Original article

In vitro inhibition of HBV replication by a novel compound, GLS4, and its efficacy against adefovir-dipivoxil-resistant HBV mutations

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Background: HBV infection continues to be an important worldwide cause of morbidity and mortality. Patients with chronic hepatitis B can be successfully treated using nucleoside/nucleotide analogues. However, drug-resistant HBV mutants frequently arise, leading to treatment failure and progression to liver disease. Here, we report the effects of GLS4, a non-nucleosidic inhibitor that exhibits a novel and highly specific anti-HBV activity.

Methods: The median inhibitory concentrations (IC50s) of GLS4 on HBV were measured by Southern blotting. HBV capsid and core protein levels were detected by immunoblotting. To determine the antiviral activity of GLS4 against adefovir dipivoxil (ADV)-resistant HBV mutants, HepG2 cells transiently transfected with PUC-HBV1.2 plasmids that contained one of three major ADV-resistant mutations (rtA181T, rtA181V and rtN236T) were treated with GLS4. Intracellular HBV replicative intermediates were detected by Southern blotting. The effect on the in vitro assembly of HBV capsid protein was examined using dynamic light scattering and electron microscopy.

Results: The IC50 of GLS4 was 0.012 μM, which is significantly lower than that of lamivudine (0.325 μM). Immunoblot analysis of HepG2.2.15 cells and transiently transfected HepG2 cells indicated that GLS4 treatment interfered with the formation of core particles (assembly). The ADV-resistant HBV mutant strains were also sensitive to GLS4. Upon examining the in vitro assembly of HBV core protein 149 by electron microscopy, increased aberrant particles were observed after GLS4 treatment.

Conclusions: GLS4 is a new and unique potential anti-HBV agent that possesses a different mechanism of action than existing therapeutic drugs.

Introduction

HBV poses a major public health problem worldwide, particularly in southeast Asia and Africa, despite the presence of effective vaccines. Currently, there are >350 million chronic carriers worldwide, among whom 1 million die each year [1]. However, substantial advances have been made in the treatment of chronic hepatitis B in the past decade. Approved treatments for chronic hepatitis B have expanded from just one to a total of seven agents: standard interferon (IFN) [2], pegylated IFN [3], and five oral drugs that belong to the class of nucleoside/nucleotide analogues (NAs; lamivudine [3TC] [4], adefovir dipivoxil [ADV] [5], entecavir [6], telbivudine [7] and tenofovir [8]). Traditional IFN-α treatment has very limited efficacy and various adverse effects [9] that are not well tolerated by a subset of patients. The emergence of drug-resistant mutations in HBV during long-term therapy is also a major problem [10]. Clearly, it is important to develop new non-NA agents with different modes of action that can be used in antiviral combination therapy.

HBV is a small enveloped DNA virus that replicates via reverse transcription [11,12]. The 3.2 kb relaxed circular DNA genome of the virus is converted within the host cell nucleus into covalently closed circular DNA, which is the template for transcription of several subgenomic and genomic RNAs. Of the two genomic RNAs, one functions
as the pregenome and the messenger RNA for the viral core protein and polymerase. The second genomic RNA serves as the messenger RNA for the precore protein, which is the precursor of hepatitis B e antigen (HBeAg), a soluble derivative of the core protein that is secreted by infected hepatocytes. Interaction of the RNA pregenome with the viral polymerase facilitates encapsidation into the viral capsid. Progeny nucleocapsids may redeliver their genomes to the nucleus for covalently closed circular DNA amplification, or interact with the surface proteins and be released into circulation. The capsid is assembled in the cytoplasm with the core protein, viral pregenomic RNA, viral reverse transcriptase, and a few host proteins. The capsid plays indispensable roles in viral DNA synthesis from the pregenome and intracellular trafficking. Thus, core assembly is likely to be a high value target for therapeutics [11]. Recently, it was discovered that heteroaryldihydropyrimidines (HAPs) affect the accumulation of HBV capsids by decreasing the yield of assembled core and HBV genomes from cells that constitutively produce HBV [12,13].

Here we report that GLS4 (ethyl 4-[2-bromo-4-fluorophenyl]-6-[morpholino-methyl]-2-[2-thiazolyl]-1,4-dihydro-pyrimidine-5-carboxylate), which is a HAP compound against HBV replication, is a potent inhibitor of the replication of both wild-type and ADV-resistant HBV in stably HBV-expressing HepG2.2.15 cells and transiently transfected HepG2 cells. GLS4 targets HBV capsid formation, which is a process that is necessary for genome replication and is assumed to be less prone to developing drug resistance.

Methods
Preparation of antiviral compounds
The structural formula of GLS4 is shown in Figure 1. The compound was provided by the HEC Pharma group (Sunshine Lake Pharma Co., Ltd, Dongguan, China). 3TC and ADV were provided by GlaxoSmithKline, Suzhou, China. Stock solutions (50 mM) of 3TC were prepared in distilled water, and ADV and GLS4 were dissolved in dimethyl sulfoxide.

Plasmid construction
The plasmid pUC18-HBV1.2-WT [14], which contains 1.2× the genome-length of wild-type HBV (genotype B, subtype adw), was used to construct the ADV-resistant mutation (rtA181T/V and rtN236T) plasmids [15]. All constructs were sequenced to verify the mutation. A C-terminal Flag-tagged hepatitis B core antigen (HBcAg), 1-149/pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), was constructed that contained the HBV core protein amino acids 1 to 149 followed by an antigenic Flag (DYKD-DDDK) epitope that was generated by PCR. Plasmid pHBc1-149 was also derived from pUC18-HBV1.2-WT.

Cell culture and transfection
HepG2.2.15 [16], a hepatoblastoma cell line stably transfected with an HBV genome (subtype ayw) was used to assess the antiviral activity of GLS4. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. In addition, the HepG2.2.15 cell maintenance medium contained 200 μg of G418 per ml. During the experimental procedures, the cells were grown in the media described above without penicillin, streptomycin or G418.

HepG2 [17] cells were maintained in DMEM/F-12 supplemented with 10% FBS at 37°C in 5% CO₂. Transient transfections were performed using the FuGENE HD transfection reagent (Roche Applied Science, Mannheim, Germany) according to the instructions provided by the manufacturer. Intracellular HBeAg expression was measured by chemiluminescent microparticle immunoassay (CMIA; Architect i2000, Abbott, Chicago, IL, USA) as an indicator of transfection efficiency.

Antiviral treatment
The cells were seeded (5×10⁵ cells per well) in 2 ml of essential medium supplemented with 10% FBS in 6-well plates precoated with 1 ml of 0.02 mg/ml collagen (rat tail) type I (Sigma, St Louis, MO, USA) reconstituted in 0.1 M acetic acid. The collagen treatment helped the cells disperse evenly over the culture plate. Once the cells reached confluency, the culture medium was replaced every 2 days with medium containing various concentrations of the indicated drug. The cells and culture supernatants were harvested for analysis on day 8 of drug treatment.

Cytotoxicity and anti-proliferation assay
To determine if the compounds were cytotoxic to HepG2.2.15 cells, the cells were plated in 96-well
were determined from the dose-response curves. Cell viability by 50% compared to the drug-free cells, (pH 7.4), 1 mM EDTA and 20 µg/ml RNAse A. The precipitated DNA was dissolved in 10 mM Tris-HCl (pH 7.4). After washing with 70% ethanol, the precipitated DNA was dissolved with 20 µl 100 mM NaCl, 0.1 mM EDTA and 10 mM Tris-HCl (pH 8.0). The samples were separated on native 1% agarose gel and transferred onto a nitrocellulose transfer membrane (pore size 0.45 µm; Whatman, Dassel, Germany) by capillary transfer using 20× SSC buffer (3 M NaCl and 300 mM sodium citrate [pH 7.0]).

HBV core proteins were detected using a monoclonal mouse anti-Flag tag antibody ( Cli-Bio, Coachella, CA, USA) from the lysates of HepG2 cells, which were transfected with pHBe1-149-Flag. A quantity of 50 µg prepared samples were loaded onto a 12% SDS-PAGE gel and transferred onto a PVDF membrane (Invitrogen) by electroblotting.

Detection of the capsids and core protein were performed by immunoblotting using the polyclonal rabbit anti-HBcAg antibody (1:5,000; DAKO) and the monoclonal mouse anti-Flag tag antibody (1:10,000; Cli-Bio), respectively. A horseradish peroxidase anti-rabbit or anti-mouse antibody (1:5,000, Santa Cruz) was used as the secondary antibody. Detection was performed by ECL (Thermo Scientific). Fluorescence signals were detected with Kodak x-ray film and quantified with Quantity One software (BioRad).

Drug susceptibility assay
HepG2 cells were transiently transfected as described above with wild-type HBV or ADV-resistant mutant (rtA181T/V and rtN236T) constructs. Treatment with drugs started 24 h after transfection and was renewed every other day for 8 days. To determine the median inhibitory concentrations (IC_{50}s), transfected cells were treated with increasing drug concentrations ranging from 0 to 20 µM of ADV and from 0 to 10 µM of GLS4. After the drug treatment, intracellular HBV DNA replicative intermediates were extracted and analysed by Southern blotting.

Antiviral effects and data analysis
Viral genomic DNA was detected by Southern blotting, and the densities of the specific bands were quantified with Quantity One software. The levels of viral replication in drug-treated samples were expressed as a
percentage of the levels of replication in drug-free controls. Data were fitted to logistic dose-response curves, and curve parameters were estimated with the aid of GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA, USA). The IC_{50}, defined as the drug concentrations required to decrease HBV replication in HepG2.2.15 cells or HepG2 cells transiently transfected with ADV-resistant mutations (rtA181T/V and rtN236T) plasmids by 50% compared to the drug-free controls, were estimated from dose-response curves. The ratio of the mutant to wild-type IC_{50} was determined for each drug.

**Protein expression and purification**

C-terminally truncated HBV capsid protein with amino acids from 1 to 149 (core protein; Cp149) was expressed and purified from *Escherichia coli* as Cp149 dimers, as previously described [19,20]. Proteins were quantitated by absorbance at 280 nm. When required, preformed capsids were assembled from concentrated Cp149 dimers overnight at room temperature in 50 mM HEPES and 150 mM NaCl and then purified by size exclusion.

**Light scattering**

The size of the HBV-like capsid was monitored by dynamic light scattering after Cp149 was incubated with different concentrations of GLS4 for 24 h. The solutions were centrifuged, and the extract was filtered through 0.45 μm mesh filters. Measurements were recorded at 25°C by using a light scattering instrument (DynaPro-801; Wyatt Technology Corp., Santa Barbara, CA, USA) equipped with a 50 mW diode-pumped laser (λ=532 nm). The scattered light was monitored at 90° to the incident beam. The particle sizes were calculated from the diffusion coefficient by using the Stokes-Einstein equation with the cumulants method. Data were collected over five intervals of 30 s for each sample and plotted as the mean particle size with the accompanying standard error (n=3).

**Electron microscopy**

Samples were visualized on a Philips CM120 (Philips Electronics, Eindhoven, the Netherlands) transmission electron microscope at an acceleration voltage of 90 kV.

**Results**

**Cytotoxicity of GLS4**

To rule out the possibility that any of the observed anti-HBV activity of GLS4 was due to an anti-cellular activity, HepG2.2.15 cells, were grown for 8 days in a concentration range of 1 nM to 100 μM of GLS4 and 3TC, and their viabilities were assessed. The TC_{50s} of GLS4 and 3TC were 73.46 μM and >100 μM, respectively (Table 1 and Figure 2). The therapeutic index (TI) is the ratio of toxicity to efficacy (TC_{50}/IC_{50}).

**Effect of GLS4 on HBV DNA**

The HepG2.2.15 cells were treated with different concentrations of anti-viral drugs for 8 days, and HBV replicative intermediates were extracted and assayed by quantitative Southern blotting. GLS4 inhibited replication of wild-type HBV in a dose-dependent manner (Figure 3A). From three independent experiments, the IC_{50} of GLS4 was 0.012 μM, whereas the IC_{50} of 3TC was 0.325 μM (Table 1 and Figure 3B), indicating that GLS4 is a potent viral inhibitor.

**Effect of GLS4 on HBV capsid and core protein**

HBV capsids from HepG2.2.15 could be detected in the cells treated with GLS4 of 0.001, 0.01 and 0.1 μM, but not 1 μM. 3TC did not show any effect on HBV capsids even at a high concentration of 10 μM (Figure 4A). We observed similar effects on the formation of ‘empty core particles’ induced by the core protein expression vector pHBc1-149-Flag transfected into exponentially growing HepG2 cells (Figure 4B, immunoblot I). HBV core proteins also were detected in transiently transfected HepG2 cells using an anti-Flag antibody. The amount of HBV core proteins declined with increasing concentrations of GLS4 in transiently transfected HepG2 cells. Core proteins were readily detected at concentrations between 1 and 10 μM of GLS4, but no core particles were detected. By contrast, 3TC did not reduce core particle or core protein levels (Figure 4B, immunoblot II).

**ADV-resistant HBV is susceptible to GLS4**

To evaluate the effects of GLS4 on ADV-resistant HBV, we assessed the susceptibility of the rtA181T/V or rtN236T mutant to antiviral drugs in HepG2 cells. HepG2 cells transiently transfected with wildtype, rtA181T/V or rtN236T vectors were treated for

### Table 1. The median toxic concentrations and median inhibitory concentrations of GLS4 and lamivudine in HepG2.2.15 cell line

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median TC_{50} μM (so)</th>
<th>Median IC_{50} μM (so)</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLS4</td>
<td>73.460 (45.86)</td>
<td>0.012 (0.001)</td>
<td>6,122</td>
</tr>
<tr>
<td>3TC</td>
<td>&gt;100</td>
<td>0.325 (0.09)</td>
<td>-</td>
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*The median toxic concentration (TC_{50}) was determined by exposing confluent monolayers of HepG2.2.15 cells to GLS4 and lamivudine (3TC) for 8 days and then determining cytotoxicity by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA). The median inhibitory concentration (IC_{50}) was determined by exposing confluent monolayers of HepG2.2.15 cells to GLS4 and 3TC for 8 days and determining HBV replicative intermediate by Southern blotting. The therapeutic index (TI) is the ratio of toxicity to efficacy (TC_{50}/IC_{50}).*
GLS4, a novel inhibitor of HBV replication

**Figure 2. Cytotoxic profile of GLS4 and lamivudine in the Hep2.2.15 cell line**

The median toxic concentrations ($TC_{50}$), the drug concentration that impairs HepG2.2.15 cell viability by 50% compared to the drug-free cells, were determined from the dose–response curves. The $TC_{50}$s of GLS4 and lamivudine (3TC) were 73.46 µM and >100 µM, respectively.

**Figure 3. GLS4 blocks HBV replication in vitro**

(A) Southern blotting of HBV replicative intermediates extracted from HepG2.2.15 cells. The positions of relaxed circular (RC), double-stranded linear (DS) and single-stranded (SS) HBV DNA are indicated. (B) Inhibition of HBV replication by treatment of HBV-producing HepG2.2.15 cells with GLS4 or lamivudine (3TC). The median inhibitory concentrations ($IC_{50}$), which are the drug concentrations required to decrease HBV replication in HepG2.2.15 cells by 50% compared to the drug-free controls, were estimated from dose–response curves. The $IC_{50}$ of GLS4 was 0.012 µM, whereas the $IC_{50}$ of 3TC was 0.325 µM.

(AV)
8 days with increasing drug concentrations ranging from 0 to 10 μM of GLS4 and 0 to 20 μM of ADV. IC_{50} values were estimated from dose-response curves, and the fold resistances were determined by comparison with wild-type.

The ADV-resistant mutant rtA181T/V and rtN236T viruses were approximately 2.6, 3.1 and 7.2-fold less susceptible to ADV than wild-type HBV (IC_{50} 5.745 μM, 6.827 μM and 15.781 μM, respectively). With GLS4, the IC_{50} values for the wild-type and rtA181T/V and rtN236T strains were 0.146 μM, 0.145 μM, 0.161 μM and 0.131 μM, respectively (Table 2 and Figure 5).

Exposure to GLS4 caused dose-dependent inhibition of replication of both the wild-type and the mutant HBV. The dose-response curves and IC_{50} values derived from them indicate that GLS4 was equally effective at inhibiting replication of ADV-resistant HBV mutants and the wild-type virus. The fold resistances were close to or <1.0, confirming that the ADV-resistant HBV was susceptible to GLS4.

GLS4 misdirects assembly to the formation of aberrant products

When the 183-amino-acid-long core protein is truncated at amino acid 149, it preferentially forms T=4 particles, which were previously used to determine the crystal structure [21]. In this work, these truncated proteins spontaneously formed capsids under favourable in vitro conditions and were used to study core assembly. Various concentrations of GLS4, 5 μM Cp149 and

<table>
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<tr>
<th>Cell line and HBV construct</th>
<th>IC_{50}, μM</th>
<th>Fold resistance</th>
<th>IC_{50}, μM</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2.2.15</td>
<td>1.011 (0.075)</td>
<td>–</td>
<td>0.012 (0.001)</td>
<td>–</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.196 (0.44)</td>
<td>1</td>
<td>0.146 (0.007)</td>
<td>1</td>
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<tr>
<td>rtA181T</td>
<td>5.745 (0.60)</td>
<td>2.6</td>
<td>0.145 (0.02)</td>
<td>1</td>
</tr>
<tr>
<td>rtA181V</td>
<td>6.827 (0.55)</td>
<td>3.1</td>
<td>0.161 (0.03)</td>
<td>1.1</td>
</tr>
<tr>
<td>rtN236T</td>
<td>15.781 (3.00)</td>
<td>7.2</td>
<td>0.131 (0.03)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Values represent the mean (±S.D) of three independent experiments. *Fold resistance = (mutant median inhibitory concentration [IC_{50}]) / (wild-type IC_{50}). **P<0.05. ADV, adefovir dipivoxil.
150 mM NaCl were coincubated for approximately 24 h at room temperature (21°C). The particle size of the Cp149 formed was measured by dynamic light scattering. The particle size changed after treatment with different concentrations of GLS4 (Figure 6). Each column in Figure 6 represents the particle size at a corresponding concentration of GLS4. Cp149 efficiently assembled into capsids in the absence of GLS4, and the particle size was approximately 28 nm. With the concentrations of GLS4 in 1.25, 2.5, 5, 10 and 20 μM, the particle sizes were determined to be approximately 31, 34, 34, 44 and 65 nm, respectively.

Examination of assembly reaction products by electron microscopy provided additional evidence for assembly misdirection. Electron microscopy examination of the samples was performed at room temperature in 5 μM Cp149, 150 mM NaCl and different concentrations of GLS4 (Figure 7). Cp149 efficiently assembled into capsids in the absence of GLS4, and all of the capsids appeared to be normal spheres. At the
Figure 6. Measurement of the size distribution of virus-like particles by dynamic light scattering

The particle size changed significantly after treatment with increasing concentrations of GLS4 from 0 to 20 μM. *P<0.05.

Figure 7. Products of assembly reactions examined by electron microscopy

Assembly reactions were performed at room temperature (21°C) with 5 μM Cp149 and 150 mM NaCl. Capsids, and non-capsid oligomers induced by misdirection, are seen in the presence of GLS4. The GLS4 concentrations (μM) were as noted on individual micrographs.
lowest concentration of GLS4 (1.25 μM), a few changes were observed in the core capsid, although the intact capsids were still detectable. With increasing concentrations of GLS4, the observed proportion of intact capsids decreased, while the anamorphic core protein masses increased. At the highest concentration of GLS4 (20 μM), no intact capsids were observed in the visual field, and only some anamorphic core protein masses were seen.

Discussion

The only genuine enzymatic activity of HBV is performed by its polymerase. Thus, NAs, which affect either the reverse transcriptase or the DNA-dependent polymerase activity, are successfully used to interrupt HBV replication. A similar reduction of the viral load can be achieved by using IFN-α, a treatment that has been established for 20 years. Although NAs are more convenient than IFN-based therapies and have fewer side-effects, sustained viral suppression is usually not achievable after withdrawal from a 48-week course of NA therapy, necessitating lengthy, and in many cases, indefinite treatment. Unfortunately, the lengthy duration of NA treatment is associated with an increased risk of development of drug resistance, leading to treatment failure and progression to liver disease. Clearly, there is a great need to diversify the therapeutic and prophylactic arsenal for HBV.

HBV persistence and transmission require HBV replication, which depends on the assembly of a core particle composed of core proteins, polymerase and pregenomic RNA. Reverse transcription to produce infectious DNA-containing particles occurs solely within the core, which resides in the cytoplasm. Thus, novel experimental approaches that target the HBV core protein and interfere with capsid formation, which is necessary for genome replication, are assumed to be less prone to inducing drug resistance.

The capsid, the protein shell of the core, is composed of 120 core protein dimers arranged with T=4 symmetry [22,23]. The HBV core protein is a 183 amino acid polypeptide that is dimeric in low ionic strength solution [24]. Assembly is nucleated by a trimer of core protein dimers and proceeds without accumulating observable populations of intermediates [25]. The interaction between the two proteins of the core protein dimer is weak, but interactions in the higher-ordered structure provide global stability to the capsid [26].

Recent drug screens have identified HAPs as a novel class of HBV inhibitors. In tissue culture and animal models, BAY 41-4109, a representative HAP compound, leads to decreased production of virions and accelerated loss of capsid protein [12,13]. Further research has shown that BAY 41-4109 enhances the rate of assembly and causes the formation of aberrant capsids at higher drug concentrations [27–29]. Our study demonstrated that GLS4, another HAP compound, had a 30-fold lower IC_{50} than 3TC (0.012 versus 0.325 μM) and possesses potent in vitro dose-dependent anti-HBV activity.

Aside from the effects of GLS4 on viral DNA, additional mechanisms of viral inhibition were investigated by testing the effect of GLS4 on viral capsids and core protein. Consistent with the observed effects on the replication of viral DNA, GLS4 decreased the number of capsids in HepG2.2.15 cells stably expressing HBV and transiently transfected HepG2 cells, whereas 3TC did not show any effect on the viral capsids even at a relatively high concentration of 10 μM. To determine whether the decline of capsids coincided with the decline of core protein, we also detected core protein in transiently transfected HepG2 cells in the presence of GLS4. Core protein was readily detected in these cells when treated with GLS4 at concentrations of 1 and 10 μM, but no core particles were detected in either HepG2 or HepG2.2.15 cells with the same concentrations of GLS4. By contrast, 3TC did not reduce core protein levels. Thus, GLS4 treatment interferes with the capsid formation (assembly) without primarily affecting core protein levels. Deres et al. [13] indicated that inhibition of capsid formation is concomitant with a reduced half-life of the core protein. So, we assumed that core protein did not become stabilized and was instead degraded when particle formation was inhibited at higher doses of GLS4. As drug resistance is an ongoing problem of the currently used drug regimens, it would be highly valuable to evaluate the potential effects of GLS4 against viruses resistant to other drugs. We found that exposure to GLS4 caused a dose-dependent inhibition of replication of both wild-type and mutant HBV. Dose-response curves and IC_{50} values indicated that GLS4 was equally effective as an inhibitor of replication of ADV-resistant HBV mutants and wild-type HBV.

This broad-spectrum activity is expected given the difference in the proposed mechanism of action for the HAP compounds. The assembly of capsid should be independent of the reverse transcriptase activity or mutations in the transcriptase machinery that do not affect the physical interactions of reverse transcriptase with the core protein. Therefore, future studies of combination protocols using ADV together with GLS4, which provides a new mechanism of antiviral action and overcomes ADV-resistant HBV, could produce a better strategy for the treatment of patients with HBV infection.

Normal capsid assembly is characterized by a slow nucleation rate and weak pairwise association energies between capsid protein dimers. Both factors are necessary to ensure the maximum yield of capsid with
minimal kinetic traps, which would be a waste of protein [25,30]. It has been reported that treatment of HBV with HAP compounds in vitro results in acceleration and misdirected assembly [28].

We used dynamic light scattering and electron microscopy to analyse the potential mechanism underlying the anti-HBV activity of GLS4. Dynamic light scattering is a well-established technique used to measure particle size ranging from a few nanometres to a few microns. Due to its small size, the core protein dimer cannot be detected by dynamic light scattering. Therefore, the particles detected in our study are assembled core protein. In our study, the size of the assembled core protein in the absence of GLS4 was 28 nm, which is similar to that reported previously. A change in the particle size was observed after treatment with different concentrations of GLS4, and the core particle sizes increased with increasing concentrations of the drug. This is consistent with a significant reduction of intact viral capsids and more misdirected assembly of core proteins occurring, observed with increasing concentrations of GLS4 by electron microscopy analysis. Our study suggests that GLS4 may inhibit virus replication by inducing inappropriate assembly and by misdirecting assembly, thereby decreasing the stability of normal capsids.

We propose a substance class for the treatment of HBV infection that displays a highly specific antiviral activity, namely, inhibition of capsid formation, can complement the current NA drugs that target the viral genome. We believe that small molecules that affect assembly could be used as a general antiviral strategy and provide an alternative treatment for patients who have developed resistance to agents that operate by other mechanisms. The candidate compound tested in this study, GLS4, may become a valuable addition to future therapy (mono- or combination therapy regimens) in light of its multi-specific mechanisms of action.

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Disclosure statement

The authors declare no competing interests.

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