A Novel Pyridazinone Derivative Inhibits Hepatitis B Virus Replication by Inducing Genome-Free Capsid Formation

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Here we first identified a novel pyridazinone derivative, compound 3711, as a nonnucleosidic hepatitis B virus (HBV) inhibitor in a cell model system. 3711 decreased extracellular HBV DNA levels by 50% (50% inhibitory concentration [IC₅₀]) at 1.5 ± 0.2 μ M and intracellular DNA levels at 1.9 ± 0.1 μ M, which demonstrated antiviral activity at levels far below those associated with toxicity. Both the 3TC/ETV dually resistant L180M/M204I mutant and the adefovir (ADV)-resistant A181T/N236T mutant were as susceptible to 3711 as wild-type HBV. 3711 treatment induced the formation of genome-free capsids, a portion of which migrated faster on 1.8% native agarose gel. The induced genome-free capsids sedimented more slowly in isopycnic CsCl gradient centrifugation without significant morphological changes. 3711 treatment decreased levels of HBV DNA contained in both secreted enveloped virion and naked virus particles in supernatant. 3711 could interfere with capsid formation of the core protein (Cp) assembly domain. A Cp V124W mutant, which strengthens capsid interdimer interactions, recapitulated the effect of 3711 on capsid assembly. Pyridazinone derivative 3711, a novel chemical entity and HBV inhibitor, may provide a new opportunity to combat chronic HBV infection.

Worldwide, approximately 240 million people suffer from chronic hepatitis B virus (HBV) infection; among them, 780,000 die per year from cirrhosis, liver failure, and hepatocellular carcinoma (1, 2).

Although there are several treatment options for chronic HBV infection, none of them is flawless. Interferon (IFN) alpha is effective in only one-third of patients and has various side effects (1, 3). Nucleot(s)ide analogues (NAs), such as telbivudine, lamivudine (LMV), and adefovir (ADV), have quickly manifesting and potent inhibitory effects on hepatitis B virus polymerase (Pol) and reverse transcriptase activity, but long-term use of NAs frequently results in the emergence of drug-resistant HBV strains (4). Although the problem of resistance to the nucleos(t)ide analogs has been solved by the use of tenofovir and to a lesser extent by the use of entecavir (ETV), the big problem with them is their expense and the need of the patient for essentially lifelong treatment (1). Thus, new non-NA agents targeting different stages in the viral life cycle are expected, and drug combinations may contribute to treatment of HBV infection (1, 5).

HBV is a small DNA virus with an envelope surrounding an icosahedral capsid. HBV capsid plays a vital role in the life cycle of HBV. The envelope not only forms the shell of the capsid but also has a direct effect on nucleic acid processing. The capsid assembly process is exquisitely timed and regulated; any disruption could be devastating to virus replication (6). HBV capsids are mostly composed of 120 core protein (Cp) homodimers arranged with $T = 4$ icosahedral symmetry; a small fraction are formed from 90 core protein homodimers and have $T = 3$ icosahedral symmetry (7). HBV Cp encompasses 183 or 185 amino acids, depending on the genotype, and is comprised of an N-terminal assembly domain (amino acids 1 to 149) and a C-terminal domain (CTD) consisting of 150 to 183 or 185 amino acids (8). The CTD is enriched in arginine and is well documented to be essential for HBV pregenome RNA (pgRNA) packaging and HBV DNA replication (9).

The assembly domain, especially the interface between HBV Cp subunits, was recently reported to be also critical for HBV capsid assembly, pgRNA packaging, and reverse transcription (RT) (6, 10, 11). Due to the importance of the HBV capsid assembly process during the HBV life cycle, it provides a potent target for research into antivirals, the development of which is still in its infancy. Thus far, assembly effectors (AEFs), including heteroaryldihydropyrimidines (HAPs) and phenylpropenamides, have been developed to disrupt the assembly of HBV capsids from Cp and inhibit HBV replication (10–12). Treatment using heteroaryldihydropyrimidines was able to both accelerate and misdirect capsid assembly *in vitro* (13–17). Phenylpropenamides, such as AT-130, blocked RNA packaging, producing genome-free capsid without affecting capsid morphology (18). Studies conducted with AT-130 showed that the phenylpropenamides inhibited HBV replication by affecting the capsid assembly reaction rate and timing (19–22). Despite the different mechanisms of the two family of compounds with respect to capsid assembly, based on some recently published reports, AT-130 and heteroaryldihydropyrimidines have been shown to strengthen hydrophobic interactions at the dimer-dimer interface by binding the same hydrophobic

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pocket on the capsid surface (10, 11, 23), which informed us that the interface between capsid proteins was a tremendous potential target for the development of anti-HBV drugs.

Here we first identified a pyridazinone derivative, 3711, as a new type of non-NA inhibitor of HBV. 3711 effectively inhibited the replication of both wild-type (WT) and nucleos(t)ide analogue-resistant strains of hepatitis B virus in cell culture. We observed that 3711 induced the formation of genome-free capsids similarly to previously reported AEFs and that the effect could be mimicked by strengthening the hydrophobic interactions at the interdimer interface of HBV capsid protein subunits (6). We proposed that 3711 could be used as a tool compound to further investigate the mechanism of HBV AEFs and throw light on anti-HBV studies of pyridazinone derivatives.

MATERIALS AND METHODS

Reagents. 3711 and AT-130 (18, 20) were synthesized in our institution. The structures of these compounds were identified using proton nuclear magnetic resonance, and the purity was determined as 99.5% using high-performance liquid chromatography (HPLC). 3TC was purchased from Tianfeng Chemical Technology Co., Ltd. (Suizhou, Hubei, China), and the purity was determined as 99.1% by HPLC. All compounds were prepared in dimethyl sulfoxide (DMSO) as a 40 mM stock solution and kept at 4°C. Dilutions of the compounds were performed on the day of medium change.

Plasmids. The pHBV1.3 plasmid contains a 1.3-mer (overall length) HBV genotype A2 genome (adw2 subtype; GenBank accession number X02763.1). Plasmid pHBV1.3 L180M/M204I, which contained a lamivudine/entecavir (3TC/ETV) dually resistant (rtL180M/rtM204I) HBV genome (20), and plasmid pHBV1.3A181T/N236T, which contained an adefovir (ADV)-resistant (rtA181T/rtN236T) HBV genome (24), were generated by using a site-directed gene mutagenesis kit (Beyotime, Haimen, Jiangsu, China) according to a method that was described before (25). The HBV genome regions encoding the C-terminally truncated Cp144, Cp156, Cp163, and Cp175 proteins and the full-length Cp185 protein were amplified by PCR using pHBV1.3 as a template, and then the PCR products were individually cloned into pcDNA3.1 vector to construct plasmids expressing C-terminally truncated and full-length core proteins (26). The pHBV1.3 plasmid was mutated to pHBV1.3-HBcV124W by use of a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies).

Cell culture and transient transfection. HepG2.2.15, a HepG2-derived cell line which stably transfected with four integrated tandem copies of the HBV genome (ayw serotype; GenBank accession number U95551) (27), was maintained in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) supplemented with 10% fetal bovine serum (FBS; HyClone) and 380 µg/ml G418 (GibcoBRL). Cells of the Huh7 cell line were grown in DMEM supplemented with 10% FBS, and transient transfections were performed in six-well plates with 1 µg plasmid DNA by the use of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. All cell lines were cultured at 37°C with 5% CO₂.

Detection of intracellular HBV DNA. HepG2.2.15 cells were cultured and treated using compounds in 6-well plates at a density of 3×10^5 cells per well for 8 days. The cells were collected and lysed with cold lysis buffer (1 mM EDTA-2Na, 50 mM Tris-Cl [pH 7.5], 0.5% [vol/vol] Nonidet P-40 [NP-40]) (Tris7.5 buffer) at 4°C for 15 min. The cellular debris was removed by centrifugation. The cell lysates were treated with proteinase K and sodium dodecyl sulfate (SDS) separately and sequentially, achieving final concentrations of 500 µg/ml of proteinase K and 0.5% SDS, and then incubated for 2 h at 56°C. The viral DNA was then purified by phenol and chloroform extractions and precipitated with 1 volume of isopropanol, and a final concentration consisting of 0.3 M NaCl and 5 µg carrier RNA was added during precipitation. Nucleic acid pellets were resuspended in

an appropriate volume of Tris-EDTA (TE) and then analyzed by real-time PCR and Southern blotting (28). The sequences of the HBV probe and a pair of primers were as follows: for the probe, 5'-6-carboxyfluorescein (FAM)-CGCAGACCAATTTATGCCTACAGCC-black hole quencher 1 (BQ1)-3'; for the forward primer, 5'-AAGACTGGGAGGAGTTGGG-3'; for the reverse primer, 5'-AGTTGCATGGTCTGGTG-3' (25). The HBV DNA probe used for Southern blot analysis was a digoxigenin (DIG)-labeled PCR fragment encompassing genome positions 463 to 1499 (DIG DNA labeling kit; Roche) (25, 29).

Detection of extracellular HBV DNA. HepG2.2.15 cells were cultured and treated in 96-well plates at a density of 3×10^3 cells for 8 days under standard conditions. HBV DNA from cell culture medium was collected and extracted by the use of a blood and tissue kit (Qiagen) and was quantified by real-time PCR as described previously (25). For detection of extracellular replication intermediates, HepG2.2.15 cells were cultured in 6-well plates for 8 days, HBV particles were precipitated from the medium samples with dry polyethylene glycol 8000 (PEG-8000; Sigma Chemical Co., St. Louis, MO) (30) and NaCl (final concentrations, 10% [wt/vol] and 2% [wt/vol], respectively), incubated overnight at 4°C, and spun for 1 h at 4,000 rpm at room temperature. Virus pellets were resuspended in cold lysis buffer, and then viral DNA was extracted as described above and HBV DNA bands were visualized by Southern blotting (28).

Detection of HBV RNAs and capsid-associated RNA by qRT-PCR and Northern blot analysis. HepG2.2.15 cells were cultured in 6-well plates at a density of 3×10^5 cells for 8 days. Total RNA was extracted from HepG2.2.15 cells by the use of TRIzol reagent (Invitrogen). For capsid-associated RNA detection, HepG2.2.15 cell lysate was digested with 20 U/ml Cyronase cold-active nuclease (TaKaRa) for 15 min at 4°C, isolated using a QIAamp MinElute virus spin kit (Qiagen) (31), including digestion with DNase Set (Qiagen), and then detected by Northern blot analysis and quantitative RT-PCR (qRT-PCR), which was performed by a one-step method using a Quantitect virus kit (Qiagen). For Northern blot analysis, the isolated total RNAs and capsid-associated RNAs of each sample were electrophoresed on a 1% formaldehyde agarose gel and transferred onto a nylon membrane following the protocol of a NorthernMax kit (Ambion). The hybridization was performed with the HBV DNA probe used in Southern blot analysis (DIG DNA labeling kit; Roche) for 16 h at 50°C (DNA:RNA hybridization).

HBV capsid and capsid-associated viral nucleic acid assay. HBV capsids and associated viral DNAs were analyzed by the use of a native agarose gel electrophoresis-based assay (32). After treatment with compounds for 8 days, cells were lysed using the cold lysis buffer as described above. A 10-µl volume of each of the samples was loaded into a native agarose Tris-acetate-EDTA (TAE) gel to detect capsid, capsid-associated DNA, capsid-associated RNA/plus-strand DNA [RNA/DNA (+)], and minus-strand DNA [DNA (-)] separately.

For the detection of capsid assembly, the capsids were transferred directly to nitrocellulose with 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by using the capillary transfer method. The filter was then probed for core antigens (nitrocellulose) by immunostaining with the antibody recognizing HBcAg (Abcam).

For the detection of capsid-associated DNA, the agarose gel was denatured twice for 15 min in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) and washed using neutralization buffer (1.5 M NaCl, 0.5 M Tris-Cl, pH 7.0) twice for 15 min. The encapsidated virus DNA was then transferred to a nylon filter with 20× SSC buffer by using the capillary transfer method. The dried filter was probed for viral DNA by Southern blotting (33). Then, capsid-associated DNA was detected by hybridization with HBV-specific DNA probe as used in Southern blot analysis.

For analysis of capsid-associated RNA/plus-strand DNA [RNA/DNA (+)] and minus-strand DNA [DNA (-)], HBV capsids were transferred directly to a nylon membrane with TNE (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.1 mM EDTA) overnight. The nucleic acid contained in these capsids was then released by wetting the membrane for 10 s in the dena-

turation buffer, followed by washing in neutralization buffer for 5 min. The RNA/DNA (+) and the DNA (–) were detected by hybridization with HBV-specific RNA probe (DIG RNA labeling kit; Roche) at 68°C (RNA:RNA hybridization). RNA probe targeting genome positions 81 to 1040 was used for hybridization and labeled with digoxigenin-UTP by *in vitro* transcription with SP6 or T7 RNA polymerase (DIG RNA labeling kit; Roche), separately. All given nucleotide positions began with the adenine of the initiation codon of the C gene.

Western blot analysis. Cell lysates were boiled in SDS sample buffer (62.5 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue) for 5 min at 100°C. The samples were applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted with primary antibodies. The primary antibody recognized HBcAg (Abcam, Cambridge, United Kingdom), HBsAg (Abcam, Cambridge, United Kingdom), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Abcam, Cambridge, United Kingdom). Bound secondary antibodies were detected with a chemiluminescence substrate (GE) and exposed to Kodak X-ray film (Kodak).

Isopycnic CsCl gradient centrifugation. For viral particle secretion assays, 11 ml of cleared HepG2.2.15 cell culture medium was used to dissolve 3.88 g of solid CsCl and spun for 40 h at 48,000 rpm and 4°C in a P55ST rotor (Hitachi), allowing the virions to reach their buoyant densities. Approximately 600 μ l per fraction was collected from the top of the reaction mixture to the bottom. HBV particles were precipitated from 200 μ l of each fraction with 10% polyethylene glycol 8000 as described above (30). The pellets were then dissolved with phosphate-buffered saline (PBS) and loaded into a 1.8% (wt/vol) agarose–Tris-acetate-EDTA (TAE) gel for separation. The level of HBsAg in 50 μ l of each fraction was measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Sino-American Biotechnology Company, Henan, China) following the manufacturer's recommendations. HBV DNA was extracted by the use of a blood and tissue kit (Qiagen) and detected by real-time quantitative PCR (qPCR).

For intracellular capsid research, HepG2.2.15 cells were collected and lysed with 0.5% NP-40 lysis buffer and then the cellular debris was removed. Cleared cell lysate (11 ml) was used for isopycnic CsCl gradient centrifugation. Approximately 200 μ l per fraction was collected, HBV capsids in each fraction were detected by 1.8% (wt/vol) agarose–Tris-acetate-EDTA (TAE) gel electrophoresis, and HBV DNA and capsid-associated RNA/DNA (+) were extracted and detected by Southern blot analysis (30) and Northern blot analysis and then separately quantified by qPCR and qRT-PCR. To address the capsid morphology in each fraction, the fractions containing capsids were separately dialyzed against 50 mM Tris7.5 buffer for electron microscopy (EM).

Electron microscopy. HepG2.2.15 cells were treated with or without 3711 for 8 days. HepG2.2.15 cells were washed with PBS and treated with 0.5% NP-40 lysis buffer. The cell culture medium was collected and subjected to a 26% sucrose cushion, and then the pellet was dissolved with PBS. The enriched extracellular capsids and cleared cell lysates were used separately for isopycnic CsCl gradient centrifugation as described above. Fractions containing capsids (detected by native agarose gel) were pooled and dialyzed against 50 mM Tris7.5 buffer for electron microscopy. Samples were adsorbed to glow-discharged carbon over paralodion copper grids and then stained with 2% uranyl acetate and visualized with a FEI Tecnai G₂ Spirit transmission electron microscope (34).

RESULTS

3711 inhibited HBV replication in cell culture. 3711, whose chemical structure is shown in Fig. 1A, is a pyridazinone derivative. The anti-HBV activity of 3711 was examined in HBV using a stably transfected HepG2.2.15 cell line. HepG2.2.15 cells were treated with gradient concentrations of 3711 for 8 days. Lamivudine (3TC) was used as a positive control. The cytotoxicity of 3711 in HepG2.2.15 cells was measured by the MTT [3-(4,5-dimethyl-

thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) method (35) (Fig. 1B). 3711 did not exhibit any significant cytotoxicity even at concentrations as high as 50 μ M. qPCR and Southern blotting were used to quantify HBV DNA replication. The inhibitory concentration of 3711 that decreased HBV DNA levels in supernatant by 50% (IC₅₀) was $1.5 \pm 0.2 \mu$ M (Fig. 1C). The IC₅₀ for intracellular capsid-associated HBV DNA was $1.9 \pm 0.1 \mu$ M (Fig. 1E). Southern blotting analyses showed that 3711 inhibited the various forms (relaxed circular [RC] and single-stranded [SS] HBV DNA) of both extracellular and intracellular HBV DNA in a dose-dependent manner (Fig. 1D and F).

Moreover, when wild-type (WT) HBV plasmid (Fig. 2A) and 3TC/ETV dually resistant mutant L180M/M204I (Fig. 2B) and ADV-resistant mutant A181T/N236T (Fig. 2C) plasmids (3) were transiently transfected into Huh7 cells and treated with compounds for 72 h, both the nucleoside analog-resistant mutants were as susceptible to 3711 as wild-type HBV. To confirm that these mutants showed a drug resistance phenotype, replicate assays were carried out in parallel with nucleoside analogs as an internal control. The results showed that the mechanism of 3711's action might be different from that seen with NAs.

3711 induced the formation of HBV DNA-free capsids without showing an influence on HBV protein expression and distribution, total capsid production, and morphology. To measure whether the decreased HBV replication seen after 3711 treatment was due to protein translation or capsid production, HBV core protein and capsids were analyzed by SDS-PAGE and native agarose gel electrophoresis, separately. 3711 had no effect on core protein expression (Fig. 3A, 4th panel) and distribution (data not shown), even at doses at concentrations of up to 10 μ M (a concentration near the IC₉₀) for 8 days of treatment. The surface protein expression and secreted antigen (HBsAg/HBeAg) levels were also not affected by 3711 (data not shown). GAPDH was used as a loading control here (Fig. 3A, 5th panel). Total capsid formation was detected by 1.2% native (nonreducing) agarose gel electrophoresis, and no change was observed after 3711 treatment (Fig. 3A, 1st panel). However, when we further analyzed capsid electrophoresis mobility and capsid-associated HBV DNA levels *in situ* on a 1.8% native agarose gel, the results showed that a portion of faster-migrating capsid (shifted to a lower band) was induced by 3711 (Fig. 3A, 2nd panel). And, consistently with the results described above, levels of HBV DNA packaged in capsid with the same electrophoresis mobility as the control were decreased by 3711 treatment in a dose-dependent manner. Notably, though, no HBV DNA was detected in the faster-migrating capsid (Fig. 3A, 3rd panel).

To investigate capsid morphology with or without 3711 treatment, HepG2.2.15 cell lysates were separated by CsCl gradient centrifugation. Capsids contained in each fraction were detected using native agarose gel electrophoresis (data not shown), and fractions enriched with capsids were collected and dialyzed into Tris7.5 buffer. In both 3711-treated and untreated samples, 30-nm-diameter core particles with similar morphologies were visualized using electron microscopy (EM) (Fig. 3B). Consistently with previous reports (36–38), two kinds of HBV virions, with either a filled or an empty-looking inner capsid, were observed by EM after negative staining. The proportion of HBV nucleic acid-

FIG 1 3711 inhibited HBV DNA replication *in vitro*. (A) Chemical structure of 3711. (B) HepG2.2.15 cells were treated with 3711 at the indicated concentrations for 8 days. The cytotoxicity was determined using the MTT assay. Due to the solubility of 3711, the concentrations of 3711 that inhibited cell viability by 50% (TC₅₀) could not be determined. Mol.Wt., molecular weight. (C and D) Extracellular HBV DNA of HepG2.2.15 was quantified by qPCR (C) and Southern blot analysis (D). The IC₅₀ of 3711 was 1.5 ± 0.2 μM. Virions from HepG2.2.15 culture medium were precipitated with 10% polyethylene glycol (PEG), and capsid-associated DNA was subjected to Southern blot analysis. (E and F) The IC₅₀ for intracellular capsid-associated HBV DNA was 1.9 ± 0.1 μM by qPCR (E). HBV replication intermediates were detected by Southern blot analysis (F). RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA. IC₅₀ values were expressed as the means $\pm$ standard deviations (SD) of the results of three independent experiments. 3711 inhibited the various forms (RC and SS) of both extracellular and intracellular HBV DNAs in a dose-dependent manner. 3-TC, a polymerase inhibitor, was used as a positive control and specifically decreased the amount of intracellular HBV DNA.

filled capsids decreased from 34.9% of untreated samples to 10.7% after 3711 treatment (Table 1).

Genome-free capsid induced by 3711 had a density profile showing lower buoyancy. To further investigate the effect of 3711 on HBV capsid assembly, we subjected HepG2.2.15 cytoplasmic capsid to CsCl buoyant density sedimentation centrifugation, yielding 25 fractions, and detected capsid in each fraction using 1.8% native agarose electrophoresis. Capsid-associated DNAs and RNAs were extracted and examined by Southern blot analysis and Northern blot analysis, respectively, and the results were confirmed by qPCR and qRT-PCR.

Capsids from untreated cell lysates sedimented characteristically in fractions 13 to 16 (Fig. 4A); in contrast, capsid assembly after 3711 treatment showed slightly slower sedimentation (shifted to fractions 11 to 15) (Fig. 4B). Capsid-associated DNAs and RNAs in each gradient were extracted and detected by Southern blot analysis and Northern blot analysis, and they were reduced to levels below the detection limit of Southern and Northern blot analyses after 3711 treatment (Fig. 4B). The result was further confirmed by qPCR (Fig. 4C) and qRT-PCR (Fig. 4D). To address the issue of whether the capsids with different sedimentation profiles showed morphological changes, we visualized cap-

FIG 2 Dose-dependent inhibition of replication of WT and nucleoside analog-resistant HBV mutants by 3711. Huh7 cells were transiently transfected with wild-type pHBV1.3 (pHBV1.3 wt) (A), 3TC/ETV dually resistant (rtM204V/rtL180M) (B), and ADV-resistant (rtA181T/rtN236T) HBV genomes (C) and then exposed to compounds at the indicated concentrations for 72 h, after which intracellular HBV replication was analyzed by Southern blotting. The various forms (RC and SS HBV DNA) of WT and nucleoside analog-resistant HBV mutants were dose-dependently inhibited by 3711 treatment.

sids from fractions of 3711-treated or untreated samples using transmission electron microscopy, and no relevant distinctions were observed (Fig. 4A and B).

Overall, the result of the density gradient analysis further strengthened the conclusion that 3711 induced the formation of genome-free capsids, which had a normal structure but lower buoyant density.

3711 treatment decreased the levels of HBV DNA contained in both secreted enveloped virions and naked virus particles. Previous reports showed that genome-free HBV capsid can be released as a naked capsid (39) or as an enveloped virion (22). We therefore asked whether the genome-free capsid induced by 3711 could be enveloped and secreted. To address this issue, media from HepG2.2.15 cells were separated by isopycnic CsCl gradient centrifugation. Each gradient was fractionated into 18 fractions, the HBsAg level in each fraction was measured by ELISA, capsids were precipitated with polyethylene glycol 8000 and detected on a 1.8% native agarose gel, and HBV DNA was quantified by qPCR. Capsids detected at a density of around 1.20 g/ml were indicative of virions, and fractions at densities of around 1.31 g/ml were considered to be indicative of naked capsids (Fig. 5A), which was in good agreement with the HBsAg distribution (Fig. 5B). Levels

FIG 3 3711 induced the formation of HBV DNA-free capsids, without influence on HBV protein expression, total capsid production, and morphology. HepG2.2.15 cells were treated with compounds at the indicated concentrations for 8 days. (A) 1.2% native agarose gel electrophoresis was used for analysis of total core particles (1st [top] panel). Capsids were further analyzed on 1.8% agarose gel (2nd panel). Capsid-associated HBV DNA was detected by the transfer of HBV capsids on a nylon sheet, followed by Southern blotting hybridization upon disruption of capsids *in situ*. The HBV DNA probe for Southern blot analysis was a DIG-labeled PCR fragment encompassing genome positions 463 to 1499 (DIG DNA labeling kit; Roche) (3rd panel). HBc (core) expression was detected by Western blotting (4th panel). The GAPDH level was used as a loading control (5th panel). (B) HBV capsid was collected from HepG2.2.15 cell lysates and visualized with a transmission electron microscope in negatively stained micrographs. Normal particles of the HBcAg with or without 3711 treatment were readily visualized. White arrowheads denote the empty virion or empty-virion-derived capsids; black arrowheads denote the filled virion or filled-virion-derived capsids.

of HBV DNA packaged in both HBV particle forms were significantly decreased by 3711 treatment (Fig. 5C). Consistently with the intracellular results, the naked capsid after 3711 treatment sedimented slower than the WT, which shifted from 15 to 17 fractions of untreated gradient to 14 to 16 fractions after 3711 treatment (Fig. 5A). Virion and naked capsid fractions were exposed separately due to the levels of the virion signals being lower than

TABLE 1 Percentage of filled capsids in HepG2.2.15 cell lysates under EM

Type(s) of capsids	No. or % of selected capsids ^a	
	Control	3711 treated
Filled	86	16
Total	246	149
Filled/total (%)	34.9	10.7

^a Capsids from HepG2.2.15 cell lysates with or without 3711 treatment were visualized under EM. In the negatively stained micrographs shown in Fig. 3 to 5, white arrows denote the empty virion or empty-virion-derived capsids and black arrows indicate the filled virion or filled-virion-derived capsids.

FIG 4 Empty faster-migrating capsid induced by 3711 had normal morphology but lower buoyant density. (A and B) HepG2.2.15 cell lysates without (A) or with (B) 3711 treatment were subjected to CsCl buoyant density sedimentation centrifugation, the samples were fractionated into 25 fractions after abandoning the first 5-ml gradient, and capsids were analyzed in each fraction by 1.8% native agarose gel electrophoresis. Capsid-associated DNA was extracted and detected by Southern blot analysis. After digestion with DNase Set (Qiagen), capsid-associated RNA was extracted by using a QIAamp MinElute virus spin kit (Qiagen) and detected by Northern blot analysis. Capsid assemblies sedimented slower (shifted to a lower density) after 3711 treatment than wild-type capsid assemblies. Capsids contained in fractions 13 to 15 of the control and capsids contained in fractions 11 to 13 of 3711-treated reaction mixtures were observed by transmission electron microscopy. White arrowheads denote the empty virion or empty-virion-derived capsids; black arrowheads denote the filled virion or filled-virion-derived capsids. (C) Graphic representa-

those seen with naked capsids. For EM observation of extracellular capsid, the stain could not penetrate the envelope of virions, thus making it difficult to determine if the capsids within the virions were filled or empty. To visualize better the capsids contained in the virions, the virion envelope was removed by detergent lysis and the released capsids were observed. Upon removal of the virion envelope by the detergent treatment, we found that the majority of the released capsids had an empty appearance consistent with their containing no nucleic acid (Fig. 5D), whereas plenty of empty capsids were also observed in the untreated sample. Taking the results together, we concluded that 3711 treatment decreased the levels of HBV DNA contained in both the secreted enveloped virion and naked virus particle forms, but whether the 3711-induced faster-migrating capsids were enveloped and secreted needs further investigation.

3711 decreased capsid-associated RNA levels without an influence on total HBV RNA production. To address whether the HBV capsid-associated DNA diminishment was due to the impact of 3711 on pgRNA encapsidation, which is the step prior to genome synthesis, total RNAs and capsid-associated RNAs were extracted and subjected to Northern blot analysis (35) 8 days after 3711 treatment. 3711 had no effect on total HBV RNA production (Fig. 6A) but dose-dependently decreased the capsid-associated RNA level (Fig. 6B), which was quantified using qRT-PCR (Fig. 6C). AT-130, a known assembly effector, was used at a concentration approximating the IC_{50} (5 μ M) as a positive control and showed activity similar to that seen with 3711 treatment. The results described above indicated that 3711 decreased HBV RNA encapsidation to inhibit HBV replication.

3711 interfered with capsid assembly independently of the Cp CTD and Pol/pgRNA complex. To encapsidate HBV RNA and initiate capsid assembly, core proteins assemble around the pregenomic RNA (pgRNA) and viral reverse transcriptase (Pol) (40). Viral replication occurs within nucleocapsids by the reverse transcription of pgRNA, and the tyrosine residue (i.e., Y63) of polymerase functions as a protein primer to initiate reverse transcription (41, 42). To determine whether the activity of 3711 in decreasing HBV RNA encapsidation was related to the presence of the Pol/pgRNA complex, we generated plasmids expressing a Pol deletion mutant and a Pol mutant with a Y63F amino acid substitution which was defective in RT activity but retained RNA binding ability (42). Huh7 cells were transfected with WT plasmid HBV1.3 or one of the Pol mutant plasmids. Transfection with the pHBV1.3 Pol Y63F mutant led to defective HBV DNA synthesis (Fig. 7A, 3rd panel, 5th lane) but did not completely block HBV pgRNA packaging (Fig. 7A, 2nd panel, 5th lane). 3711 was able to inhibit the encapsidation of pgRNA from both WT HBV and the Pol Y63F mutant plasmids. Moreover, results from the 1.8% agarose electrophoresis assay showed that 3711 induced the faster-migrating capsid assembly from the WT strain and both mutants, independently of the function of Pol/pgRNA complex (Fig. 7A). So we concluded that the loss of HBV RNA packaging was not the direct result of 3711 treatment but was presumably a secondary consequence of capsid assembly without viral RNA.

tation of qPCR data from capsid-associated DNA from gradients of samples that were left untreated or treated with 5 μ M 3711. (D) Graphic representation of qRT-PCR data from capsid-associated RNA from gradients of untreated or 5 μ M 3711-treated samples. The analyses of the DNA and RNA content of the capsids were performed in triplicate.

FIG 5 3711 treatment decreased HBV capsid-associated DNA levels in both enveloped and naked HBV particles in supernatant. HepG2.2.15 cells were treated with 5 μ M 3711 or without 3711 for 8 days, and the culture media were separated by the use of isopycnic CsCl gradients. Fractions were taken from the top. (A and B) Capsids were precipitated from each gradient with polyethylene glycol 8000, and the pellets were dissolved with mild detergent and electrophoresed on a 1.8% native agarose gel. Capsids detected at a density of around 1.20 g/ml were indicative of virions, and fractions at densities of around 1.31 g/ml were considered to be naked capsids (A), an interpretation supported by the detection of HBsAg of each fraction by ELISA as shown (B). Virion and naked capsid fractions were exposed separately due to the virion signals being lower in quantity than the naked capsids. (C) Capsid-associated HBV DNA was extracted from each fraction and quantified by qPCR. (D) Both 3711-treated and untreated fractions enriched with capsids were collected from extracellular isopycnic CsCl gradients and dialyzed into Tris7.5 buffer. Capsid was visualized with a transmission electron microscope in negatively stained micrographs. White arrowheads denote the empty virion or empty-virion-derived capsids; the black arrowhead denotes the filled virion or filled-virion-derived capsids.

To define the specific core protein domain critical for 3711's effect on capsid assembly, a series of plasmids expressing C-terminally truncated HBcAgs with different C-terminal lengths (Cp183, Cp175, Cp163, Cp156, Cp144, and Cp140), as shown in Fig. 7B, were transiently transfected into Huh7 cells. Capsids that consisted of these truncated HBcAgs were analyzed in the presence of

3711 by 1.8% native agarose gel electrophoresis, and formation of faster-migrating capsids was induced in all five groups of recombinant hepatitis B core particles. The results implied that the effect of 3711 on capsid assembly was irrelevant to the CTD of core proteins; instead, its activity might in part represent a link to the Cp assembly domain.

FIG 6 3711 treatment decreased capsid-associated RNA levels without exerting an influence on the 3.5-kb, 2.4-kb, and 2.1-kb HBV-specific transcripts. HepG2.2.15 cells were treated with compounds at the indicated concentrations for 8 days. (A) Total RNA was extracted and detected by Northern blotting hybridization. (B) After digestion with DNase Set (Qia-gen), capsid-associated RNA was extracted by using a QIAamp MinElute virus spin kit and detected by Northern blot analysis. (C) Graphic representation of RT and qPCR data of total RNA and capsid-associated RNA from HepG2.2.15 cells after treatment with compounds at indicated concentrations.

3711's effect is recapitulated by strengthening the hydrophobic interactions at the interdimer interface of HBV capsid. To explore the mechanism of 3711, we compared the effects of 3711 and AT-130 on capsid assembly. Plasmids expressing Cp183 and Cp144 were transiently transfected into Huh7 cells. The result showed that both 3711 and AT-130 induced the production of HBV faster-migrating capsids that consisted of Cp183 and Cp144 (Fig. 8A).

Previous studies identified AT-130 as an assembly effector which effectively blocks virus production by simply affecting the timing or the rate of normal, on-path assembly (19). It was reported that AT-130 bound with a hydrophobic pocket on the capsid surface and that use of a plasmid expressing core protein with an amino acid substitution (V124W) which strengthened hydrophobic interaction at the dimer-dimer interface resulted in activity that resembled AT-130-bound WT capsid assembly (10, 11). To substantiate our expectation that 3711 might interact with the same pocket of capsid as AT-130, we studied the effect of 3711 on V124W mutant assembly in cell culture. Huh7 cells were transfected with pHBV1.3 (WT) and core V124W mutant plasmids, and 1:1 coexpression of core V124W mutant plasmid and the full-length core protein (Cp185)-expressing plasmid was performed. Expression of the core V124W mutant recapitulated the

effects of 3711 and AT-130 on inducing production of faster-migrating "empty" capsids. A 1:1 coexpression of the core V124W mutant and HBc185 plasmids resulted in production at approximately 30% of the total DNA level seen with the WT plasmid alone and reduced assembly of faster-migrating capsids compared to the level seen with the core V124W mutant plasmid (Fig. 8B). Notably, HBV replication from the 1:1 coexpression experiments was inhibited by 3711 and AT-130 treatment (Fig. 8C). The data presented here revealed that the results seen with core V124W mutant, which strengthened hydrophobic interactions at HBV Cp interdimer interface, resembled 3711/AT-130-bound wild-type capsid assembly. The results suggested that the activity of 3711 as an assembly effector was at least partly related to strengthening the hydrophobic interactions at the interdimer interface of HBV capsid.

DISCUSSION

We first reported a pyridazinone derivative, compound 3711, as an HBV inhibitor by inducing genome-free capsid assembly. In a cell model system, 3711 demonstrated antiviral activity effects on both wild-type and nucleos(t)ide analogue-resistant HBV replication at levels far below those associated with toxicity (Fig. 1 and 2).

3711 treatment induced the formation of genome-free capsids. The induced genome-free capsids showed different levels of electrophoresis mobility on a 1.8% native agarose gel (Fig. 3) and different density profiles in isopycnic CsCl gradient centrifugation (Fig. 4). However, as shown in the electron micrographs, we observed no aberrant capsid assembly after 3711 treatment (Fig. 3B, 4, and 5D). Therefore, we hypothesized that 3711 treatment prevents formation of virions by initiating genome-free capsid assembly to "escape" the normal capsid assembly path. Moreover, HepG2.117 cells, a stably HBV-replicating TetOFF cell line, were treated with chemicals in doxycycline (Dox; 100 ng/ml)-containing or Dox-free medium. The presence of 100 ng/ml Dox has been shown to lead to no detectable production of nascent HBV particles (43). In the presence of doxycycline, 3711 had no effect on the preexisting capsids (data not shown). But, comparably, 3711 treatment induced formation of the faster-migrating capsids in Dox-free medium. The results indicated that 3711 interfered with nascent formation of capsids from newly synthesized HBcAg, which was confirmed by the inhibition effect of 3711 on HBV DNA replication.

We compared the activity of 3711 with that of members of other, better-studied classes of compounds, such as AT-130 (18), Bay38-7690 (14), and 8-1 (44), which were previously reported to disrupt capsid assembly (data not shown). The results showed us that the effect of 3711 treatment on capsid assembly was similar to that of AT-130; 3711 and AT-130 both induced genome-free capsid formation with normal morphology seen using an EM but did not affect the expression and distribution of viral protein (Fig. 3A, 4th panel). Thus, AT-130 was used as a control in our experiment to substantiate our hypothesis that 3711 acted as an assembly effector. Consistently with our expectation, we found that both 3711 and AT-130 could induce the production of faster-migrating capsids.

To further explore the profiles of 3711-induced capsids, a series of C-terminally truncated HBcAg and $\Delta\text{Pol}/\text{Pol}$ Y63F mutant plasmids were transiently transfected into Huh7 cells. The results showed that genome-free capsid assembly without 3711 treatment

FIG 7 3711 treatment interfered with capsid assembly independently of Cp CTD and Pol/pgRNA complex. Huh7 cells were transiently transfected with various plasmids and then treated with 3711 for 72 h. Intracellular HBV capsids were loaded on a 1.8% native agarose gel. (A) Huh7 cells were transiently transfected with plasmids containing a Pol deletion mutant and a Pol mutant with a Y63F amino acid substitution. Capsid-associated RNAs with plus-strand DNA [RNA/DNA (+)] and minus-strand DNA [DNA (-)] were detected *in situ* by hybridization with an HBV-specific RNA probe (DIG RNA labeling kit; Roche) at 68°C. (B) Huh7 cells were transiently transfected with a series of plasmids expressing C-terminally truncated HBcAg with different C-terminal lengths (Cp183, Cp175, Cp163, Cp156, Cp144, and Cp140). Capsids were analyzed on a 1.8% agarose gel (top panel). HBc (core) expression was detected by Western blotting (middle panel). The GAPDH level was used as a loading control (bottom panel).

exhibited normal electrophoresis mobility (Fig. 7), which suggested that the effect of 3711 in inducing faster-migrating capsids was not due only to the formation of genome-free capsid. In addition, we found that the effect of 3711 on capsid assembly was irrelevant to CTD of core protein and Pol/pgRNA complex. So we

assumed that 3711's activity might in part represent a link with the Cp assembly domain. The notion was supported by experiments performed on a core protein mutant, V124W, which showed strengthened hydrophobic interaction at the dimer-dimer interface and activity that resembled 3711-bound WT capsid assembly.

FIG 8 3711's effect in inducing the faster-migrating capsid assembly could be recapitulated by AEF and Cp V124W mutation. (A) Huh7 cells were transiently transfected with plasmids expressing Cp183 and Cp144 and then exposed to 3711 and AT-130, respectively, after which intracellular capsids were loaded into a 1.8% native agarose gel. (B and C) pHBV1.3 wt alone, pHBV1.3 core V124W mutant alone, and mixed plasmids (1:1) of the pHBc185 and pHBV1.3 core V124W mutant were transfected separately into Huh7 cells and then exposed to compounds at the indicated concentrations for 72 h. Capsids were analyzed on a 1.8% native agarose gel (B), and capsid-associated DNA was detected by Southern blot analysis (C).

It is possible that 3711 treatment changed the tertiary and quaternary structures of partial-genome-free capsids and was responsible for the formation of faster-migrating capsids. But the subtle changes seen in the tertiary and quaternary structures of capsids could not be observed in negatively stained micrographs. 3711's effects on the capsid assembly reaction rate and thermodynamic aspects have not yet been investigated, and other explanations cannot be excluded. Moreover, it was previously reported that the core V124W mutant and HBc185 coassembled into capsids (11). Our findings presented in Fig. 8A showed that upon treatment of WT and V124W core protein coassembled capsids, more faster-migrating capsids were induced by 3711 than by AT-130. So we assumed that, although both 3711 and AT-130 modulated capsid assembly and the modulation was partly related to strengthening the hydrophobic interactions at the interdimer interface, due to their being different chemical entities, the responses of the coassembled capsids to these two compounds were slightly different.

HBV secretes three major types of viral particles in cell culture medium, including enveloped virion and subviral particles and nonenveloped "naked" capsid (36, 45, 46). According to a recent study (36), the comigration of the capsid and envelope proteins on the agarose gel and their cofractionation on the density gradient indicated the capsids detected at the virion position were enveloped. As shown in Fig. 5, we found that 3711 treatment resulted in a significant reduction in the levels of HBV DNA contained in

both secreted enveloped virions and naked virus particles but had little effect on the HBV capsid level in both viral particle forms (Fig. 5A). Thus, our results were consistent with the single-strand blocking model for virion morphogenesis of pararetrovirus and were incompatible with the prevailing model of hepadnavirus morphogenesis, suggesting that HBV nucleocapsids with no genome could be enveloped and secreted as empty virions (36). The model was supported by a recent observation that genome-free hepatitis B virions were present in the serum of chronic hepatitis B patients at high levels (47). However, due to the low level of enveloped capsid in HepG2.2.15 culture medium, we could not determine here whether 3711-induced faster-migrating HBV capsids were enveloped and released from host cells. Moreover, our findings supported the notion that the release of naked (nucleo)capsids is not a byproduct of HBV-replicating liver cell lines but could be mimicked in cells expressing core protein alone.

HBV capsid assembly process is exquisitely timed and regulated, and any disruption could be devastating to virus replication (6), making it a very attractive target for the development of new anti-HBV therapies. The capsid can maintain its structural integrity with tertiary and quaternary conformational changes, but even small changes can significantly disrupt the normal life cycle and replication of the virus (10). Previous reports had demonstrated that AEFs, such as HAPs and phenylpropenamides, bind a hydrophobic pocket at the interdimer interface and enhance assembly kinetic and thermodynamics (6, 10, 11). We here provided a pyridazinone derivative, 3711, as an addition to AEFs and analyzed capsid physical profile changes caused by AEFs in cell model system.

Taking the results together, we first identified a pyridazinone derivative, 3711, as an HBV inhibitor. 3711 not only will provide an additional tool compound for HBV capsid assembly effector development but also can be used to combat chronic HBV infection in the future.

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