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Discovery of novel hepatitis B virus nucleocapsid assembly inhibitors

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Abstract

Hepatitis B virus (HBV) core protein is a small protein with 183 amino acid residues and assembles the pre-genomic (pg) RNA and viral DNA polymerase to form nucleocapsids. During the last decades, several groups have reported HBV core protein allosteric modulators (CpAMs) with distinct chemical structures. CpAMs bind to the hydrophobic HAP pocket located at the dimer-dimer interface and induce allosteric conformational changes in the core protein subunits. While type I CpAMs, heteroaryldihydropyrimidine (HAP) derivatives, misdirect core protein dimers to assemble non-capsid polymers, Type II CpAMs, represented by sulfamoylbenzamides, phenylpropenamides and several other chemotypes, induce the assembly of empty capsids with global structural alterations and faster mobility in native agarose gel electrophoresis. Through high throughput screening of an Asinex small molecule library containing 19,920 compounds, we identified 8 structurally distinct CpAMs. While 7 of those compounds are typical Type II CpAMs, a novel benzamide derivative, designated as BA-53038B, induced the formation of morphologically "normal" empty capsids with slow electrophoresis mobility. Drug resistant profile analyses indicated that BA-53038B most likely bound to the HAP pocket, but obviously modulated HBV capsid assembly in a distinct manner. BA-53038B and other CpAMs reported herein provide novel structure scaffolds for the development of core protein-targeted antiviral agents for the treatment of chronic hepatitis B.

Keywords

Hepatitis B virus; Core protein allosteric modulator; Antiviral; Capsid assembly; Nucleocapsids

Although an effective vaccine is available, there are approximately 250 million people chronically infected by hepatitis B virus (HBV) worldwide[1–2]. The currently available antiviral therapeutics against HBV include pegylated interferon-alpha (IFN-α) and seven nucleos(t)ide analogue viral DNA polymerase inhibitors that can efficiently reduce viral load and prevent progression to cirrhosis and hepatocellular carcinoma in a large fraction of patients treated with these drugs [3–4]. However, a "functional cure", characterized by HBV surface antigen loss or seroconversion, is rarely achieved with the current therapies [5–6].

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Therefore, development of novel antiviral agents that more efficiently inhibit HBV replication and accomplish a functional cure of chronic hepatitis B as monotherapeutic agents or components of combination therapy is a demanding medical need [7–9].

While the vast majority of currently available antiviral agents target virus-encoded enzymes [10], viral capsid proteins have been considered to be promising antiviral targets with high selectivity in recent years. Indeed, small molecular compounds specifically targeting the capsid proteins of human immunodeficiency virus [11–12], HBV [13–14] and dengue virus [15], have been shown to disrupt capsid assembly and/or disassembly (uncoating) and are currently under preclinical or clinical development.

As shown in Fig. 1, several chemotypes of HBV core protein allosteric modulators (CpAMs) were reported in the last two decades. These compounds bind to a hydrophobic pocket (the HAP pocket), formed at the interface between core protein dimers and disrupt capsid assembly and packaging of the HBV pregenomic RNA (pgRNA) and DNA polymerase [16–20]. Based on their pharmacological phenotypes, the CpAMs are categorized into two types. Type I CpAMs, which are heteroaryldihydropyrimidine (HAP) derivatives (represented by Bay 41–4109) [21–23], misdirect core protein dimers to form non-capsid polymers that can be either degraded intracellularly or accumulated in the nuclear PML bodies of hepatocytes [24–25]. On the contrary, Type II CpAMs, including phenylpropenamides (PPAs) [26], sulfamoylbenzamides (SBAs) [27], benzamides [28] and others [29–30], induce the formation of empty capsids without packaging viral pgRNA and DNA polymerase and thus preclude viral genome replication. Moreover, both type I and type II CpAMs have been demonstrated to induce mature nucleocapsid uncoating [31] and inhibit *de novo* synthesis of covalently closed circular (ccc) DNA [31–3233].

In order to identify novel chemotypes of CpAMs as development leads of antiviral agents and as molecular probes to investigate the molecular mechanisms of HBV nucleocapsid assembly and disassembly, we screened 19,920 compounds from an in-house library for their ability to reduce the amount of HBV DNA in AML12HBV10 cells, an immortalized mouse hepatocyte-derived stable cell line supporting high levels of HBV DNA replication in a tetracycline inducible manner [34]. Our screening effort identified six new chemotypes of compounds that, as previously reported type II CpAMs, induced the assembly of empty capsids devoid of pgRNA with faster electrophoresis mobility in a native agarose gel-based particle gel assay [35]. However, a novel benzamide derivative, designated as BA-53038B, induced the formation of empty capsids with slow electrophoresis mobility. Mechanistic studies demonstrated that like other CpAMs, BA-53038B disrupted pgRNA encapsidation and associated core protein dephosphorylation [36], most likely by binding to the HAP pocket of the dimer-dimer interface. These new CpAMs reported herein provide novel structure scaffolds for the development of core protein-targeted antivirals for the treatment of chronic hepatitis B.

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Results

Identification of novel CpAMs

Using an AML12HBV10 cell-based assay described previously [27], 19,920 compounds from an in-house library were tested for their ability to suppress HBV DNA replication. The primary screening identified 89 compounds that reduced HBV core DNA by greater than 60% at 10 μM concentration, compared to the mock treated controls. In order to identify compounds that modulate HBV capsid assembly, taking advantage of our recent finding that CpAMs either induce the decay of mis-assembled core protein aggregates (Type I CpAMs) or assembly of capsids devoid of pgRNA with faster electrophoresis mobility (Type II CpAMs) [28, 35], all the primary "hit" compounds were tested for their effects on capsid mobility in a native agarose gel electrophoresis-based particle gel assay and identified 8 new compounds that altered the capsid electrophoresis mobility. As shown in Fig. 2A, by increasing the agarose concentration from 1% to 1.8%, HBV capsids in mock-treated cells can be separated into two species, a predominantly slow and a minor fast migrating capsid. Despite the reduction of HBV core DNA that co-migrated with the slow migrating capsids, viral DNA polymerase inhibitor entecavir (ETV) did not change the electrophoresis pattern of HBV capsids. Also as expected, Bay 41–4109 treatment induced decay of the core protein and abolished the formation of capsids. However, similar to DVR-23, a SBA chemotype of CpAM [27], treatment of cells with compounds 1 to 7 reduced the amount of capsids with slow migrating rate, but increased the amount of capsids with fast migrating rate. Also similar to DVR-23, those seven compounds reduced the amount of capsid-associated viral DNA (Fig. 2A) and core protein with hypophosphorylation (Fig. 2B). The results suggest that the seven compounds are typical type II CpAMs. However, treatment of cells with compound 8, a novel benzamide derivative and renamed as BA-53038B after re-synthesis, reduced the amount of fast migrating capsids and formed slow migrating capsids. Like other CpAMs, but distinct from viral DNA polymerase inhibitor ETV, BA-53083B treatment reduced the amount of hypophosphorylated core protein (Fig. 2B and C), suggesting the inhibition of viral pgRNA encapsidation [36]. Hence, these results indicated that unlike other type II CpAMs, BA-53083B treatment promoted the assembly of empty capsids with slow electrophoresis mobility. The structure, antiviral activity and cytotoxicity of the novel CpAMs discovered in this study are presented in Table 1.

BA-53038B inhibits HBV nucleocapsid assembly

Our recent studies showed that HBV capsid electrophoresis mobility is determined by its surface charge, which is, in turn, determined by the ionization of selected charged amino acid side chains in the core protein assembly domain[35]. Hence, changes in capsid mobility most likely reflect capsid global structural alteration that affects the exposure and/or ionization of amino acid with charged side chains. We thus speculated that unlike other type II CpAMs, BA-53038B might uniquely interact with HBV core protein and disrupt HBV capsid assembly *via* distinct mechanisms. To test this hypothesis, we first showed that BA-53038B demonstrated selective antiviral activity against HBV in both AML12HBV10 and HepDES19 cells (Fig. 3). We then determined its effect on every step of the HBV replication cycle from pgRNA production to rcDNA synthesis. In this experiment, Bay 41–4109 and DVR-23, the representative type I and II CpAMs were also included. As shown in

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Fig. 4, the particle gel assay revealed that Bay41–4109 treatment completely abolished capsid formation (Panel C) and thus pgRNA encapsidation and DNA synthesis (panels B, C and D). On the contrary, DVR-23 treatment induced the formation of fast migrating capsids (panel C), but reduced the amount of encapsidated pgRNA (panel B) and core-associated HBV DNA replication intermediates (panels C and D). These results are in consistent with the reported antiviral mechanism of Bay 41–4109 and DVR-23. Similar to DRV-23, BA-53038B treatment did not affect the levels of pgRNA (panel A), but reduced the amount of encapsidated pgRNA (panel B) as well as core-associated HBV DNA replication intermediates (panels C and D). Interestingly, in agreement with results presented in Fig. 2, BA-53038B treatment promoted the assembly of capsids with slow electrophoresis mobility, and reduced the amount of capsids with fast electrophoresis mobility (panel C, upper). Those findings were further validated in HepDES19 cells with additional type II CpAMs (BA-38017 and AT-61) and viral DNA polymerase inhibitor ETV as positive controls (Fig. 5). Taken together, like other type II CpAMs, BA-53038B inhibited pgRNA encapsidation into nucleocapsids and consequentially suppressed viral DNA replication. However, unlike other type II CpAMs examined thus far, BA-53038B induced the assembly of structurally distinct empty capsids, as indicated by its slower electrophoresis mobility.

Physical properties of capsids derived from BA-53038B-treated cells

In order to determine the physical properties of HBV capsids assembled in the presence of BA-53038B, the sedimentation velocity of HBV capsids from AML12HBV10 cells with or without BA-53038B treatment were determined. As shown in Fig. 6A, similar to that from mock-treated cells, capsids assembled in the presence of BA-53038B sedimented in a sucrose gradient centrifugation with a flat peak ranging from fractions 5 to 10, and DNA-containing capsids peaking at fraction 6. Although the total amount of capsids was similar in mock-treated and BA-53038B treated cells, the amount of HBV DNA-containing capsids was significantly reduced in BA-53038 treated cells. In agreement with our recent report that core proteins are hyper-phosphorylated in empty capsids, but hypophosphorylated in pgRNA- and DNA-containing capsids [36], BA-53038B treatment inhibited pgRNA encapsidation and consequentially reduced the amount of DNA-containing capsids and hypophosphorylated core protein (Fig. 6B). Moreover, visualization of HBV capsids with electronic microscopy revealed that the capsids from mock-treated and BA-53038B treated AML12HBV10 cells are similar in morphology and size (Fig. 6C). Obviously, more advanced physics technology, such as cryo-EM structure analysis, is required to reveal the structural differences of capsids assembled in the presence of BA-38058B and other CpAMs.

Antiviral activity of BA-53038B may be chirality and/or polarity dependent

As shown in Fig. 7A, BA-53038B is a fused bicyclo[4.1.0]heptane-7-carboxamide, potentially with exo- and endo- isomers. Because many protein allosteric modulators, including CpAMs, interact with their target proteins in a chirality-dependent manner [37], we intended to separate and determine the antiviral activity of each BA-53038B enantiomer. However, the separation was not successful, we thus synthesized compounds 62031A and 62031B by substitution of the potential chiral center at C7 of BA-53038B with a nitrogen atom to eliminate the chirality at that position. Interestingly, antiviral assay performed in

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HepAD38 cells indicated that the two new compounds completely lost activity to inhibit HBV replication (Fig. 7B), but BA-53038B and BA-38017 demonstrated similar antiviral activities as observed in HepDES19 cells (Fig. 3) [28]. These results imply that not only chirality but also polarity is important for modulators to interact with core protein dimers and disrupt nucleocapsid assembly, since 62031B (ClogP 2.62) is more polar than BA53038B (ClogP 4.15).

Genetic evidence suggesting that BA-53038B may interact with core protein at HAP pocket

Core protein free dimer is the building block of capsids. Assembly of capsids is driven by hydrophobic interactions at the dimer-dimer interface of core protein. Recent crystal structure and cry-EM studies indicated that CpAMs bind to a hydrophobic pocket, designated as the HAP pocket, at the dimer-dimer interfaces [19]. Part of the wall of the HAP pocket is formed by residue V124 of core protein. Mutation at V124, especially substitutions with amino acid residues with larger hydrophobic side chain, results in partially occupation of the HAP pocket and consequentially interferes with the assembly of capsid as well as the interaction between capsid and CpAMs[38–39]. To investigate whether BA-53038B disrupts capsid assembly and pgRNA encapsidation by binding to the HAP pocket, HepG2 cells transfected with pHBV1.3-derived plasmids with V124A, V124L, V124F or V124W mutations of core protein were used to examine the effect of BA-53038B on the capsid assembly and HBV replication. As a control, a natural occurring core protein mutation F97L, which is located beyond the dimer-dimer interface, was also included [40]. As shown in Fig. 8, consistent with our previous report, Bay 41–4109 interfered with the accumulation of capsids and inhibited viral DNA replication in the cells transfected with pHBV1.3-derived plasmids with substitution of F97L or V124A, but not that with V124W. Interestingly, Bay 41–4109 treatment efficiently induced assembly of slower migrating capsids in cells transfected with pHBV1.3-derived plasmid with V124F mutation, without reducing the amount of HBV DNA. However, while DVR-23 and BA-53038B treatment induced the assembly of faster and slower migrating capsids in the cells transfected with pHBV1.3 or pHBV1.3 with F97L or V124A substitution, both compounds failed to alter the mobility of capsids assembled in cells transfected with plasmid with V124F or V124W substitution. Moreover, by using real-time PCR assay to quantify HBV DNA, we have demonstrated that only pHBV1.3/core-V124W supported HBV DNA replication was resistant to both DVR-23 and BA-53038B (Table 2). These experimental data thus suggest that like the SBA derivative DVR-23 and other type II CpAMs, BA-53038B modulates HBV capsid assembly by binding to the HAP pocket.

Discussion

The lack of cellular homologues of HBV core protein makes HBV capsid assembly and disassembly highly selective antiviral targets for the inhibition of HBV replication [13]. Furthermore, due to targeting distinct viral proteins, CpAMs and viral DNA polymerase inhibitors have no cross resistance to each other's drug-resistant variants and are thus ideal partners for combination therapy toward more potent or complete inhibition of viral replication, which cannot be achieved by monotherapy with either antiviral agents alone [9, 41]. Currently, several type I and type II CpAMs are in clinical trials and demonstrated

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potent antiviral activity [42–45]. It will be interesting to see if complete inhibition of HBV replication for an extended period of time will result in the exhaustion of cccDNA pool and cure of chronic hepatitis B, particularly for the patients with high basal level of antiviral immune response [9].

Extensive biophysical studies showed that although all the CpAMs bind to HAP pocket at the core protein dimer-dimer interface to disrupt capsid assembly, different CpAMs differentially alter core protein dimer assembly and disassembly [31, 46]. Type I CpAMs induce core protein dimers to assemble non-capsid aggregates that are either degraded in the cytoplasm or accumulated in PML bodies in HBV infected hepatocytes [24–25]. It will be interesting to investigate whether the decay of HBV core protein enhances viral antigen presentation and consequentially, the antiviral immune response. In addition, due to the important role of PML body in HBx protein regulation of cccDNA transcription [47], the possibility that accumulation of core protein aggregates in PML bodies may suppress cccDNA function should also be examined. On the contrary, binding of type II CpAMs at the HAP pocket changes the tertiary structure of the core protein dimer, which results in the assembly of empty capsids with global alteration of quaternary structure [17–18]. However, how the structurally diversified type II CpAMs induce the assembly of empty capsids without encapsidation of viral pgRNA and DNA polymerase has not been fully understood. In agreement with our recent report [35], like other type II CpAMs examined thus far, compounds 1 to 7 treatment induced the assembly of capsids with increased electrophoresis mobility (Fig. 2A). The altered electrophoresis mobility is likely due to the change of surface charge of capsids, which is resulted from altered exposure and/or ionization of charged amino acid side chains in the assembly domain of core protein in capsids [35]. This finding indicates that in addition to accelerating the kinetics of core protein dimers to assembly empty capsids and indirectly reducing pgRNA encapsidation [48], it is also possible that type II CpAMs induce the assembly of structurally altered nucleocapsids that do not favor the encapsidation of viral pgRNA and DNA polymerase complex [35].

Practically, type II CpAM-induced capsid electrophoresis mobility shift provides a very convenient diagnostic marker for compounds targeting core protein assembly. This assay allowed us to identify CpAMs from the “hits” of cell-based primary screening with great confidence. Moreover, the distinct electrophoresis mobility of empty capsids assembled in the presence of BA-53038B indicates that this compound induces the assembly of capsids with distinct structure feature, and thus is an important chemical probe for dissecting the capsid structure response to different CpAMs.

Conclusions

The novel chemotypes of CpAMs discovered in this screening campaign provide new leads for developing antiviral agents against HBV infection. It is particularly interesting to identify the most active chiral isomer of BA-53038B as a new chemical scaffold of next generation of CpAMs.

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Materials and Methods

Cell lines and compounds.

HepG2 cell line was purchased from ATCC. HepAD38 was obtained from Dr. Christoph Seeger (Fox Chase Cancer Center, Philadelphia, USA) [49]. HepDES19 and AML12HBV10 cell lines were established as we described previously [34, 50]. All three cell lines support HBV pgRNA transcription and DNA replication in a tetracycline (tet)-inducible manner. DVR-23, BA-38017, BA-53038B, 62031A, 62031B and AT-61 were synthesized in-house [28]. Entecavir (ETV) is a gift from Dr. William S. Mason (Fox Chase Cancer Center, Philadelphia, USA). Bay41–4109 is a gift of Dr. Lai Wei (Hepatology Institute, People's Hospital, Peking University, China).

Plasmids.

Wild-type HBV replicon pHBV-1.3, pHBV-1.3-derived plasmids expressing core proteins with substation of F97L, V124A, V124F and V124W were described previously [28]. The plasmids pCI-HBc-WT, pCI-HBc-3A and pCI-HBc-7A were reported previously [51].

Screening of compound library from Asinex

AML12HBV10 screening assay in 96-well plate was performed as previously described [27]. The 19,920 small molecular compounds were purchased from Asinex and tested at 10 μM concentration. Forty-eight h post treatment, the amounts of HBV DNA in the cell lysates were determined by dot-blot hybridization with $\alpha-^{32}$P-UTP-labelled riboprobe to detect minus strand HBV DNA. The compounds that reduced HBV DNA by greater than 60% were deemed as primary "hits".

Determination of antiviral activity of primary "hit" compounds

AML12HBV10 cells were seeded in 96-well plates in the absence of tetracycline and treated with a serial of 2-fold dilution of test compounds, ranging from 10 μM to 0.08 μM. Intracellular HBV core DNA was determined by dot blot hybridization. Quantification of dot blot was used to determine the concentration that reduces the amount of HBV DNA by 50% (EC_{50}). Cytotoxicity of the "hit" compounds was determined by an MTT assay (Sigma) and expressed as the concentration of compound that reduced the viability of the cells by 50% (CC_{50}).

Analysis of cytoplasmic HBV capsids by particle gel electrophoresis and ultracentrifugation.

Cells were lysed with buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and 0.1% NP-40. Cell debris were clarified by centrifugation at 12,000 g for 10 min. followed by electrophoresis through nondenaturing 1.8% agarose gels and transferring to a nitrocellulose filter. HBV capsids were detected with an antibody against HBV core protein (DAKO, Cat. No. B0586) [28]. Capsid associated HBV DNA were detected by hybridization with an $\alpha-^{32}$P-UTP (800Ci/mmol, Perkin Elmer) labeled full-length riboprobe in positive strand polarity[28].

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Analyses of HBV core DNA and RNA.

Cytoplasmic core DNA from HBV replicating cell lines or replicon plasmid transfected HepG2 cells were extracted as described previously [28]. HBV DNA replicative intermediates were analyzed by Southern blot hybridization with an α–^{32}P-UTP labeled positive-stranded riboprobe [28]. HBV DNA were quantified by quantitative real-time PCR assay [52]. Total cellular RNA was extracted with TRIzol reagent (Invitrogen). Encapsidated HBV RNA was extracted as previously described [34]. HBV mRNA and encapsidated HBV RNA were analyzed by Northern blot hybridization with an α–^{32}P-UTP labeled full-length riboprobe in minus strand polarity.

Western blot assay.

Cells were lysed in 1 × LDS loading buffer (Invitrogen). Cell lysate was resolved in a NuPAGE™ 12% Bis-Tris Protein Gel (Invitrogen). HBV core protein was probed with a rabbit polyclonal antibody against peptide spanning the C-terminal aa 170 to 183 of HBV core protein, (GenScript, NJ, USA) [36]. β-actin was detected by an antibody from Cell Signaling Technologies.

Electron microscopic analysis of capsids.

HBV capsids were purified from AML12HBV10 cells, with or without treatment with 5 μM of BA-53038B for 2 days, by sucrose gradient centrifugation [53].Electron microscopy analysis was performed after negatively stained with Uranyless (Electron Microscopy Sciences) on a FEI Tecnai 12 Spirit/Biotwin (LaB6 filament), operating at 100kV, with an AMT 2k × 2k CCD camera.

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Abbreviations

HBV	hepatitis B virus
HBsAg	HBV surface antigen
pgRNA	pre-genomic RNA
rcDNA	relaxed circular DNA
cccDNA	covalently closed circular DNA
CpAM	core protein allosteric modulator
HAP	heteroaryldihydropyrimidine
SBA	sulfamoylbenzamide
PPA	phenylpropenamide

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ETV	entecavir
tet	tetracycline

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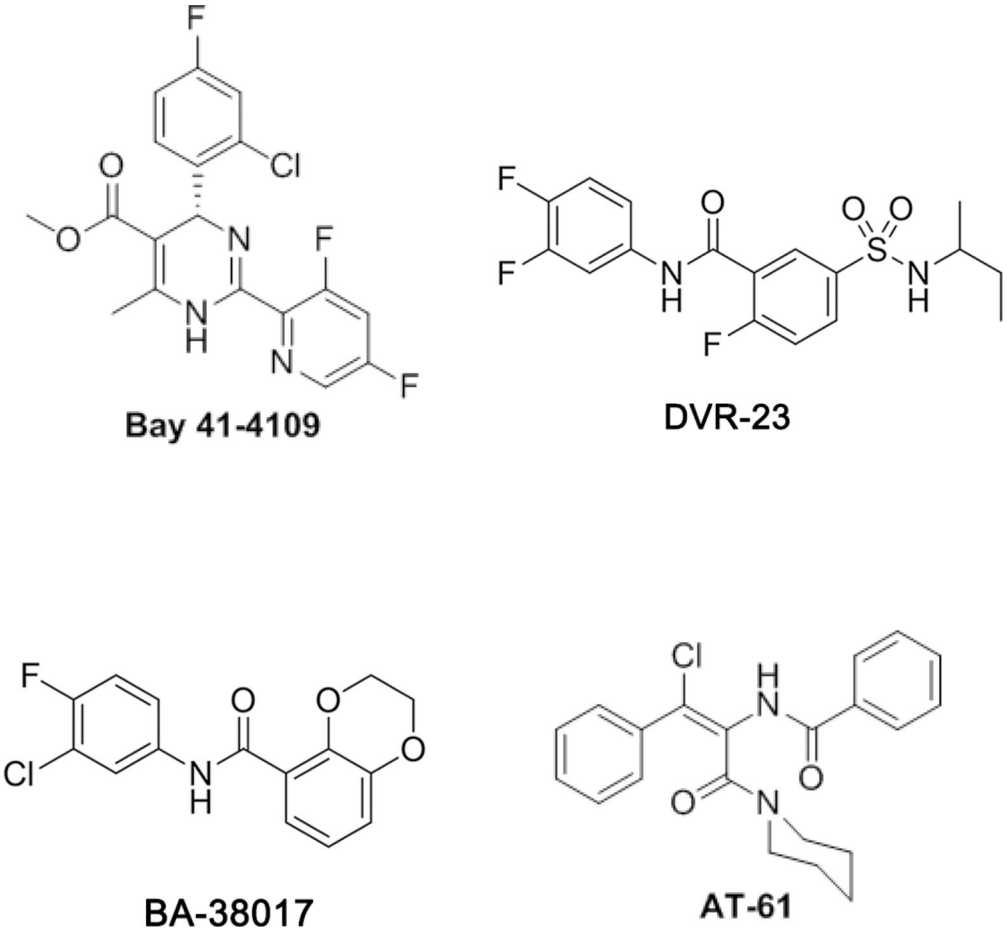

Fig. 1.
Structure of representative HBV core protein allosteric modulators (CpAMs).

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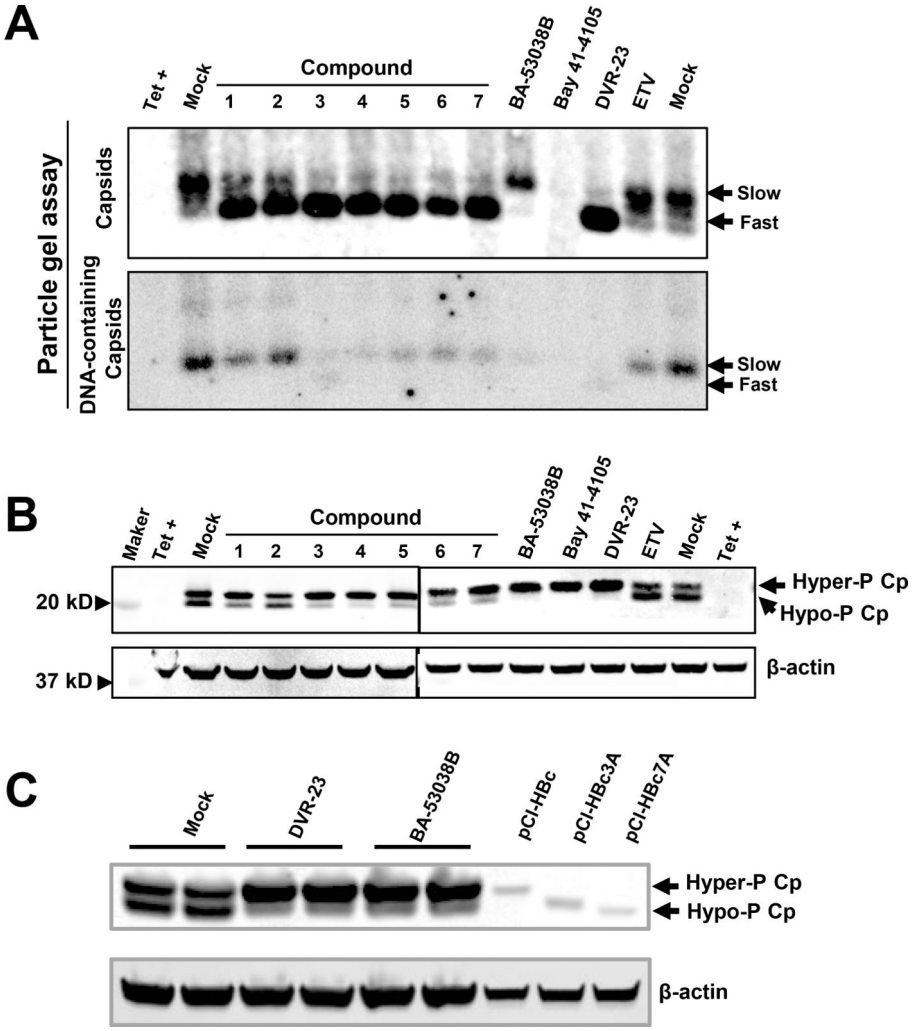

Fig. 2. The novel antiviral compounds induced the capsid/nucleocapsid mobility shift and reduced the amount of hypophosphorylated core protein.

(A and B) AML12HBV10 cells were cultured in the absence of tetracycline (tet) and treated with 5 μM of compounds 1 to 7, BA-53038B, DVR-23 or 2 μM of Bay 41–4109, 1 μM of ETV. Two days post treatment, cytoplasmic HBV capsids were resolved by a 1.8 % agarose gel electrophoresis followed by transferring on to a nitrocellulose membrane. core protein was detected by antibody Dako (A, upper panel). Capsid-associated HBV DNA was detected by using a ^{32}P-labeled riboprobe to detect minus strand HBV DNA. (A). Intracellular core protein was detected with antibody HBc-170A in a Western blot assay. (B). **(C)** AML12HBV10 cells were cultured in the absence of tet and treated with 5 μM of DVR-23 or BA-53038B for 2 days. Cell lysates from HepG2 cells transfected with plasmids expressing wild-type or mutant core proteins (pCI-HBc-WT, pCI-HBc3A or pCI-HBc7A) were run in parallel to serve as size markers for core protein with different level of phosphorylation. Intracellular core protein was detected with antibody HBc-170A in a Western blot assay. β-actin served as a loading control.

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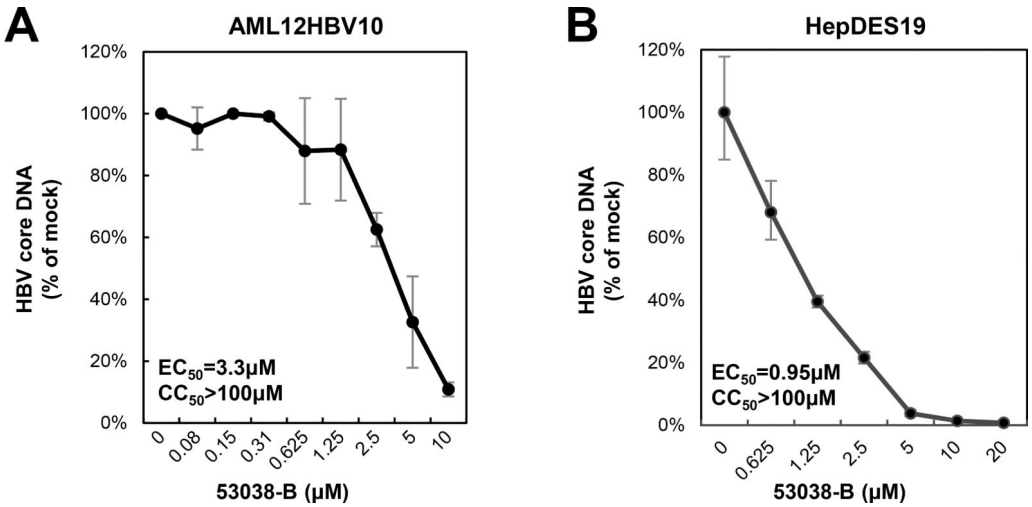

Fig. 3. BA-53038B inhibited HBV replication in both AML12HBV10 and HepDES19 cells.

AML12HBV10 (**A**) and HepDES19 (**B**) cells were cultured in the absence of tet and treated with BA-53038B for 2 and 6 days, respectively. (A) Cytoplasmic HBV core DNA were quantified by dot blot hybridization. (B) Cytoplasmic HBV core DNA was detected by a real-time PCR assay. Data were plotted as a fraction of that in mock-treated cells (n=3). Cytotoxicity of the compound were determined by a MTT assay.

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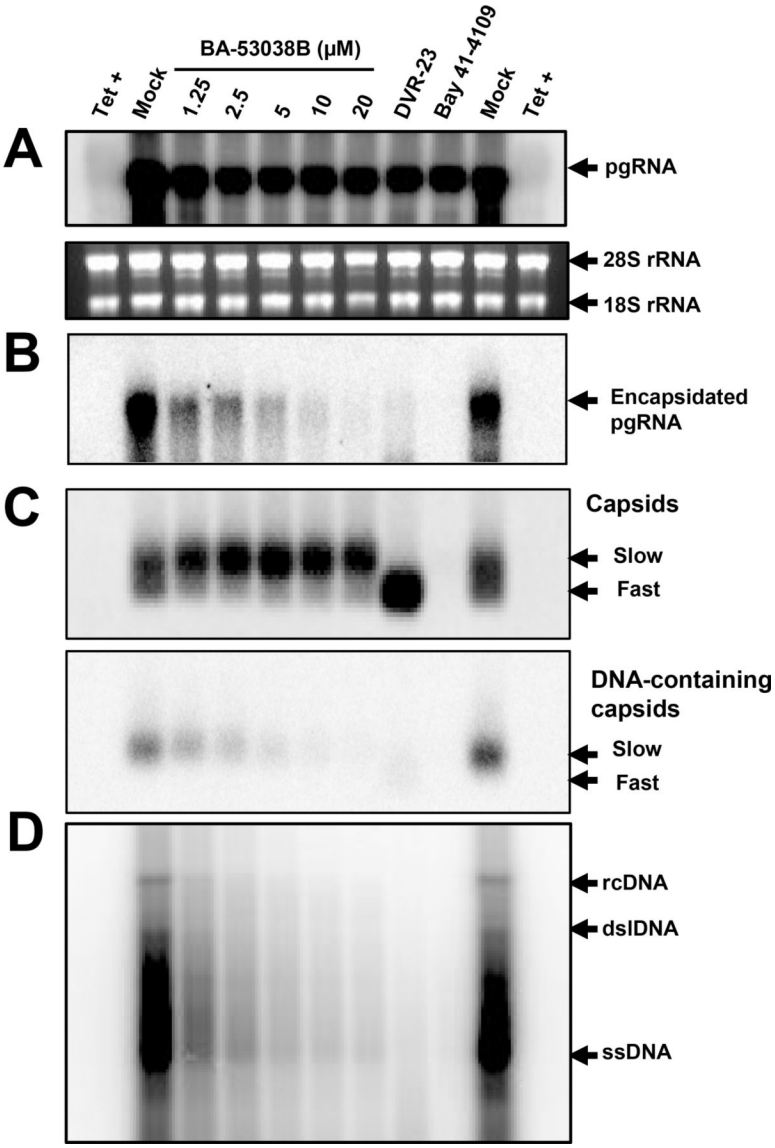

Fig. 4. BA-53038B inhibited the assembly of pgRNA-containing nucleocapsids.

AML12HBV10 cells were cultured in the absence of tet and treated with the indicated concentrations of BA-53038B, 5 μM of DVR-23, 2 μM of Bay 41–4109 or 1 μM of ETV. Two days post treatment, total HBV RNA **(A)** and encapsidated pgRNA **(B)** were detected by Northern blot hybridization with a ^{32}P-labeled riboprobe to detect minus-strand HBV DNA. 28S and 18S rRNA served as loading controls. **(C)** Cytoplasmic HBV capsids were resolved by a 1.8 % agarose gel electrophoresis followed by detection of capsid with antibody against core protein and HBV DNA, as described in Fig.2A legend. **(D)** HBV DNA replication intermediates were detected by Southern blot hybridization. rcDNA, dslDNA and ssDNA are relaxed circular, double-stranded linear and single-stranded forms of HBV DNA, respectively.

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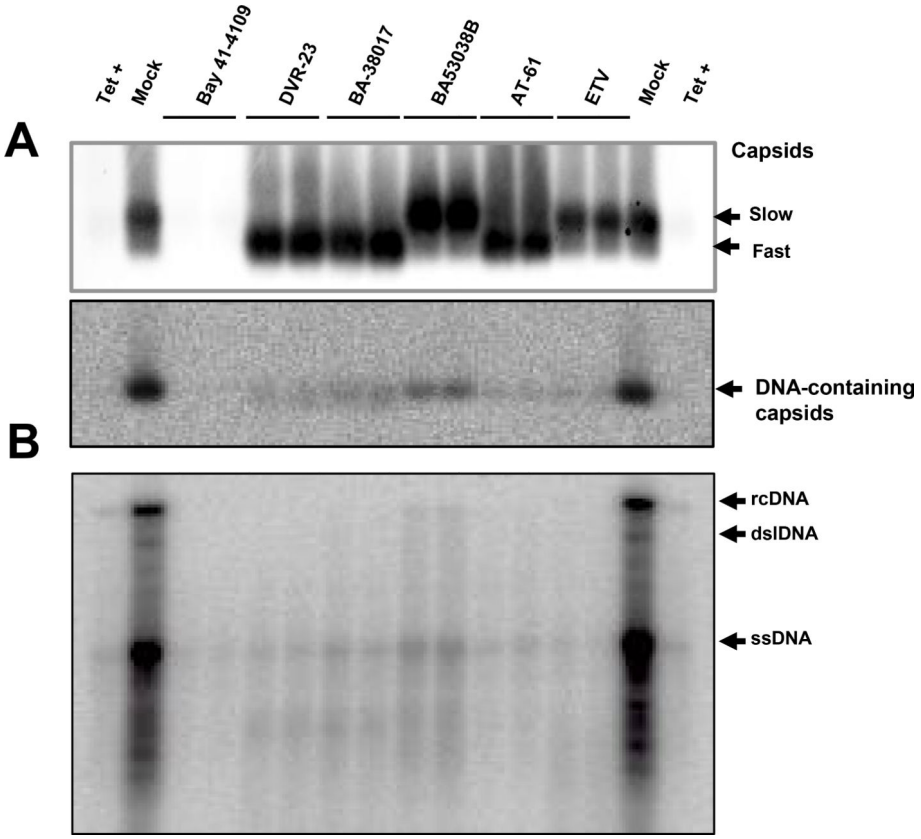

Fig. 5. BA-38058B induced the formation of distinct HBV capsids in HepG2 cells.

HepDES19 cells were culture in the absence of tetracycline and treated with Bay 41–4109 (2 μM), DVR-23 (5 μM), BA-38017 (5 μM), BA-53038B (5 μM), AT-61 (25 μM) or ETV (1 μM) for 6 days. **(A)** The total amounts of capsids and the capsid-associated HBV DNA were determined using a particle gel assay in a 1.8 % agarose gel electrophoresis as described in Fig. 2A legend. **(B)** HBV DNA replication intermediates were determined by Southern blot analysis. rcDNA, relaxed circular DNA. dslDNA, double-stranded linear DNA. ssDNA, single-stranded DNA.

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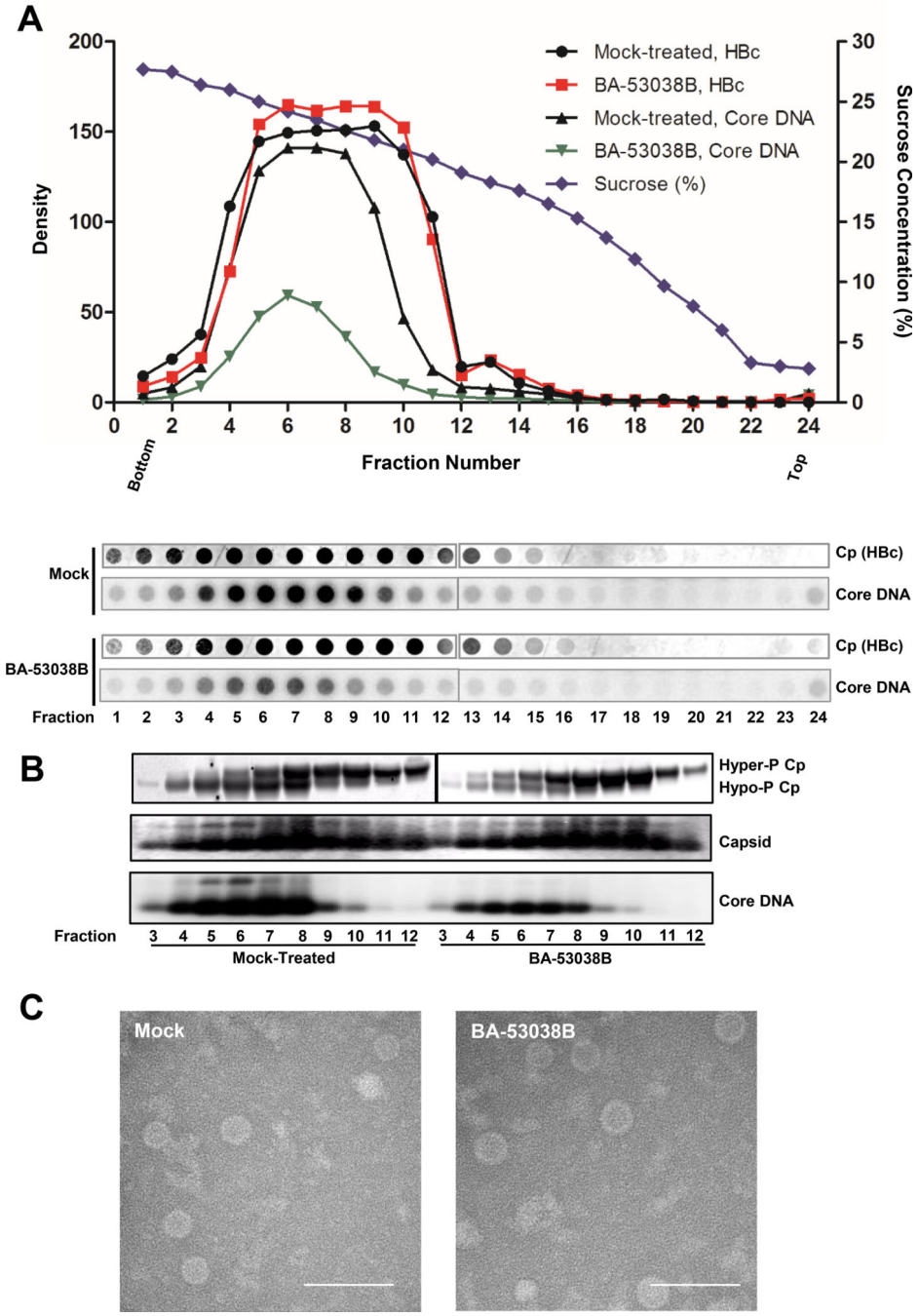

Fig. 6. Physical properties of capsids derived from BA-53038B treated cells.

(A) AML12HBV10 cells were cultured in the absence of tetracycline and treated with 5 μM of BA-53038B. Two days poster treatment, the intracellular capsids were sedimented on a 15% to 30% sucrose. Total of 24fractions were collected from the bottom. 1/30 volume of each fraction was spotted on Nylon membrane for detection of HBV core protein (HBc) with Dako antibody followed by detection of minus strand HBV DNA using HBV riboprobe. **(B)** HBV capsids from fractions 3–12 were purified by ultracentrifugation and subjected to Western blot to detect core protein with antibody HBc-170A and analyses of

capsids and their associated HBV DNA in 1.8 % native agarose gel assay. **(C)** Electronic microscopic graphs of the capsids purified from AML12HBV10 cells in presence and absence of 5 μM of BA-53038B treatment. Scale bar is 100 nm.

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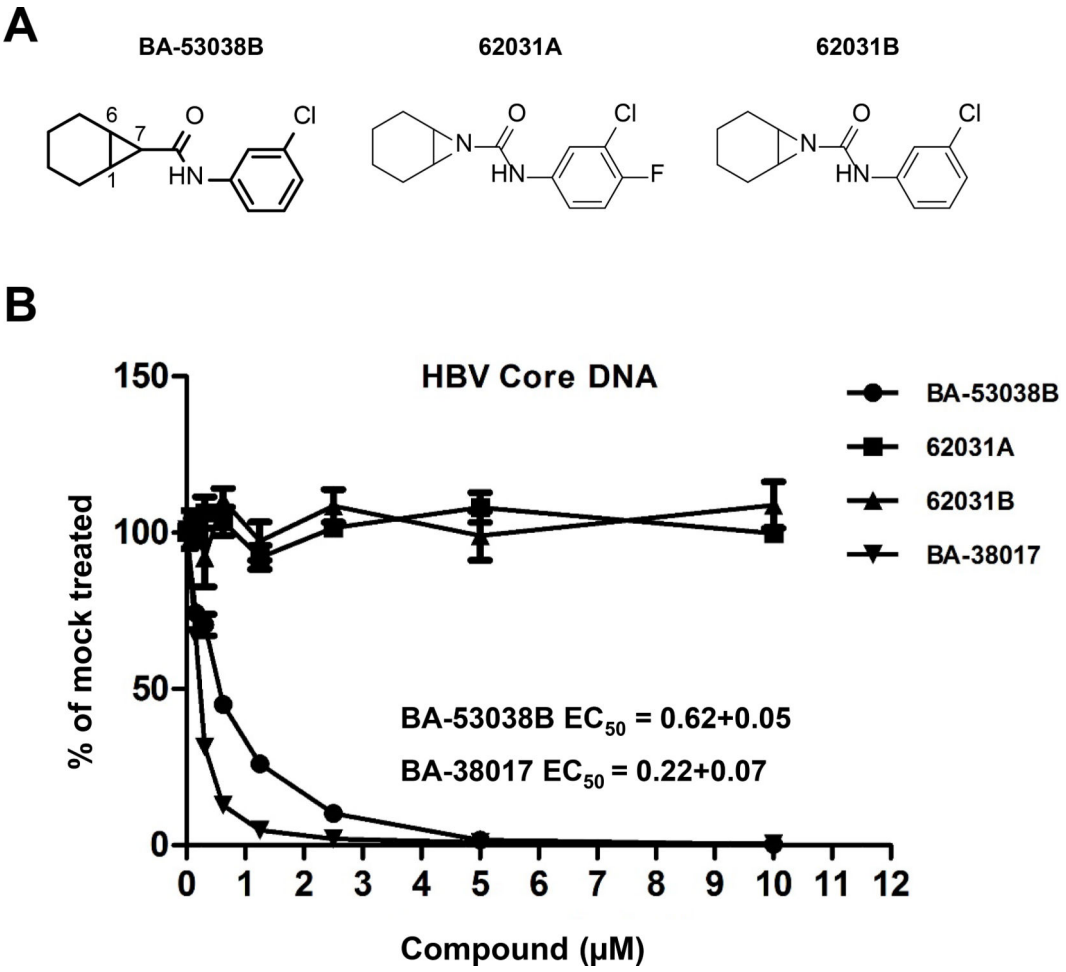

Fig. 7. Effects of BA-53038B derivatives on HBV replication in HepAD38 cells.

(**A**) Structure of BA-53038B and derivatives. (**B**) HepAD38 cells were cultured in the absence of tetracycline and treated with the indicated concentration of compound for 6 days. Cytoplasmic HBV core DNA was determined by a real-time PCR assay and plotted as a fraction of that in mock-treated cells (n=3).

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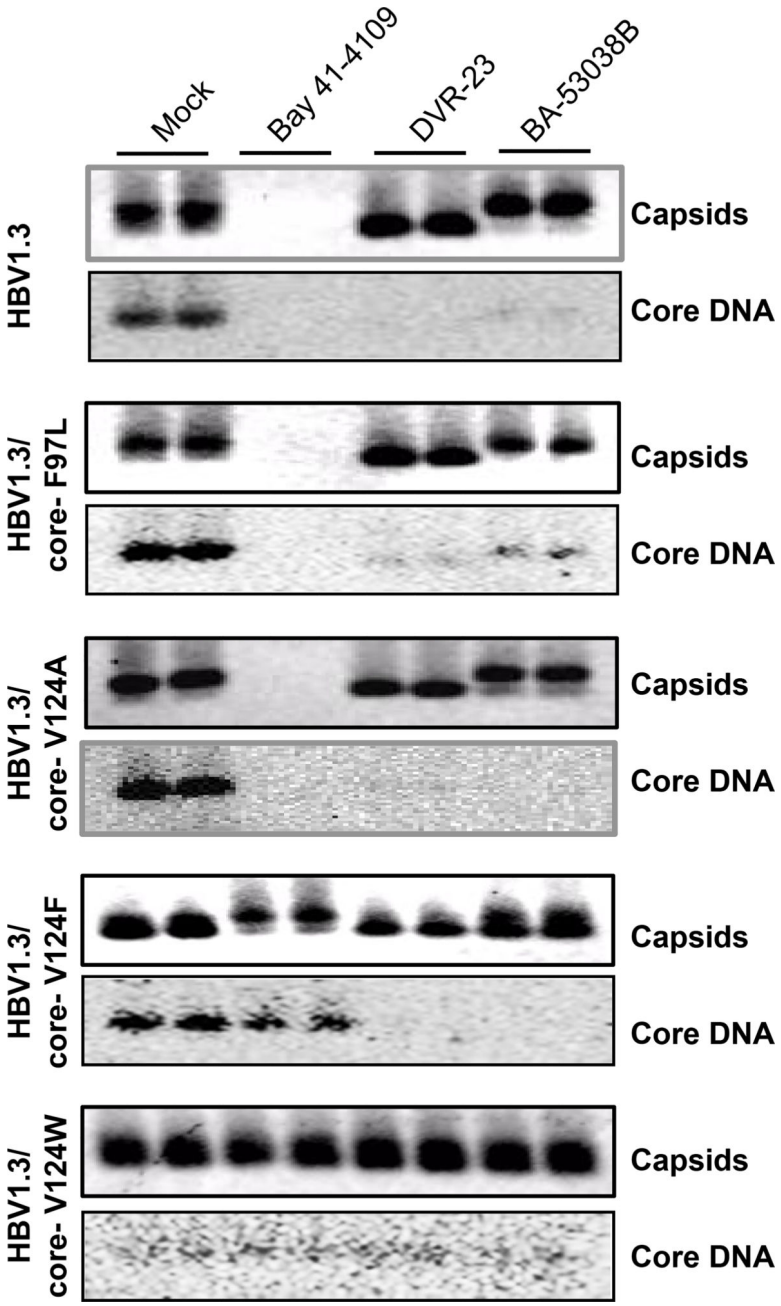

Fig. 8. Substitution of core protein valine (V) 124 with tryptophan (W) conferred resistant to BA-53038B induced capsid mobility shift and HBV DNA synthesis.

HepG2 cells were transfected with pHBV1.3 or pHBV1.3-derived plasmids expressing core protein with indicated substitution at V124. The cells were treated with 2 μM of Bay 41–4109, 5 μM of DVR-23 or BA-53038B starting at 6 h post transfection. Cytoplasmic capsids were analyzed 3 days post treatment by a particle gel assay. Capsid-associated minus-strand HBV DNA was detected with a ^{32}P-labeled riboprobe.

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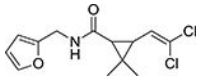
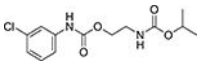
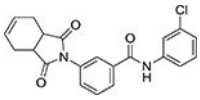
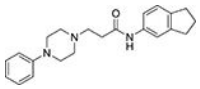
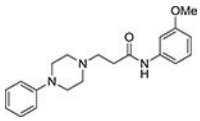
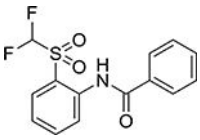
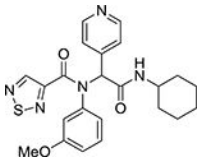
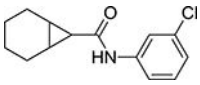
Table 1.

The structure and antiviral activity of novel HBV nucleocapsid assembly inhibitors

Chemotype	Compound Name	Structure	EC_{50} (μM)	CC_{50} (μM)
I	**Comp. 1**		6.68	>10
II	**Comp. 2**		2.77	>10
III	**Comp. 3**		0.83	>10
IV	**Comp. 4**		0.85	>10
	Comp. 5		1.47	>10
V	**Comp. 6**		2.25	>10
VI	**Comp. 7**		2.25	>10
VII	**BA-53038B**		3.32	>100

EC_{50} was determined by dot blot hybridization assay. CC_{50} was determined by MTT assay.

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Table 2.

The effect of BA-53038B on HBV capsid (HepG2 cells transiently transfected)

Compound	EC50 (μM)				
	HBV1.3	HBV1.3/core-F97L	HBV1.3/core-V124A	HBV1.3/core-V124F	HBV1.3/core-V124W
DVR23	0.37 ± 0.002	0.45 ± 0.06	1.61 ± 0.05	1.57 ± 0.15	>10
BA-53038B	0.67 ± 0.17	2.18 ± 0.81	0.40 ± 0.13	0.32 ± 0.06	>10

EC_{50} was determined by real-time PCR assay.