at concentrations up to 100 μM (Fig. 2).

We demonstrated that compounds 17–20 have anti-HBV activity, but the actual drug target was unknown. HPDs have been shown to inhibit not only HIV-1 RNH but also HIV-1 integrase and polymerase functions (18, 20–22). Therefore, we probed whether the observed HBV antiviral effect was due to RNH inhibition or inhibition of another target. This was accomplished with the use of a previously described Southern blot-based assay (10, 11). HepAD38 cells were treated with a large amount of compound (40 μM) to ensure that viral replication was significantly suppressed, and HBV core-associated nucleic acid was purified as described earlier. Samples were split into two aliquots, mock treated or treated with *Escherichia coli* RNH, separated by agarose gel electrophoresis, and transferred to a positively charged nylon membrane (Roche). The membrane was subjected to Southern blot analysis using a 500-bp digoxigenin (DIG)-labeled HBV-specific probe synthesized from HepAD38 cells using 5'-GGCCTTTCTGTG TAAACAATACCTGAACC-3' and 5'-GTAATCGAGCTCCGGTGGTCTCCATGCGAC-3' primers with the PCR DIG Probe synthesis kit (Roche). The assay is based on the observation of RNA:DNA heteroduplexes that accumulate due to RNH inhibition migrating like double-stranded DNAs on agarose gels but that appear as faster-migrating species upon treatment with exogenous *E. coli* RNH before electrophoresis. Nucleic acid produced in the absence of RNHs is unaffected by exogenous RNH treatment because the double-stranded species are exclusively DNA (Fig. 3). When the cells were treated with compounds 17 through 20, however, there was a clear shift from double-stranded nucleic acids to the faster-migrating species upon exogenous RNH treatment of samples (Fig. 3), indicating that these compounds do indeed inhibit HBV RNH activity.

Currently, NRTIs are the only reasonable compounds available for treatment of HBV. Our present work suggests that RNHIs can be developed to provide antiviral activity and presents a novel chemical class that may be used for this purpose. Compounds 17

FIG 3 Compounds 17-20 inhibit HBV RNase H activity in cells. HepAD38 cells were treated with compounds (40 μ M), and HBV core-associated nucleic acid was mock treated or treated with *E. coli* RNase H and subjected to Southern blot analysis. DS, double-stranded; SS, single-stranded.

and 18 are of particular interest due to the antiviral effect with no observable toxicity; RNHIs of this nature have thus far not been reported. Combinatorial therapies consisting of multiple drug classes have proved very successful in the treatment of infections such as those caused by HIV and hepatitis C virus. Optimization of RNHIs may help improve treatments for HBV infection.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00245-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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