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Pyrimidotriazine derivatives as selective inhibitors of HBV capsid assembly

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is currently treated with nucleoside/nucleotides analogs. They are potent inhibitors of HBV DNA polymerase, which also functions as reverse transcriptase. Although nucleoside/nucleotide analogs efficiently suppress HBV replication in liver cells, they cannot eradicate HBV DNA from liver cells and cure the disease. Therefore, it is still mandatory to identify and develop effective inhibitors that target a step other than reverse transcription in the viral replication cycle. HBV capsid assembly is a critical step for viral replication and an attractive target for inhibition of HBV replication. We conducted *in silico* screening of compounds expected to bind to the HBV capsid dimer-dimer interaction site. The selected compounds were further examined for their anti-HBV activity *in vitro*. Among the test compounds, novel pyrimidotriazine derivatives were found to be selective inhibitors of HBV replication in HepG2.2.15.7 cells. Among the compounds, 2-[(2,3-dichlorophenyl)amino]-4-(4-*tert*-butylphenyl)-8-methyl-4H,9H-pyrimido[1,2-a][1,3,5]triazin-6-one was the most active against HBV replication. Studies on its mechanism of action revealed that the compound interfered with HBV capsid assembly determined by a cell-free capsid assembly system. Thus, the pyrimidotriazine derivatives are considered to be potential leads for novel HBV capsid assembly inhibitors.

1. Introduction

Hepatitis B virus (HBV) infection is a serious health problem with more than 240 million chronically infected individuals worldwide (Ott et al., 2012). Chronic HBV infection is closely associated with liver fibrosis, cirrhosis, and hepatocellular carcinoma, resulting in 300,000 deaths per year (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Interferons (IFNs) and nucleoside/nucleotide analogs, such as lamivudine (3TC), adefovir dipivoxil (ADF), entecavir (ETV), telbivudine (LdT), tenofovir disoproxil fumatate (TDF), and tenofovir alafenamide (TAF) are currently approved for treatment of chronic HBV infection. IFNs are capable of eliminating HBV in a certain proportion of patients. However, the potency of IFNs is limited, and treatment with high-dose IFNs is often associated with intolerable adverse effects in patients (Wursthorn et al., 2006). The nucleoside/nucleotide analogs are potent inhibitors of HBV DNA polymerase, which also functions as reverse transcriptase. Long-term treatment with these analogs is required to prevent viral reactivation, thereby drug-resistant mutants may emerge during treatment (Gish et al., 2012; Lim et al., 2002). This is mainly due to the presence of highly stable and covalently closed circular HBV DNA (cccDNA) in the nucleus of infected hepatocytes (Guo and Guo, 2015). The current nucleoside/nucleotide and IFN therapies are unable to eliminate cccDNA from the cells. Therefore, it seems still mandatory to identify and develop potent and selective inhibitors of HBV replication with a novel mechanism of action.

HBV is a member of *Hepadnaviridae* family and has a small viral DNA (3.2 kb) with an envelope surrounding viral capsid. The process of viral capsid assembly, which is generated by self-assembly of HBV core protein (Cp), plays a critical role in polymerase-bound pregenomic RNA (pgRNA) packaging and reverse transcription, leading to viral DNA synthesis and subsequent infectious viral particle formation. In addition, HBV Cp also modulates cccDNA transcription or post-transcriptional events (Guo and Guo, 2015; Tan et al., 2015; Bock et al., 2001; Fernandez et al., 2003; Kwon and Rho, 2003; Pollicino et al., 2006; Guo

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et al., 2011; Xiang et al., 2015; Hu et al., 2019). Therefore, capsid formation and pgRNA packaging are assumed to be potential targets for inhibition of HBV replication. Two different classes of capsid assembly modulators (CAMs) have been developed. The class I CAMs represented by heteroarylpyrimidine (HAP) derivatives, such as Bay 41-4109 and GLS4, induce misassembled non-capsid core polymers (Stray et al., 2005; Stray and Zlotnick, 2006; Bourne et al., 2008). The class II CAMs represented by phenylpropenamide (PPA) and sulfamoylbenzamide (SBA) interfere with pgRNA packaging and accelerate the formation of immature empty capsid-like particles, respectively (Feld et al., 2007; Perni et al., 2000; Campagna et al., 2013). At present, several CAMs, including JNJ-379, GLS4, ABI-H0731, and NVR 3-778, are under clinical trials (Xia and Liang, 2019).

In this study, we attempted to find novel small molecules that effectively inhibit HBV capsid assembly. To this end, we established an *in silico* screening system to identify small-molecule compounds expected to bind to the HBV capsid dimer-dimer interaction site. Among the compounds selected by *in silico* screening, novel pyrimidotriazine derivatives were found to be selective inhibitors of HBV replication in cell cultures. Furthermore, they proved to inhibit HBV capsid assembly in a cell-free capsid assembly system.

2. Materials and Methods

2.1. Compounds and reagents

Two pyrimidotriazine derivatives (compounds **2b** and **2c**) and GLS4 were synthesized in-house. 3TC, ETV, and AB-423 were purchased from AdooQ BioScience (Irvine, CA) and MedChemExpress (Monmouth Junction, NJ). Other pyrimidotriazine derivatives with *in silico* high docking scores were purchased from Molport (Riga, Latvia). These compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM or higher to exclude the cytotoxicity of DMSO and stored at -20 °C until use.

2.2. Cell cultures

HepG2.2.15.7 cells, a HepG2.2.15 clone producing a higher amount of viral particles, were maintained in DMEM/F12 medium supplemented with GlutaMAX (Gibco, Thermo Fischer Scientific, Waltham, MA), 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fischer Scientific), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 50 μM hydrocortisone (Sigma-Aldrich), 10 mM HEPES (Gibco, Thermo Fischer Scientific), 400 μg/ml G418 (Nacalai Tespue, Kyoto, Japan), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Ogura et al., 2014). Huh-7 cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

2.3. Transient transfection

The plasmids pUC19/C_JPNAT and its mutant (L180 M/S202 G/M204 V) containing a 1.24-fold over-length HBV genome (isolate C_JPNAT, genotype C, accession number: AB246345) were previously described (Sugiyama et al., 2006). Huh-7 cells were transiently transfected with the plasmid DNA using Lipofectamine 3000 reagent (Thermo Fischer Scientific), according to the manufacturer's protocol.

2.4. Conformer generation and in silico screening of compounds

Conformer generation was performed, as described previously (Hamasaki et al., 2013). *In silico* screening of compounds was performed using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada). First, model structures of monomer and dimer were constructed by the X-ray crystal structure of HBV capsid protein (HBc1-149) (PDB ID: 1QGT) (Wynne et al., 1999), which was available from the database at the Research Collaboration for Structural Bioinformatics (RCSB) (https://www.rcsb.org/), using MOE software. After addition of hydrogen atoms and assignment of atomic charges, the constructed monomer and dimer models were subjected to energy minimization using the Merck Molecular Force Field 94x (MMFF94x) (Halgren, 1999a, 1999b; Weiner et al., 1984). Second, Alpha Site Finder, a function of MOE, was used to search the target sites for *in silico* screening, where compounds could bind in the capsid model. We selected a pocket containing the dimer-dimer interaction as a target site. Finally, MOE-ASEDock 2005 (Ryoka Systems, Tokyo, Japan) was used for docking drug-like compounds to the target site (Goto et al., 2008). The docking state was evaluated based on the docking energy calculated by MOE software, and compounds with high docking energy scores were selected for anti-HBV evaluation in cell cultures. The selected compounds were purchased from MolPort.

2.5. Antiviral assay

HepG2.2.15.7 cells were seeded in a collagen type-I coated 96-well plate (Corning Inc., corning, NY) at a density of 1.0×10^4 cells/well. After incubation for 24 h, the cells were cultured in the presence of various concentrations of test compounds. Every 3 days, the culture medium was replaced by fresh medium containing appropriate concentrations of the compounds. After 9 days of incubation, culture supernatants were collected and lysed with an equal volume of SideStep Lysis and Stabilization Buffer (Agilent Technologies, Santa Clara, CA). HBV DNA was quantified by real-time PCR using the primer pair 5'-ACTCACCAACCTCCTGTCCT-3' and 5'-GACAAACGGGCAACATACCT-3' and the fluorescent probe 5'-FAM-TATCGCTGGATGTGTCTGCGGCGT-TAMRA-3' (Liu et al., 2007). The number of viable cells was determined by a tetrazolium dye method (DOJINDO, Kumamoto, Japan). The real-time PCR was performed at 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min with TaqMan® Gene Expressions Master Mix (Thermo Fischer Scientific) and the primer pair and probe, as described above.

2.6. Analysis for capsid-associated HBV RNA

Total RNA was extracted from HepG2.2.15.7 cells with Trizol reagent (Invitrogen), according to the manufacturer's protocol. Capsid-associated HBV RNA in the cells were purified by the following procedures. The cells were treated with lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, and 1% NP-40), and their nuclei were removed by centrifugation. The cell lysate was incubated with 10 U of micrococcus nuclease (Takara Bio Inc., Shiga, Japan) and 6 mM $CaCl_2$ for 30 min at 37 °C. Capsid-associated RNA was extracted by adding 1 ml of Trizol reagent. For northern blot analysis, 5 μg of total RNA or a half volume of capsid-associated RNA was separated in 1.2% agarose gel with 1% formaldehyde. Total RNA or capsid-associated RNA was transferred onto a positive charge membrane with 20 × saline sodium citrate (SSC) buffer for 12 h by a capillary transfer method and cross-linked using a UV cross-linker. The membrane was hybridized with a digoxingenin (DIG)-labeled HBV DNA (PCR product of the HBV X region corresponding to nucleotides 1481-1837) probe.

2.7. Analysis for intracellular core HBV DNA

HepG2.2.15.7 cells exposed to test compounds were treated with lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, and 1% NP-40), and their nuclei and debris were removed by centrifugation. The cell lysate was treated with 6 mM MgOAc, 200 μg/ml DNase I (Worthington Biochemical, Lakewood, NJ), and 100 μg/ml RNase A (Nacalai Tespue) for 2 h at 37 °C, and the reaction was stopped by adding 10 mM EDTA and incubated for 15 min at 65 °C. The cell lysate was treated with 0.5 mg/ml proteinase K and 1% sodium dodecyl sulfate (SDS) and incubated for 2 h at 37 °C. The intracellular core HBV

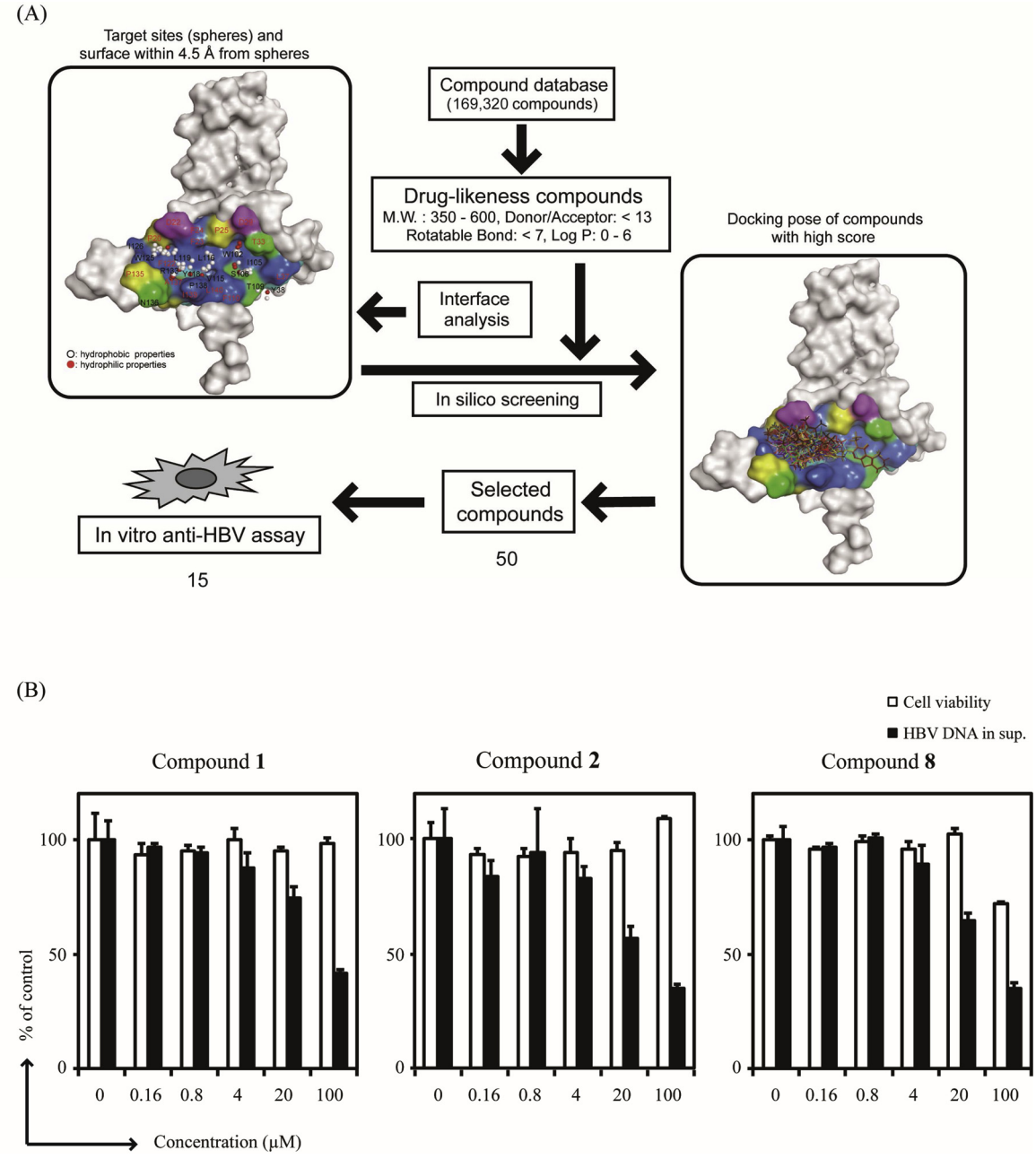


Fig. 1. Flow chart of *in silico* screening and anti-HBV activity of lead compounds. (A) Compound databases were filtered with the following conditions: molecular weight = 350 to 600; log P = 0 to 6; number of hydrogen bond donors or acceptors < 13; and number of rotatable bonds < 7. MOE-ASEDock was used for docking drug-like compounds to the target site. Compounds with high docking energy scores were selected and purchased for evaluating their anti-HBV activity in cell cultures. (B) HepG2.2.15.7 cells were treated with compound **1**, **2**, or **8** at various concentrations for 9 days. Every 3 days, culture medium was replaced by fresh culture medium containing appropriate concentrations of the compound. Cell viability was measured by a tetrazolium dye method (open column). The culture supernatant was collected and treated with lysis buffer, and HBV DNA was quantified by real-time PCR (closed column). All experiments were carried out in triplicate and repeated 3 times in HepG2.2.15.7 cells.

DNA was purified by phenol/chloroform/isoamylalchohol (25:24:1) extraction. For southern blot analysis, one-third of the extracted intracellular core DNA was loaded and separated with 1.0% agarose gel. The agarose gel was denatured in denaturation buffer (0.5 M NaOH and 1.5 M NaCl) for 30 min and washed in neutralization buffer (0.5 M Tris-HCl [pH7.5] for 30 min and 1.5 M NaCl). The DNA was transferred onto a positive charge membrane with 20 × SSC buffer by a capillary transfer method. The membrane was hybridized with a DIG-labeled full-length HBV DNA probe.

2.8. Analysis for extracellular HBV DNA

HepG2.2.15.7 cells were seeded in a collagen type-I coated 12-well plate (Corning Inc.) at a density of 1.0×10^5 cells/well. After incubation for 24 h, the cells were cultured in the presence of various concentrations of test compounds. Every 3 days, the culture medium was replaced by fresh medium containing appropriate concentrations of the compounds. After 9 days of incubation, culture supernatants were collected, and viral particles were precipitated with 10% polyethylene glycol 8000 (PEG8000) (Sigma-Aldrich) for 1 h at 4 °C and centrifuged for 20 min at 5,000 × *g* at room temperature. Viral pellets were

Table 1
Anti-HBV activity of selected compounds.

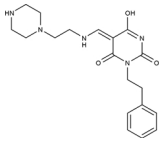
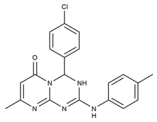
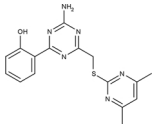
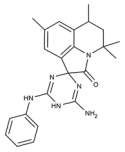
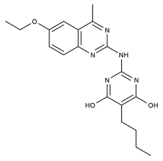
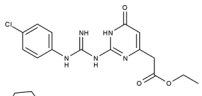
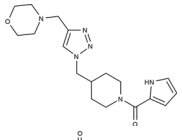
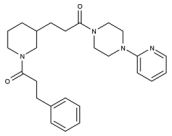
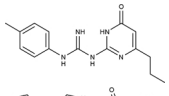
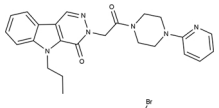
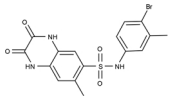
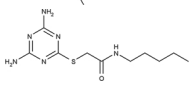
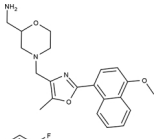
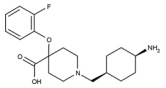
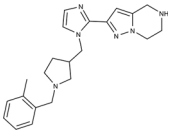
Compound No.	Chemical structure	EC_{50} (μM)[a]	CC_{50} (μM)[b]
1		66.9	> 100
2		25.6 ± 13.4	> 100
3		> 100	> 100
4		> 100	> 100
5		> 100	> 100
6		> 100	> 100
7		> 100	> 100
8		44.0	> 100
9		> 100	> 100
10		> 100	> 100
11		> 100	> 100
12		> 100	> 100
13		> 45.0	45.0
14		> 100	> 100

Table 1 (*continued*)

Compound No.	Chemical structure	EC_{50} (μM)[a]	CC_{50} (μM)[b]
15		> 100	> 100

[a] EC_{50}: 50% effective concentration based on the inhibition of HBV DNA levels in culture supernatants.
[b] CC_{50}: 50% cytotoxic concentration based on the reduction of viable cell number.

resuspended in lysis buffer, and HBV DNA was extracted and subjected to southern blot analysis, as described above.

2.9. Analysis for capsid formation and capsid-associated HBV DNA

HepG2.2.15.7 cells exposed to test compounds were treated with lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, and 1.0% NP-40), and their nuclei and debris were removed by centrifugation. The cell lysate was treated with 6 mM MgOAc, 200 μg/ml DNase I (Worthington Biochemical), and 100 μg/ml RNase A (Nacalai Tesque) for 2 h at 37 °C, and the reaction was stopped by adding 10 mM EDTA and incubated for 15 min at 65 °C. Ten μl of the cell lysate was separated with 1.0% agarose gel and transferred onto a nitrocellulose membrane with 1 × TNE (10 mM Tris, 150 mM NaCl, and 1 mM EDTA; pH 7.6) buffer by a capillary transfer method. The presence of HBV capsid was detected by western blot analysis using an anti-HBcAg antibody (Abcam, Cambridge, UK) (1:1,000 dilution). Capsid-associated HBV DNA was analyzed by southern blot analysis, as described above.

2.10. Western blot analysis

HepG2.2.15.7 cells exposed to test compounds were treated with RIPA buffer (Nacalai Tesque) for 30 min on ice. After incubation, the cell lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was collected and stored at -20 °C until analysis. For analysis, the samples were separated by 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane. Detection was performed with an enhanced chemiluminescence prime Western Blotting Detection Reagent (ECL prime; GE Healthcare). Anti-HBcAg antibody (Abcam) and anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for analysis.

2.11. Cell-free transcription/translation in rabbit reticulocyte lysate (RRL)

DNA templates were prepared by following procedures. Total RNA was extracted from HepG2.2.15.7 cells with Trizol reagent, according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA (1 μg) using SuperScript™ IV VILO™ Master Mix (ThermoFisher Scientific), followed by PCR with Q5® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA). Primers used in this experiment were as follows: forward primer; GGATCC-TAATACGACTCACTATAGGG-AGCCACC-ATG-GACATCGACCCTTATAAAGA ($N_{6\text{-}10}$-T7 promoter region-spacer-Kozak sequence-HBc start codon-HBc sequence) and reverse primer; ACCTTATGAGTCCAAGGAATA-CTA-ACATTGAGGTTCCCGAGA ($N_{0\text{-}20}$-HBc stop codon-HBc sequence). The cycling parameters were as follows: 98 °C for 30 sec, then 30 cycles of 98 °C for 5 sec, 68 °C for 10 sec, 72 °C for 20 sec, and final extension of 72 °C for 2 min. The product DNA (606 bp) was confirmed in a 2% agarose gel.

TNT® T7 Quick for PCR DNA cell-free transcription/translation system was used to express HBc protein, according to the manufacturer's protocol with some modifications (Promega, Fitchburg, WI). Briefly, translation reaction mixture was composed of 40 μl of TNT® T7

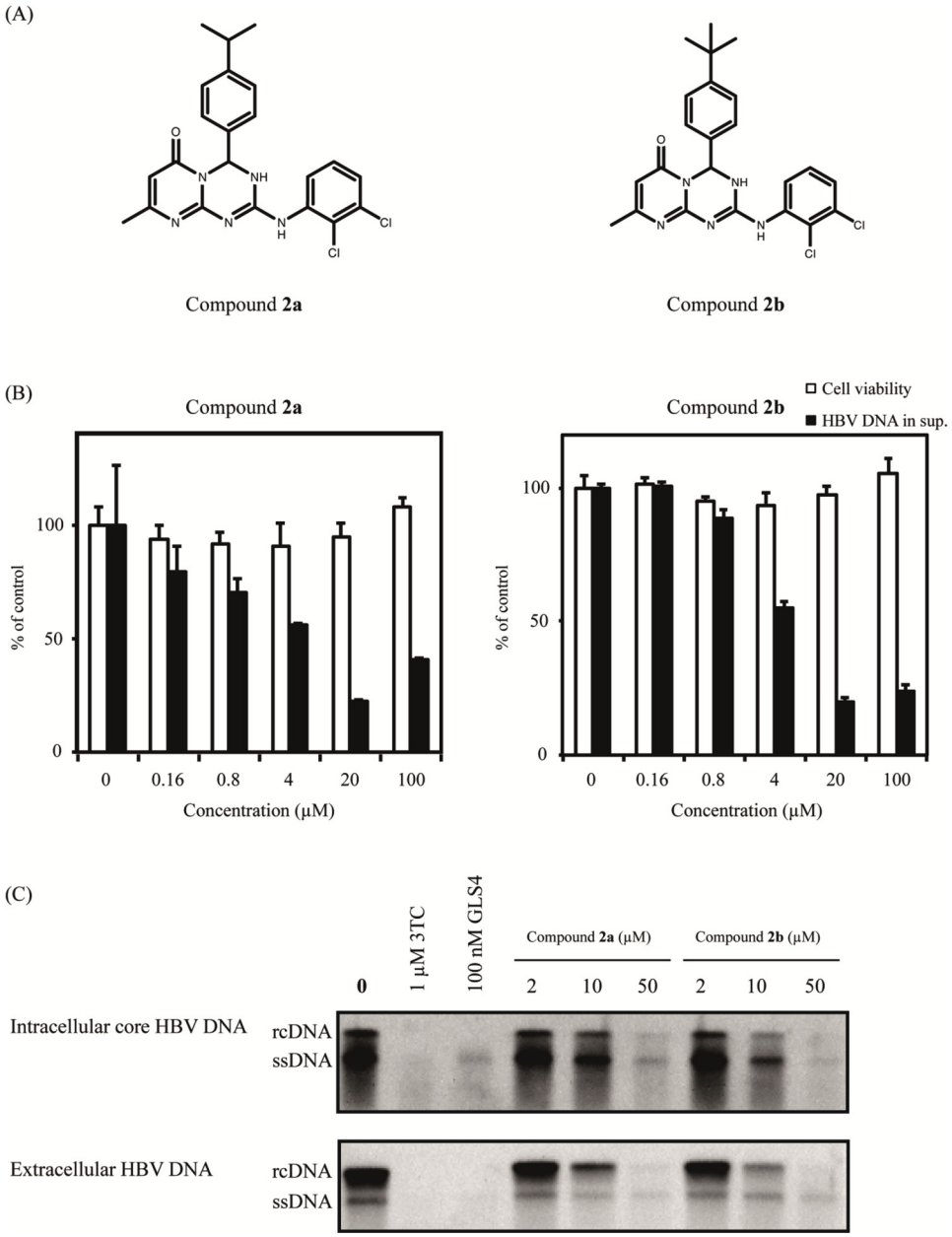


Fig. 2. Inhibitory effect of pyrimidotriazine derivatives on HBV replication. (A) Chemical structures of compounds **2a** and **2b**. (B) HepG2.2.15.7 cells were treated with compound **2a** or **2b** at various concentrations for 9 days. Every 3 days, culture medium was replaced by fresh culture medium containing appropriate concentrations of the compound. The cell viability was measured by tetrazolium dye method (open column). The culture supernatant was collected and treated with lysis buffer, and HBV DNA was quantified by real-time PCR (closed column). All experiments were carried out in triplicate and repeated 3 times in HepG2.2.15.7 cells. (C) HepG2.2.15.7 cells were treated with compound **2a**, **2b**, or the reference compound (3TC or GLS4) at various concentrations for 9 days. Every 3 days, culture medium was replaced by fresh culture medium containing appropriate concentrations of the compound. The intracellular and extracellular HBV DNA was extracted and analyzed by southern blotting.

PCR Quick Master Mix, 1 μl of 1 mM Methionine, 5 μl of PCR-product DNA template, and 4 μl of nuclease-free water. The reaction mixture was incubated for 90 min at 37 °C. After incubation, the mixture was stored at -80 °C until further use.

2.12. Analysis for cell-free capsid assembly

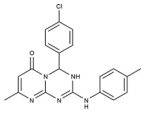
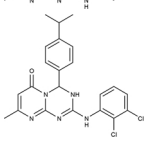
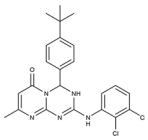
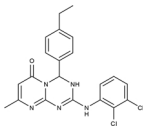
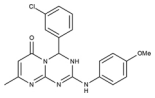
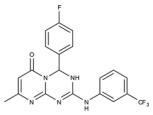
HBV capsid assembly was conducted by a previously described method with some modifications (Ludgate et al., 2016). Briefly, the reaction mixture for capsid assembly was composed of 2-3 μl of translated products per 10 μl of 1 × NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM DTT, pH 7.9; NEB) supplemented with 1 × EDTA free protease inhibitor cocktail (Nacalai Tesque), 0.8 U/μl RNasin® Plus RNase Inhibitor (Promega), 1 U/μl calf intestinal alkaline phosphatase (CIAP) (Promega), and 0.5-10% DMSO. The reaction mixture was incubated for 16 h at 37 °C, and 100 μg/ml RNase A (Nacalai Tesque) was added and incubated for 1 h at 37 °C. The reaction mixture was resolved by 1% agarose gel electrophoresis in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). The gel was transferred onto a nitrocellulose membrane with 1 × TNE buffer for 40 min using a downward capillary transfer method. The translation products were subjected to western blot analysis with a mouse monoclonal antibody (clone; T2221, Institute of Immunology Co., LTD., Tokyo, Japan) or a rabbit polyclonal antibody targeting HBc protein (Abcam).

3. RESULTS

3.1. In silico screening and in vitro anti-HBV activity

To identify HBV capsid assembly inhibitors, we decided to perform *in silico* screening of compounds targeting the HBV capsid dimer-dimer interaction site (Fig. 1A), as described in Materials and Methods. Among 169,320 compounds in a chemical database, 50 compounds with high docking energy scores were selected, and 15 compounds were commercially available (Table 1). We examined the compounds for their inhibitory effect on HBV replication in HepG2.2.15.7 cells. Three compounds, (5Z)-6-hydroxy-3-(2-phenylethyl)-5- ({[2-(piperazin-1-yl)

Table 2
Anti-HBV activity of pyrimidotriazine derivatives.

Compound No.	Chemical structure	EC_{50} (μM)[a]	CC_{50} (μM)[b]
2		25.6 ± 13.4	> 100
2a		6.9 ± 1.8	> 100
2b		5.8 ± 0.9	> 100
2c		16.5 ± 4.0	> 100
2d		35.6 ± 4.3	> 100
2e		40.4 ± 6.4	43.9 ± 1.5
GLS4		0.0164 ± 0.0015	31.5 ± 16.2

All data represent the mean ± standard deviation for 3 independent experiments.

[a] EC_{50}: 50% effective concentration based on the inhibition of HBV DNA levels in culture supernatants.

[b] CC_{50}: 50% cytotoxic concentration based on the reduction of viable cell number.

ethyl]amino}methylidene)pyrimidine-2,4-dione (compound **1**) [50% effective concentration (EC_{50}) = 66.9 μM, 50% cytotoxic concentration (CC_{50}) > 100 μM)], 4-(4-chlorophenyl)-8-methyl-2-[(4-methylphenyl) amino]-4H,6H,9H-pyrimido[1,2-a][1,3,5]triazin-6-one (compound **2**) (EC_{50} = 25.6 ± 13.4 μM, CC_{50} > 100 μM), and 3-[1-(3-phenylpropanoyl)piperidin-3-yl]-1-[4-(pyridin-2-yl)piperazin-1-yl]propan-1-one (compound **8**) (EC_{50} = 44.0 μM, CC_{50} > 100 μM), selectively inhibited HBV production in culture supernatants (Fig. 1B and Table 1). Based on their anti-HBV activity, compound **2** was selected for further experiments.

To find more active compounds, 30 derivatives of compound **2** were purchased. Among them, 2-[(2,3-dichlorophenyl)amino]-4-(4-isopropylphenyl)-8-methyl-4H,9H-pyrimido[1,2-a][1,3,5]triazin-6-one (compound **2a**) was found to be a more potent and selective inhibitor of HBV production than compound **2** (Fig. 2A and B and Table S1). The EC_{50} and CC_{50} of compound **2a** were 6.9 ± 1.9 μM and > 100 μM, respectively. To investigate the structure-activity relationship (SAR) of the series, we synthesized the compounds of which 4-isopropylphenyl molecule of pyrimido[1,2-a][1,3,5]triazine-6-one was substituted by 4-*tert*-butylphenyl molecule (compound **2b**) or 4-ethylphenyl molecule (compound **2c**). Compound **2b** displayed slightly higher anti-HBV activity (EC_{50} = 5.8 ± 0.9 μM, CC_{50} > 100 μM) than compound **2a**, while compound **2c** was less active (EC_{50} = 16.5 ± 4.0 μM, CC_{50} > 100 μM) than compound **2a** (Table 2). In addition, southern blot analysis revealed that, like 3TC (HBV DNA polymerase inhibitor) and GLS4 (class I CAM), both compound **2a** and compound **2b** reduced intracellular and extracellular HBV DNA in a dose-dependent fashion (Fig. 2C).

3.2. Inhibition of capsid formation

To confirm that the anti-HBV activity of the pyrimidotriazine derivatives is attributed to the inhibition of capsid formation, capsid particle blotting was performed to determine the level of capsid formation after treating HepG2.2.15.7 cells with the compounds. As a positive control, the cells were treated with GLS4, a known inhibitor of capsid formation. As shown in Fig. 3, compounds **2a**, **2b**, and GLS4 inhibited capsid formation. In addition, the amounts of capsid-associated HBV RNA and DNA were also reduced. In contrast, northern blot and western blot analyses revealed that pgRNA, sRNA, and HBc protein levels were not affected by these compounds (Fig. 3). Treatment of the cells with the reverse transcriptase inhibitor 3TC could reduce only capsid-associated HBV DNA. Since the pyrimidotriazine derivatives behaved similarly to GLS4, they were considered to be capsid assembly inhibitors.

3.3. Inhibition of capsid assembly

We investigated whether the pyrimidotriazine derivatives inhibited capsid assembly using a cell-free capsid assembly method. Dr. Hu and his colleagues previously reported that HBV capsid could assemble efficiently in RRL using TnT® Coupled Reticulocyte Lysate System (Promega, Fitchburg, WI) (Ludgate et al., 2016). Therefore, their inhibitory effect on capsid assembly was examined in TNT® T7 Quick for PCR DNA cell-free transcription/translation system, which is a product similar to TnT® Coupled Reticulocyte Lysate System (Fig. 4A).

HBc protein expression was confirmed by western blotting, when the protein was translated in RRL. Western blot analysis showed that the translated HBc protein had the same size as that of recombinant HBc protein (rHBc) purified from bacteria (ab49013, Abcam, Cambridge, MA) (Fig. 4B). It was also revealed that approximately 1.25 to 2.5 ng of HBc was made per 1 μl of translation mixture. To determine whether the translated HBc protein is assembled into HBV capsid, the protein was incubated in various amounts of the reaction mixture and analyzed by particle blotting. As shown in Fig. 4C, capsid formation was detectable in 1 μl of the reaction mixture and did increase with increasing the amount of the reaction mixture up to 3 μl. Since the compounds were dissolved in DMSO, we examined its effect on capsid assembly. DMSO did not affect capsid formation at concentrations up to 10% (Fig. 4D). Therefore, the following conditions were used for further experiments; 2 μL of the translated HBc protein, up to 10% for DMSO concentration, and a total volume of 10 μl for the reaction mixture.

The HAP derivatives Bay 41-4109 and GLS4 are selective inhibitors of HBV capsid formation. These compounds have pleomorphic non-capsid structures, and they displayed potent *in vitro* and *in vivo* antiviral activity (Stray et al., 2005; Stray and Zlotnick, 2006; Bourne et al., 2008; Deres et al., 2003; Weber et al., 2002). Therefore, the inhibitory effect of GLS4 on capsid assembly was examined in RRL. As expected, GLS4 strongly inhibited HBV capsid formation in this system (Fig. 4E). GLS4 completely inhibited HBV capsid formation at a concentration of 2.5 μM but not 1 μM, indicating that the inhibitory effect of the class I CAMs on HBV capsid formation can be evaluated in the RRL system. In contrast, the sulfamoylbenzamide derivative AB-423 classified with class II CAMs did not affect the capsid formation (Fig. 4F). When compound **2b** was examined for its inhibitory effect on HBV capsid assembly, it inhibited the capsid formation at a concentration of 500 μM (Fig. 4G). Thus, compound **2b** appeared to be a class I CAM, although it remains to be elucidated whether the pyrimidotriazine derivatives indeed bind to the HBV capsid dimer-dimer interaction site and inhibit HBV replication through blocking HBV capsid assembly.

3.4. Inhibition of an ETV-resistant HBV mutant

Long-term treatment with nucleoside/nucleotide analogs is required to prevent viral reactivation, thereby drug-resistant mutants may

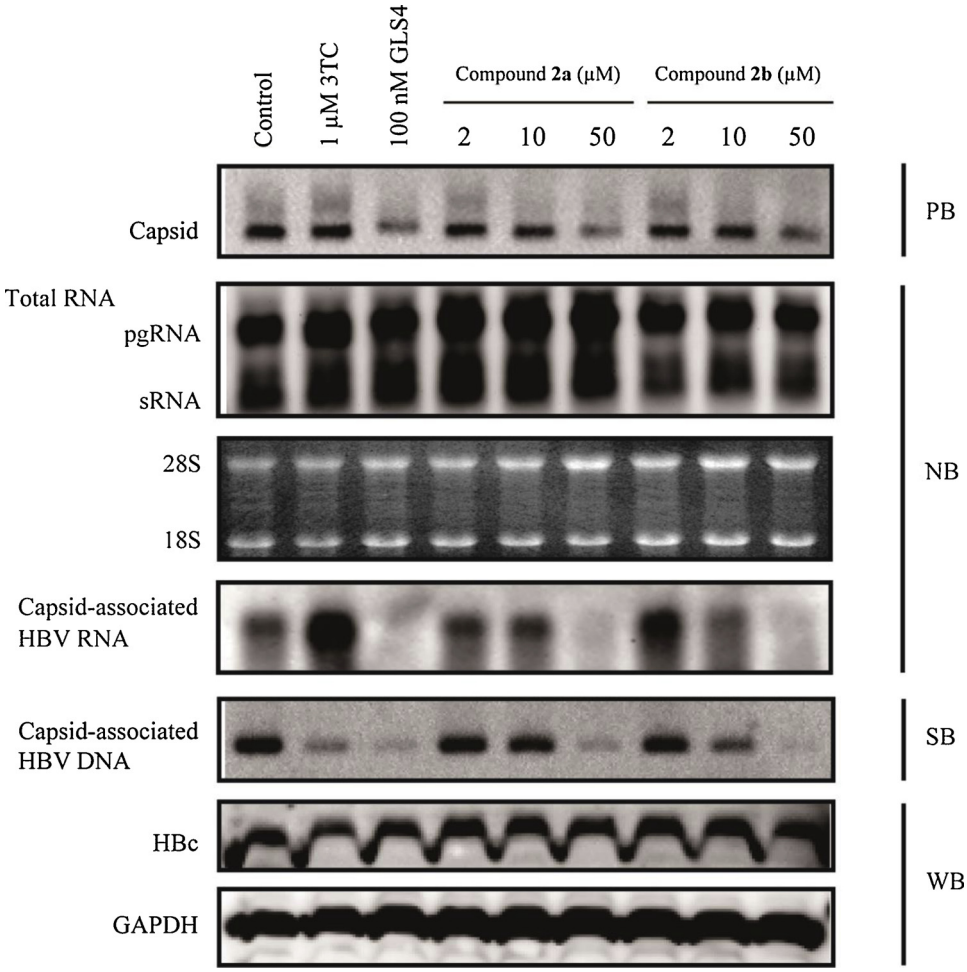


Fig. 3. Inhibitory effect of pyrimidotriazine derivatives on HBV capsid, HBV RNA, capsid-associated HBV RNA and DNA, and HBc protein. HepG2.2.15.7 cells were treated with compound **2a**, **2b**, or the reference compound (3TC or GLS4) at various concentrations for 9 days. Every 3 days, culture medium was replaced by fresh culture medium containing appropriate concentrations of the compound. The HBV capsid, total RNA, capsid-associated HBV RNA and DNA, and whole proteins of HepG2.2.15.7 cells were extracted and analyzed by particle blotting (PB), northern blotting (NB), southern blotting (SB), and western blotting (WB), respectively.

emerge during treatment. Drugs with novel mechanisms of action are capable of preventing the emergence of HBV mutants resistant to nucleoside/nucleotide analogs in patients. Therefore, we examined the antiviral activity of compound **2b** against an ETV/3TC-resistant HBV (genotype C) carrying L180 M/S202 G/M204 V mutations in Huh-7 cells. Unlike ETV, compound **2b** could equally suppress the intracellular DNA levels of the wild-type and ETV/3TC-resistant mutant in a dose-dependent fashion (Fig. 5).

4. Discussion

In this study, we conducted *in silico* screening of a variety of compounds and identified novel pyrimidotriazine derivatives as selective inhibitors of HBV replication in cell cultures. Among the derivatives, compound **2b** exhibited the anti-HBV activity against not only the wild-type of HBV but also a nucleoside/nucleotide analogue-resistant mutant. In addition, using the RRL system, we proved that the compound inhibited HBV capsid assembly.

HBV core protein plays multiple roles in the HBV life cycle. CAMs have a higher barrier to emergence of drug resistance than nucleotide/nucleoside analogs. Therefore, the core protein is an attractive target for inhibition of HBV replication. To date, a number of CAMs have been identified as potent and selective inhibitors of HBV replication in cell cultures, and some of them are currently under clinical trials. They include HAP, PPA, SBA, sulfamoylpyrrolamide (SPA), and glyoxamoylpyrroloxamide (GPA) and inhibit HBV replication at nanomolar concentrations. However, their clinical use has not been approved yet. Furthermore, HAP is the only class I CAM (Corcuera et al., 2018). Therefore, we attempted to find another class I CAM. Consequently, the pyrimidotriazine derivatives were found to be selective inhibitors of capsid assembly. Although their anti-HBV activity was lower than that of existing CAMs, the derivatives appeared to be potential leads for novel HBV inhibitors.

It is known that heterocyclic compounds having a pyrimido[1,2-α][1,3,5]triazine moiety have various biological activities, such as anticancer, antimicrobial, and antifungal activities (Sachdeva et al., 2012). However, their anti-HBV activity has not been reported. The SAR analysis for the pyrimido[1,2-α][1,3,5]triazine derivatives suggests that the alkylphenyl group at position 4 is a key modification target for increasing their anti-HBV activity. In this study, however, we could only evaluate the effect of side chains at position 2 (aminophenyl group) or position 4 (phenyl group) on anti-HBV activity. Therefore, to further increase the activity of the pyrimidotriazine derivatives, substitutions with various molecules at different positions will be required.

Inhibition of HBV capsid assembly is in general determined by size exclusion chromatography, electron microscopy, fluorescence self-quenching, or surface plasmon resonance. Although these methods are useful for developing HBV capsid assembly inhibitors, cell-free capsid assembly needs much higher concentrations (40 to 80 μM) of purified HBc protein than its physiological concentrations. Furthermore, the purified protein contains the N-terminal domain (NTD) but not the C-terminal domain (CTD) of HBc. It was reported that the HBc protein lacking CTD failed to assemble into capsid in mammalian cells (HEK293 and hepatoma cells). In addition, the full-length HBc is able to assemble into capsid at a 1,000-fold lower concentration than CTD-lacking HBc in mammalian cells or RRL (Ludgate et al., 2016). We demonstrated that the RRL system could translate PCR-amplified full-length HBc sequences to a twice amount of HBc protein as compared to that

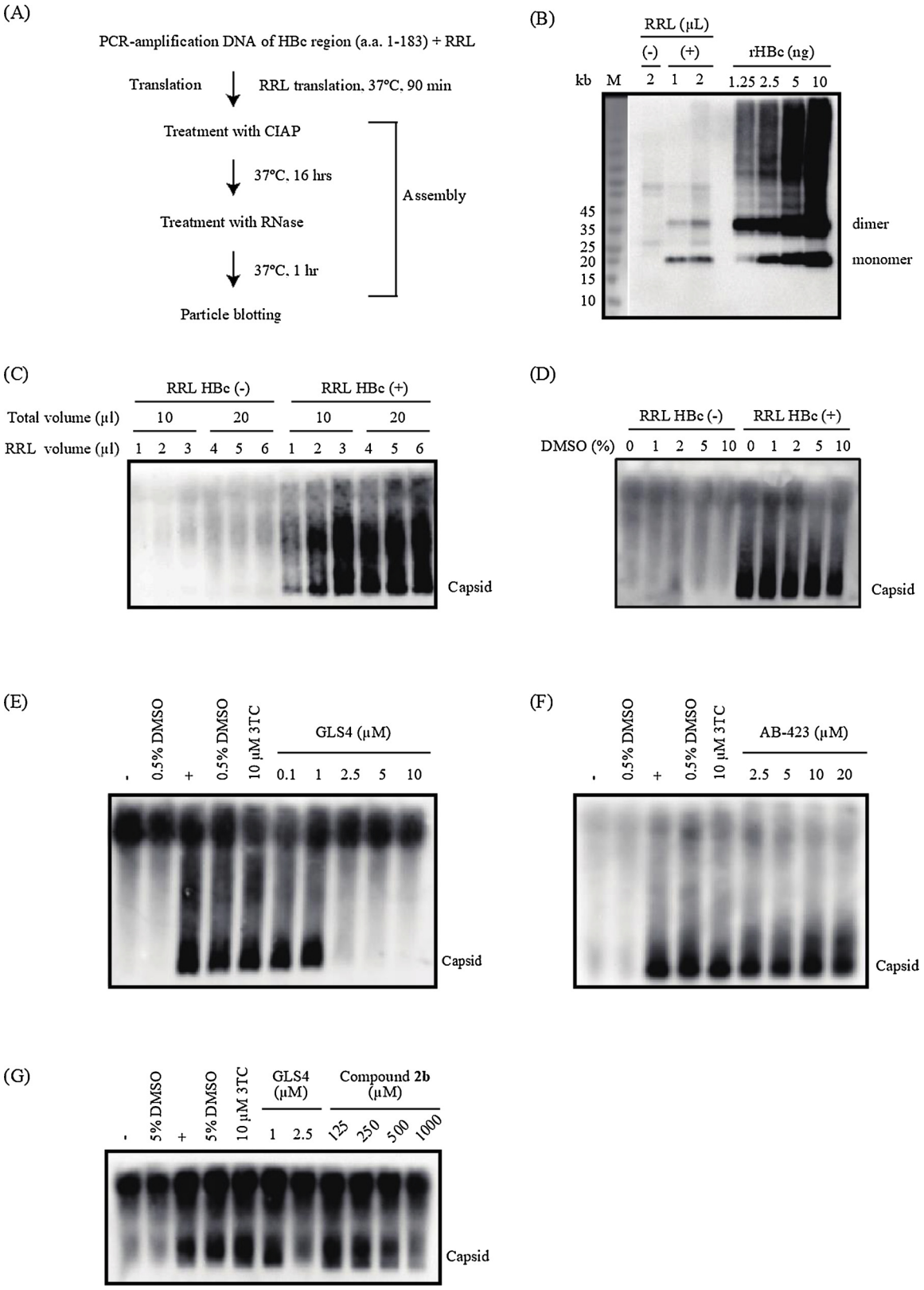


Fig. 4. HBV capsid assembly in RRL and effects of compounds on capsid assembly. (A) Scheme for translation in RRL and capsid assembly. (B) After translation in RRL, 1 or 2 μl of translation reaction mixture with (+) or without (–) template was analyzed by western blotting with the mouse anti-HBc mAb (clone T2221). The capsid assembly reaction was performed at the indicated volume of (C) reaction mixture, (D) DMSO, or the indicated concentrations of (E) GLS4 or (F) AB-423 and analyzed by particle blotting. (G) The capsid assembly reaction was performed in 2 μl of translation reaction mixture containing 3TC, GLS4, or compound **2b** at the indicated concentrations. After stopping reaction, capsid formation was analyzed by particle blotting.

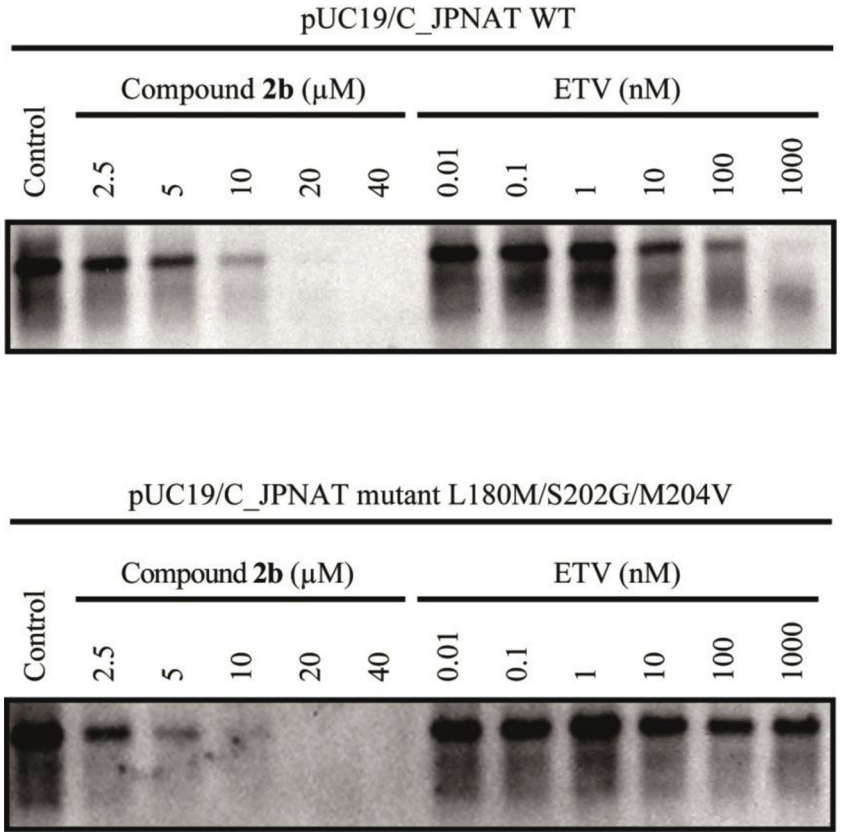


Fig. 5. Inhibitory effect of compound 2b on the replication of ETV/3TC-resistant HBV mutant. The wild-type HBV genotype C clone plasmid pUC19/C_JPNAT WT or the ETV/3TC-resistant clone plasmid pUC19/C_JPNAT L180 M/S202 G/M204 V was transiently transfected into Huh-7 cells. After 24 h of transfection, the cells were treated with compound **2b** or ETV at the indicated concentrations. After 48 h of incubation, the intracellular HBV DNA was extracted and analyzed by southern blotting.

previously reported (0.5 to 1.0 ng/μl) and that the translated HBc protein consequently assembled to capsid (Fig. 4B and Ludgate et al., 2016). Furthermore, it was clear that GLS4 (class I CAM) inhibited capsid assembly in RRL, but AB-423 (class II CAM) did not (Fig. 4E and F). The pyrimidotriazine derivatives also inhibited capsid assembly in RRL (Fig. 4G), although it remains to be elucidated whether the compounds bind to the capsid dimer-dimer interaction site or not. Nevertheless, the RRL system appears to be useful for the development of anti-HBV agents targeting capsid assembly. In addition, electron microscopy is also a useful tool for demonstrating the inhibition of capsid formation and should be performed in future studies.

A series of novel pyrimidotriazine derivatives were prepared and evaluated for their inhibitory effect on HBV replication in cell cultures. The compound **2b** exhibited selective anti-HBV activity against the wild-type and nucleoside/nucleotide analogue-resistant mutant. Although the class I CAMs could be identified in the RRL system, further experiments are required to elucidate whether the system is also applicable to identifying the class II CAMs. Taken together, the pyrimidotriazine derivatives described herein should be further pursued for their chemotherapeutic potential as novel anti-HBV agents.

Acknowledgements

N.S. has passed away on March 12, 2018 and this paper is written in his memory and tribute to his devoted work to finding cures for human diseases. We thank M. Maeda and M. Tsumagari for their secretarial work and A. Hidaka for her technical assistance. This research is supported in part by the Research Program on the Innovative Development and Application of New Drugs for Hepatitis B from the Japan Agency for Medical Research and Development (AMED).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197677.

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