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Direct Inhibition of Hepatitis B e Antigen by Core Protein Allosteric Modulator

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Hepatitis B e antigen (HBeAg) is an important immunomodulator for promoting host immune tolerance during chronic hepatitis B (CHB) infection. In patients with CHB, HBeAg loss and seroconversion represent partial immune control of CHB infection and are regarded as valuable endpoints. However, the current approved treatments have only a limited efficacy in achieving HBeAg seroconversion in HBeAg-positive patients. Hepatitis B virus (HBV) core protein has been recognized as an attractive antiviral target, and two classes of core protein allosteric modulator (CpAM) have been discovered: the phenylpropenamides (PPAs) and the heteroaryldihydropyrimidines (HAPs). However, their differentiation and potential therapeutic benefit beyond HBV DNA inhibition remain to be seen. Here, we show that in contrast to PPA series compound AT-130, a HAP CpAM, HAP_R01, reduced HBeAg levels in multiple *in vitro* and *in vivo* HBV experimental models. Mechanistically, we found that HAP_R01 treatment caused the misassembly of capsids formed by purified HBeAg *in vitro*. In addition, HAP_R01 directly reduces HBeAg levels by inducing intracellular precore protein misassembly and aggregation. Using a HAP_R01-resistant mutant, we found that HAP_R01-mediated HBeAg and core protein reductions were mediated through the same mechanism. Furthermore, HAP_R01 treatment substantially reduced serum HBeAg levels in an HBV mouse model. *Conclusion:* Unlike PPA series compound AT-130, HAP_R01 not only inhibits HBV DNA levels but also directly reduces HBeAg through induction of its misassembly. HAP_R01, as well as other similar CpAMs, has the potential to achieve higher anti-HBeAg seroconversion rates than currently approved therapies for patients with CHB. Our findings also provide guidance for dose selection when designing clinical trials with molecules from HAP series. (Hepatology 2019;70:11-24).

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family and is a small DNA virus with a relaxed-circular, partially double-stranded DNA genome approximately 3.2 kB in length. Despite the availability of a safe and effective vaccine, chronic hepatitis B (CHB) infection continues to be a major public health issue worldwide. Recent estimates suggest that, globally, there are approximately 250 million people currently living with CHB infection (global hepatitis B surface antigen [HBsAg] seroprevalence rate of 3.61%).[(1)] CHB infection leads to the development of severe liver diseases, including liver cirrhosis and hepatocellular carcinoma. In 2013, HBV infection caused 686,000

Abbreviations: cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CpAM, core protein allosteric modulator; DMSO, dimethyl sulfoxide; EC_{50}, 50% effective concentration; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; ETV, entecavir; FBS, fetal bovine serum; HA, hemagglutinin; HAP, heteroaryldihydropyrimidine; HBc, hepatitis B core; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IF, immunofluorescence; pgRNA, pregenomic RNA; PPA, phenylpropenamide; RM, HAP_R01 resistant mutation; SEC-MALS, size exclusion chromatography with multi-angle light scattering.

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deaths worldwide, placing it in the top 20 causes of human mortality.[2] Currently approved drugs have made substantial progress in treating CHB; however, the cure rate remains lower than 10%.[3]

During the natural course of CHB infection, transition from the immune clearance phase to the inactive carrier phase is associated with a loss of serum hepatitis B e antigen (HBeAg) and the development of anti–hepatitis B e antibodies (HBeAg seroconversion).[4] A shorter HBeAg seropositive phase or earlier HBeAg seroconversion, either spontaneous or treatment-induced, correlates to a higher chance of sustained remission, a lower rate of HBeAg reversion, slower progression of liver disease, and a higher probability of HBsAg loss and seroconversion, which is considered as a permanent clinical remission of liver disease.[5-9] The HBV precore protein and HBeAg have been regarded as key viral tolerogens, which can modulate the host innate and adaptive immune responses and promote mother-to-child transmission and chronic infection.[10-15] Therefore, HBeAg loss, with or without anti-hepatitis B e seroconversion, in HBeAg-positive patients with CHB is regarded as a valuable endpoint, as it often represents partial immune control of CHB infection.[16]

Currently approved treatments, however, have only a limited efficacy in achieving HBeAg seroconversion in HBeAg-positive patients, with 29%-32% achieving seroconversion at 6 months following 48 or 52 weeks of pegylated interferon-alpha (PEG-IFNα) therapies and 10%-22% achieving seroconversion at 48 or 52 weeks of nucleos(t)ide analogue therapies.[16]

HBeAg (p17) is a secreted protein with 159 residues and is approximately 18 kDa in size. It shares 149 common residues with the HBV core protein (p21, 183 residues, ~21 kDa), except for the presence of 10 amino acids and the absence of 34 amino acids at the amino-terminus and carboxy-terminus, respectively. HBeAg is generated from a precore precursor (p25, ~25 kDa) protein. On translation from the preC-C gene, the N-terminal 29 signal residues lead the protein to the endoplasmic reticulum, where the first 19 residues are cleaved, producing a 22-kDa precore protein (p22). Next, approximately 34 residues from the C terminal are cleaved by a furin-like protease, leading to the generation and secretion of HBeAg.[10,17,18]

HBV capsid assembly is a highly choreographed process. Several small molecule core protein allosteric modulators (CpAMs) have been identified, which can affect this process. One class of compounds, the phenylpropenamides (PPAs; AT-130), has been found to accelerate capsid assembly, possibly at an inappropriate time and place, thereby preventing pregenomic RNA (pgRNA) encapsidation.[19,20] In contrast to PPAs, another class of compounds, heteroaryldihydropyrimidines (HAPs), have been found to misdirect capsid assembly to form aberrant noncapsid polymers, leading to the degradation of core protein.[21,22] Interestingly, the intracellular 22-kDa precore protein, as well as the bacteria-purified HBeAg protein, can also form capsids.[23-27] However, it is unclear whether CpAM has any effect on the precore protein or HBeAg. In the current study, we aimed to investigate the effect of a CpAM (HAP_R01) on precore/HBeAg proteins and to determine the underlying mechanism of action.[28] Our results suggest that HAP_R01 not only interferes with pgRNA encapsidation, leading to the inhibition of HBV DNA levels, but also directly reduces HBeAg by inducing precore (p22) protein misassembly. Therefore, HAP_R01 represents an agent with the potential to achieve higher anti-HBeAg

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seroconversion rates than currently approved therapies in patients with CHB.

Materials and Methods

CELL LINES

The human hepatoma–derived cell line HepG2 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under humidified air containing 5% CO_2. HepG2.2.15 (Aybio, Shanghai, China) was cultured in DMEM/F12 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 300 μg/mL geneticin, and 1% dimethyl sulfoxide (DMSO).

The tetracycline-inducible HBV stable cell lines HepAD38 (kindly provided by Christoph Seeger, Fox Chase Cancer Center, Philadelphia, PA), HepDES19 (kindly provided by Prof. Ju-Tao Guo, Drexel University College of Medicine, Philadelphia, PA), and HepBHAe82 (kindly provided by Prof. Haitao Guo, Drexel University College of Medicine) were cultured as described.[29-31]

ANIMAL STUDY

All procedures in this study were in compliance with local animal welfare legislation and the *Guide for the Care and Use of Laboratory Animals*.

Male FVB/N mice (6-7 weeks old) were obtained from Vital River Laboratories Co., Ltd. (Beijing, China). The animals were grouped on the basis of day −1 body weights. On day 0, all animals underwent hydrodynamic injection (HDI) within 5 seconds through the tail vein of 5-20 μg DNA in a volume (milliliters) of saline equivalent to 8% of the body weight (grams).[32] Blood samples were collected at the indicated time post-HDI injection.

For compound treatment, entecavir (ETV) was diluted in saline from stock solutions on treatment days. HAP_R01 was diluted with 1% RC-591 and 0.3% Tween 80 in water. The vehicle was 1% RC-591 and 0.3% Tween 80 in water. All test compounds were orally administered at the indicated dose and frequency.

STATISTICAL ANALYSIS

Statistical analysis was performed using Prism software (GraphPad). A value of $P < 0.05$ was considered statistically significant.

A description of other methods used in this study is provided in the Supporting Information.

Results

HAP_R01 INDUCES HBeAg MISASSEMBLY

To further improve on the originally identified HAP compounds represented by Bay 41-4109, we identified a HAP inhibitor, HAP_R01, with improved anti-HBV activity and enhanced drug-like properties (Fig. 1A).[28] Similar to the HBV core protein, purified HBeAg can form capsid-like structures under reducing conditions.[24,27] To evaluate the effect of HAP_R01 on the HBeAg assembly products, negative staining electron microscopy (EM) was performed. The NaCl-treated control appeared as regular spheres with a diameter of approximately 30 nm. In contrast, HAP_R01 treatment led to irregular particles that were substantially larger (Fig. 1B). A similar phenotype was reported when purified core protein was used.[33] We also observed a dramatic elution volume shift and molar mass increase on HAP_R01 treatment in size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis (Fig. 1C). These results suggest that HAP_R01 may induce the misassembly of both core and precore/HBeAg proteins, prompting us to investigate whether they were depleted through the same mechanism.

HAP_R01 TREATMENT LEADS TO HBeAg REDUCTION

Next, the anti-HBV activity of HAP_R01 was investigated in HepG2.2.15 cells. Profound inhibition on HBV DNA was observed even at the lowest concentration (30 nM) (Fig. 2A). Interestingly, we observed that at higher drug concentrations, HBeAg levels in cell culture supernatant were also substantially reduced by HAP_R01 treatment in a dose-dependent manner, as determined by anti-HBeAg enzyme-linked immunosorbent assay (ELISA) (50%

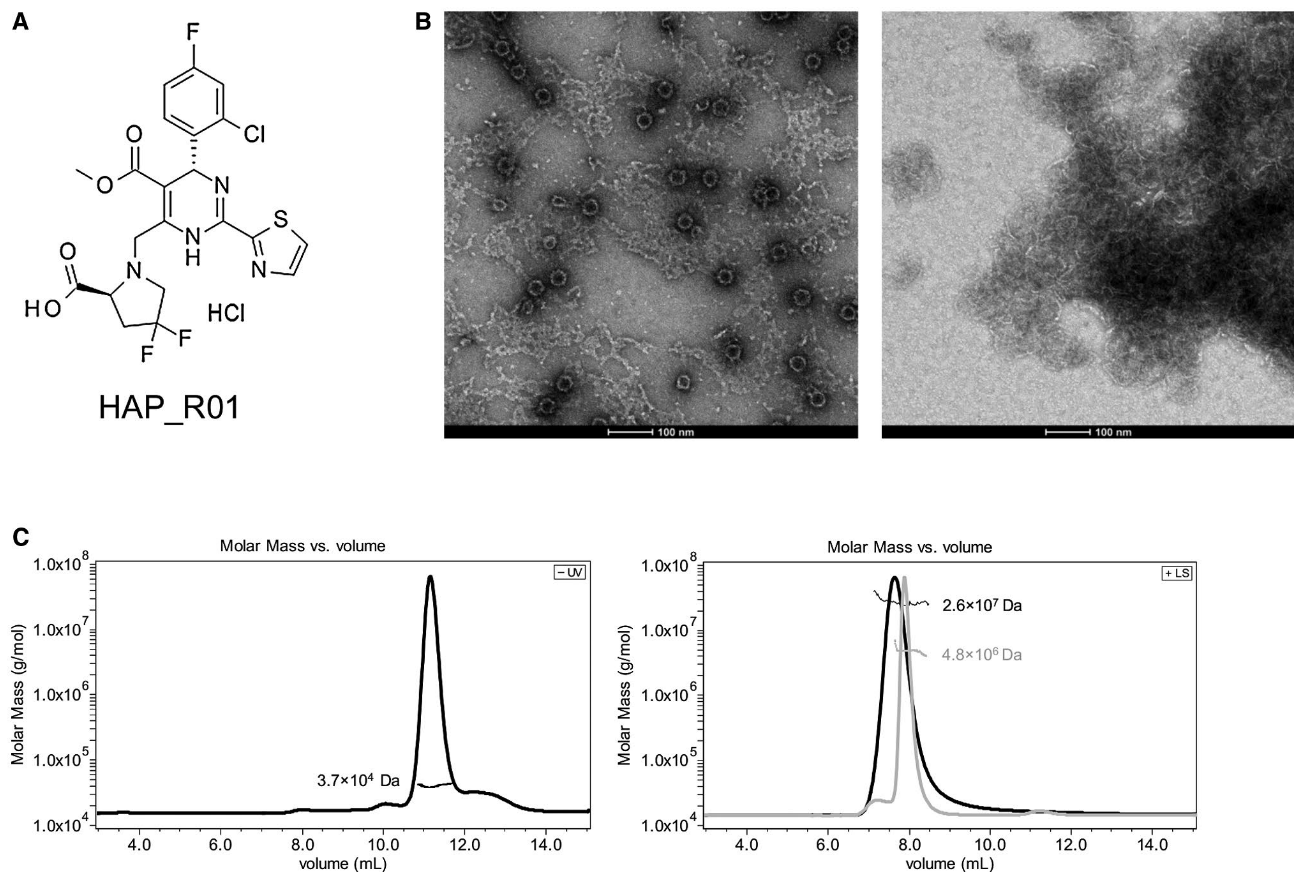

FIG. 1. Disruption of the HBeAg-formed capsid-like structure by HAP_R01. (A) Chemical structure of HAP_R01. (B) EM images of HBeAg-formed capsid. Left: 5 μM HBeAg protein dimer was incubated with 250 mM NaCl. Right: 5 μM HBeAg protein dimer was incubated with both 250 mM NaCl and 20 μM HAP_R01. (C) SEC-MALS analysis of the molar mass of HBeAg protein dimer and capsid assembly. Left: HBeAg protein dimer only. Right: HBeAg protein dimer incubated with 250 mM NaCl (gray line). HBeAg protein dimer incubated with 250 mM NaCl plus 20 μM HAP_R01 (black line).

effective concentration [EC_{50}] = 1.12 μM), whereas HBsAg levels were not affected, suggesting a specific inhibitory effect on HBeAg (Fig. 2B). In contrast, no significant HBeAg reduction was observed after AT-130 or ETV treatment (Supporting Fig. S1).

HBeAg shares 149 common residues with the HBV core protein; therefore, the HBeAg ELISA method may cross-react with both proteins. To further confirm this HBeAg reduction effect, after denaturation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation, supernatant HBeAg was detected by western blot with a polyclonal anti-core antibody that recognizes denatured HBeAg, the core protein and the native capsid. Consistent with the ELISA results, HAP_R01 dramatically decreased both the core protein and HBeAg levels, as indicated by the reduced intensity of the core protein (p21, ~21 kDa) and HBeAg (p17, ~18 kDa) bands (Fig. 2B). In addition, as expected, reductions in intracellular HBV capsid, encapsidated HBV DNA, and core protein levels were observed in cell lysates. In contrast, there was no reduction in intracellular HBV precore protein/pgRNA levels after HAP_R01 treatment (Fig. 2C). Similar reductions in HBeAg were also observed in HepG2 cells transiently transfected with HBV circle DNA, as well as in HBV-infected primary human hepatocytes (Supporting Figs. S2 and S3). Collectively, these results suggest that in addition to its expected effects on HBV DNA and the capsid, HAP_R01 can also reduce HBeAg levels through a posttranscriptional mechanism.

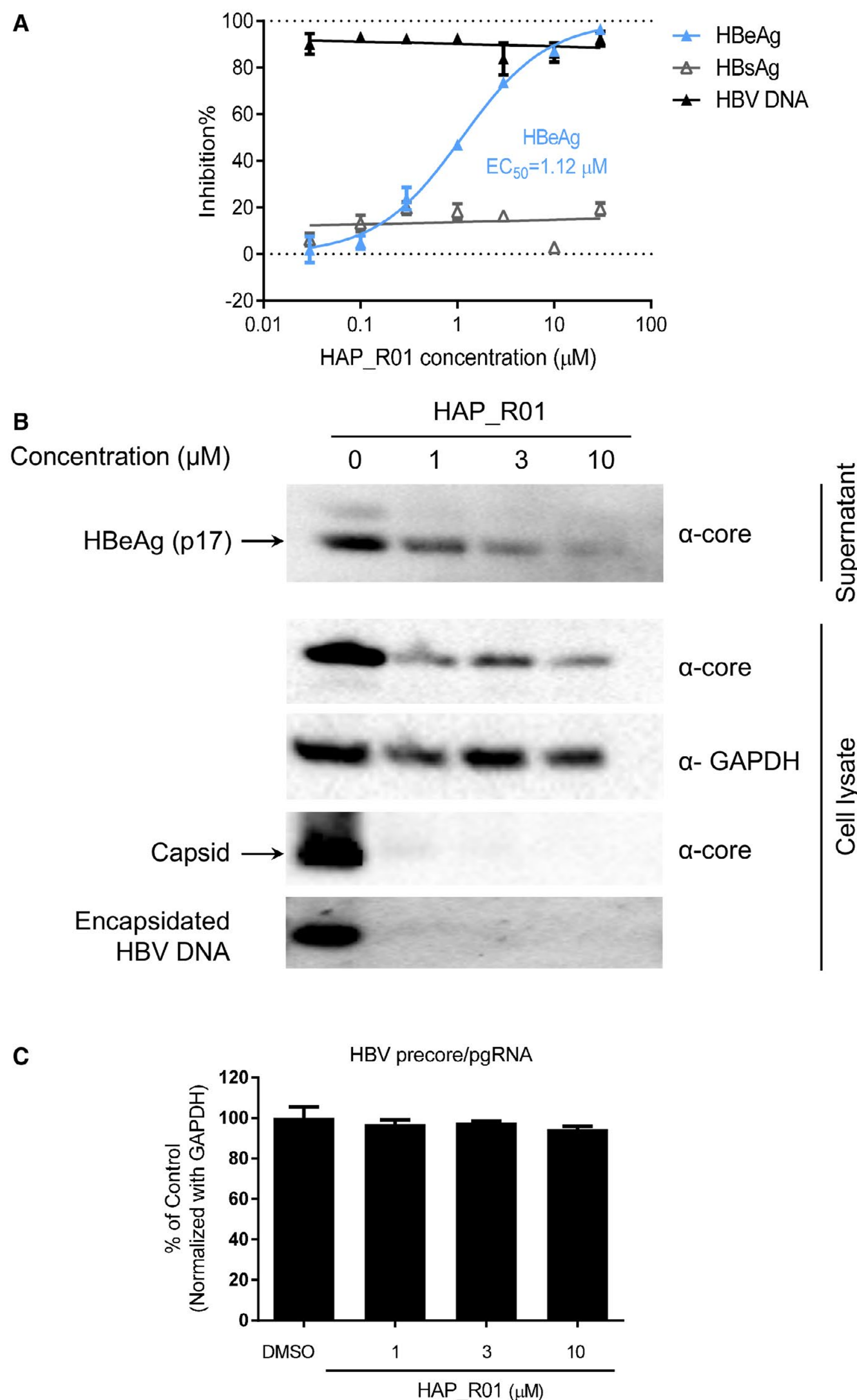

FIG. 2. HAP_R01 inhibits HBV DNA replication and reduces HBeAg levels. (A) HepG2.2.15 cells treated with the indicated concentrations of HAP_R01 for 5 days. Supernatants were collected to measure HBV DNA, HBeAg, and HBsAg. The percentage inhibition compared with DMSO treatment is plotted. Data are the means ± SD of triplicate samples from one experiment and are representative of at least three independent experiments. (B) HepG2.2.15 cells were treated as above. HBeAg levels in supernatant were detected by western blot with a polyclonal anti-core antibody. Cell lysates were subjected to western blot analysis of HBV capsid, HBc protein, and GAPDH with specific antibodies and to Southern blot analysis of encapsidated HBV DNA. (C) HepG2.2.15 cells were treated as above, and cell lysates were subjected to HBV precore protein/pgRNA detection using the QuantiGene assay. Data are the means ± SD of triplicate samples from one experiment and are representative of at least three independent experiments. Abbreviation: GADPH, glyceraldehyde 3-phosphate dehydrogenase.

HAP_R01 DOES NOT AFFECT ESTABLISHED COVALENTLY CLOSED CIRCULAR DNA LEVELS

Previous studies suggest that the HBV core protein may bind and modulate covalently closed circular DNA (cccDNA) functions.[34-36] Therefore, the depletion of core protein by HAP_R01 may also indirectly affect HBeAg levels through cccDNA. To test this hypothesis, the HepDES19 cell line was used, in which HBeAg production is dependent on cccDNA on the induction of HBV replication.[30] The cells were first induced for 9 days and then treated with HAP_R01 for 5 days. Consistent with the effect observed in HepG2.2.15 cells, HAP_R01 treatment resulted in a clear dose-dependent decrease in secreted HBeAg levels, as confirmed by both ELISA and western blot (Fig. 3A,B and Supporting Fig. S4A). Intracellular HBV cccDNA levels were not significantly affected (Fig. 3C and Supporting Fig. S4B). These results suggest that HAP_R01 does not affect established cccDNA levels in this system, further indicating that a direct mechanism at the protein level may be involved in the HAP_R01-induced HBeAg inhibition process.

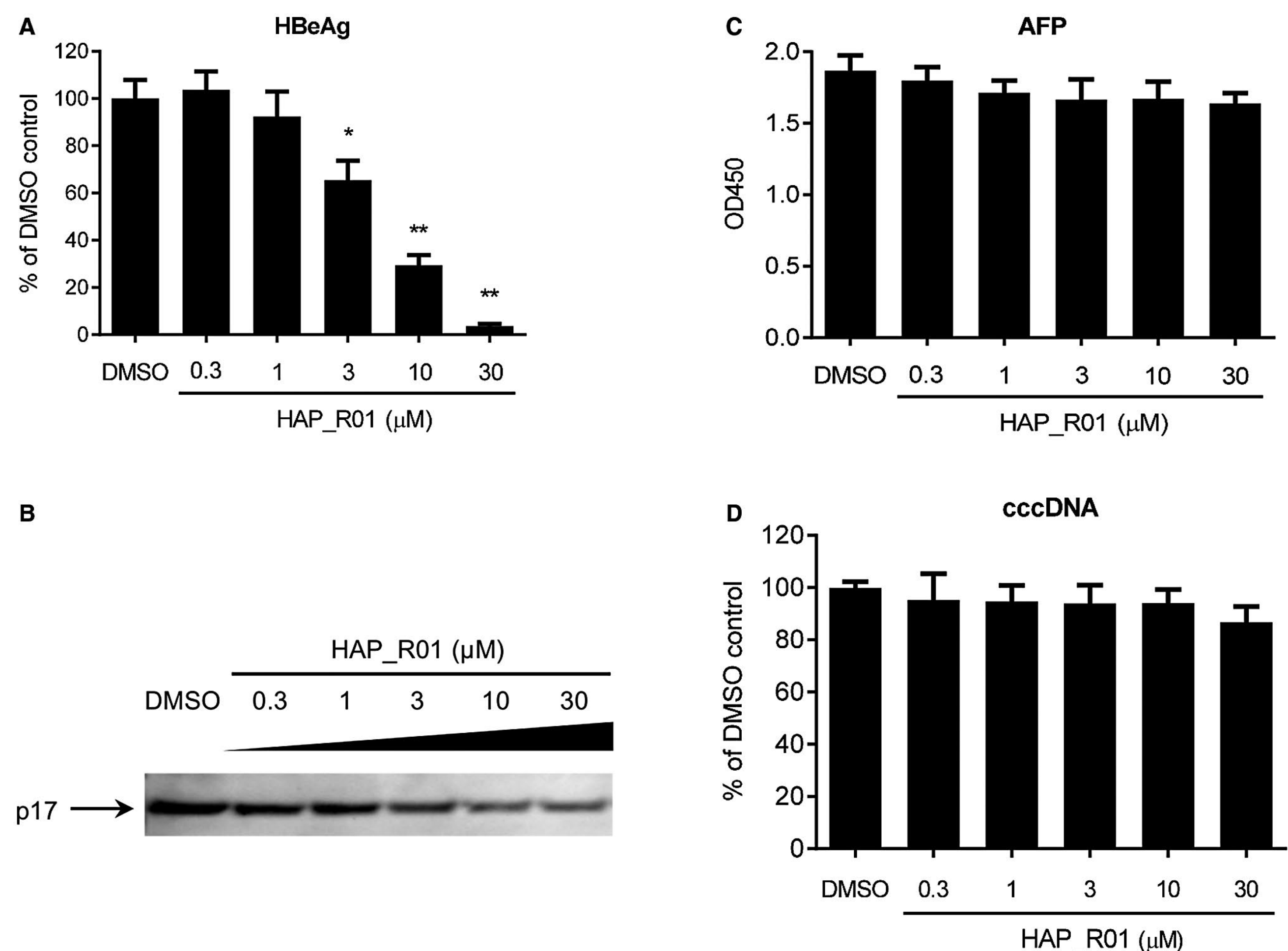

FIG. 3. HAP_R01 does not affect established cccDNA levels. HepDES19 cells treated with the indicated concentrations of HAP_R01 for 5 days. Supernatant was collected for (A) HBeAg ELISA detection (data are plotted as a percentage of the DMSO control), (B) western blot using an anti-core pAb (Dako, Carpinteria, CA), and (C) cell viability assay using AFP ELISA. (D) HBV cccDNA was isolated from cell lysate and quantified by qRT-PCR (data are plotted as a percentage of the DMSO control). (A,C,D) Data are the means ± SD of triplicate samples from one experiment and are representative of at least three independent experiments. *P* values were determined using Student *t* test, $^{**}P < 0.001$; $^{*}P < 0.01$. Abbreviations: AFP, alpha fetoprotein; pAb, polyclonal antibody; qRT-PCR, quantitative real-time polymerase chain reaction.

HAP_R01 REDUCES PRECORE PROTEIN CAPSID FORMATION AND INDUCES AGGREGATION IN THE NUCLEUS

Based on the above findings, we speculated that HAP_R01 may disrupt intracellular precore protein capsid formation and therefore lead to HBeAg reduction. To test this hypothesis, HepBHAe82 cells were used, which carry an in-frame hemagglutinin (HA) epitope tag in the precore open reading frame (ORF).[29] Similar to wild-type HBeAg, purified HA-tagged HBeAg was capable of forming capsid-like structures, which could be disrupted by HAP_R01 (Supporting Fig S5). In HepBHAe82 cells, on HAP_R01 treatment, substantial reductions of intracellular and secreted capsids, as well as HBeAg and core proteins, were observed (Fig. 4A and Supporting Fig. S6). In contrast, neither ETV nor AT-130 treatment resulted in such effects. Interestingly, we clearly detected overlapping HA and core protein signals in the capsid bands from both cell lysate and supernatant samples, suggesting that the precore protein can form capsids either by itself or in complex with the core protein. In addition, we also observed increased levels of HBV DNA after immunoprecipitation with anti-HA antibody from the cell culture supernatant, indicating that the precore protein is present in HBV DNA–containing nucleocapsids (Supporting Fig. S7).

Interestingly, on HAP_R01 treatment, we detected the formation of precore protein aggregates in the nucleus by immunofluorescence (IF) staining with anti-HA antibody, which colocalized with core protein aggregates (Fig. 4B). We further separated nuclear and cytoplasmic fractions and observed that, in contrast to the effect in the cytoplasmic fraction, HAP_R01 induced a substantial accumulation of HA-tagged precore and core proteins in the nuclear fraction, which is consistent with the IF staining observations (Fig. 4C and Supporting Fig. S8). The nuclear and cytoplasmic extractions were further analyzed with density gradient centrifugation. In DMSO-treated cells, the majority of intracellular capsids, as well as core and HA-tagged precore proteins, were detected in the seventh and eighth fractions in both nuclear and cytoplasmic extractions, which correlates well with the high levels of HBV DNA (Fig. 5 and Supporting Fig. S9). HAP_R01 treatment substantially reduced the capsid, core, and HA-tagged precore protein levels in the cytoplasmic fractions. However, in the nuclear fractions, the core and HA-tagged precore protein signals appeared to be shifted and distributed across the top fractions without a detectable capsid signal, indicating that the formed aggregates were of reduced and nonuniform density and therefore sedimented more slowly than normal capsids in the density gradient (Fig. 5).

Together, these results suggest that HAP_R01 can reduce the levels of all capsids formed by core and precore proteins and can induce their depletion in the cytoplasm and aggregation in the nucleus. The greatly reduced levels of cytoplasmic precore protein are likely to be the key factor leading to the reduction of secreted HBeAg in the cell culture supernatant.

PRECORE/CORE MUTANT ABOLISHES HAP_R01-INDUCED HBeAg REDUCTION

Our previous structural analysis showed that HAP_R01 is in close contact with core T33, and core T33N mutation leads to HAP_R01 resistance, as determined by an over 67-fold HBV DNA EC_{50} increase.[33] We explored whether this mutation would affect HAP_R01-mediated HBeAg reduction. For this aim, a plasmid carrying this mutation, pBR322-HBV1.3 RM (HAP_R01 resistant mutation, core T33N), was constructed and transfected into HepG2 cells. We observed that this mutation led to the abolishment of HAP_R01-induced core protein and HBeAg reduction, suggesting that the core protein and HBeAg reductions are mediated through the same mechanism (Fig. 6A).

Next, we investigated whether HAP_R01-mediated HBeAg reduction is dependent on the expression of the core protein. For this purpose, the precore precursor gene, p25, was cloned into a pcDNA3.1 vector with a Kozak sequence added to facilitate its expression. To avoid any residual core protein expression, an additional p25 construct with a mutated core start codon, p25-core(-), was also generated. On transfection into HepG2 cells, HBeAg, but not precore or core protein, was detected in the supernatant (Fig. 6B). HAP_R01 treatment resulted in a substantial reduction in HBeAg levels in cells expressing the wild type and the p25-core(-) mutant, suggesting that HAP_R01-mediated HBeAg reduction is independent of core protein expression. Furthermore, HAP_R01-induced HBeAg reduction was not observed in cells

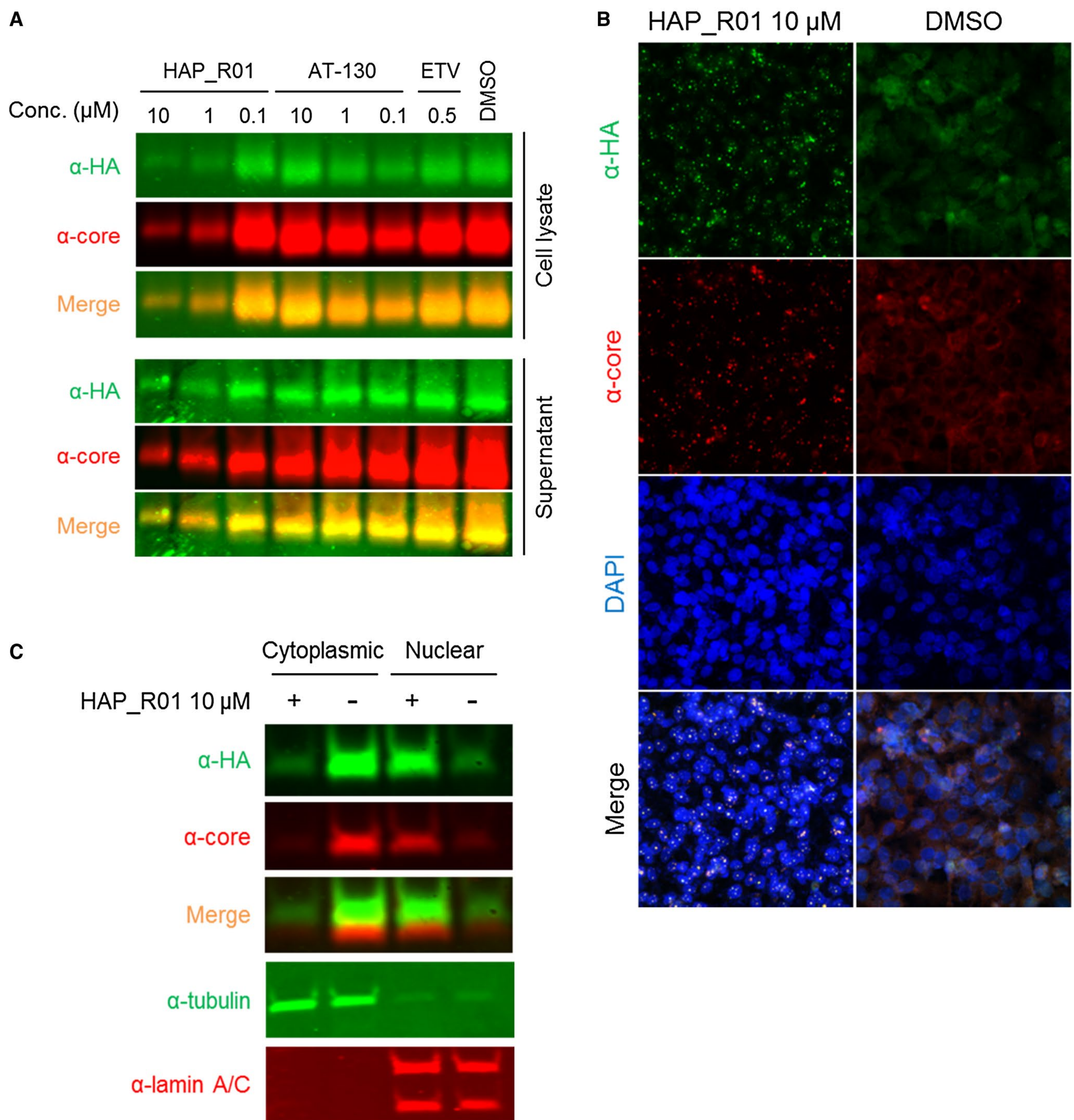

FIG. 4. HAP_R01 reduces precore protein capsid formation and induces aggregation in the nucleus. (A) HepBHAe82 cells were treated with the indicated compounds for 5 days. Supernatant and cell lysate were collected for capsid detection by western blot after native agarose electrophoresis. (B,C) HepBHAe82 cells were treated with 10 μM HAP_R01 or 1% DMSO for 5 days. (B) IF staining and (C) western blot of subcellular fractionations were performed with the indicated antibodies.

expressing the p25-RM mutant (Fig. 6B). In addition, the resistant phenotype was also confirmed with purified HBeAg carrying this mutation. On HAP_R01 treatment, HBeAg-RM formed capsid-like structures that could still be observed under EM (Supporting Fig. S5). Interestingly, in contrast to p21, we could not detect capsid in the supernatant on a native capsid gel when p25 was expressed alone, suggesting that p25

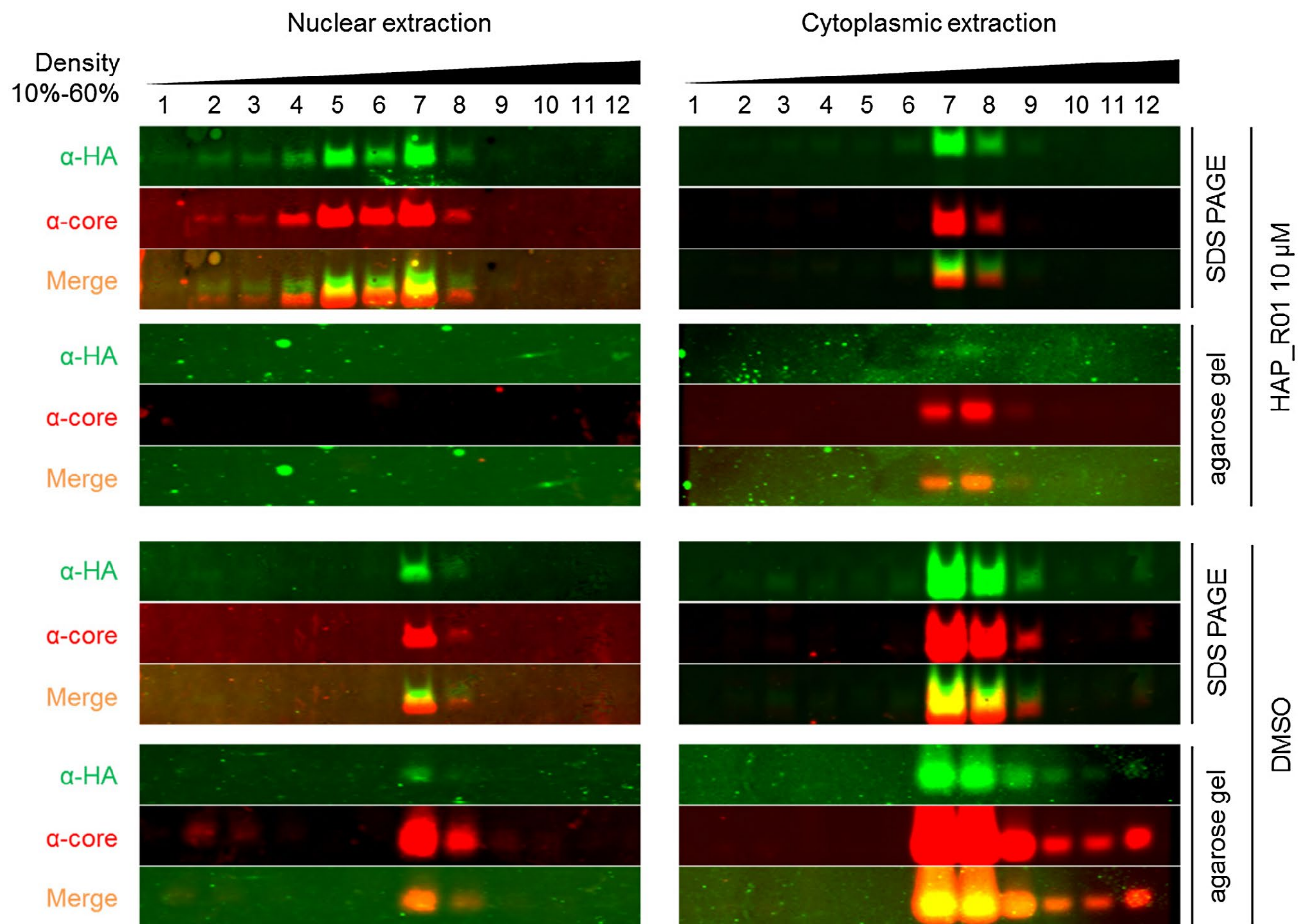

FIG. 5. Characterization of HAP_R01-reduced core and precore protein aggregates. HepBHAe82 cells were treated with 10 μM HAP_R01 or 1% DMSO for 5 days. Nuclear (right) and cytoplasmic (left) extractions were prepared and loaded onto 20%-60%–density gradients for centrifugation. Aliquots of fractions were subjected to western blot analysis for HBV capsid, core, and HA-tagged precore proteins with the indicated antibodies.

is predominantly processed into HBeAg (p17) and secreted, rather than being assembled into the capsid (Fig. 6B). Nevertheless, HAP_R01 treatment still substantially reduced HBeAg levels in cells expressing p25 without p21 coexpression. Together, these results indicate that HAP_R01 treatment may directly trigger the misassembly of precore protein, presumably p22, and lead to its aggregation and depletion, thereby reducing subsequent HBeAg processing and secretion.

HAP_R01 REDUCES HBeAg LEVELS *IN VIVO*

To determine whether HAP_R01 can reduce HBeAg levels *in vivo*, pHBV1.3-B6.2 plasmid, which contains a 1.3-fold overlength of a clinically isolated genotype B HBV genome from Taiwan, was used.[37] The plasmid DNA was hydrodynamically injected into FVB/N mice, and the injected mice exhibited high and prolonged serum levels of HBeAg (Supporting Fig. S10). The mice were given daily oral treatment with HAP_R01, ETV, or vehicle for 28 days and were then followed for an additional 9 days. Compared with the ETV- and vehicle-treated groups, the HAP_R01 treatment group exhibited significantly reduced serum HBeAg levels, while no significant weight loss or adverse effects were observed (Fig. 7). Furthermore, the HBeAg reduction effect was durable, without significant rebound during the 9-day follow-up

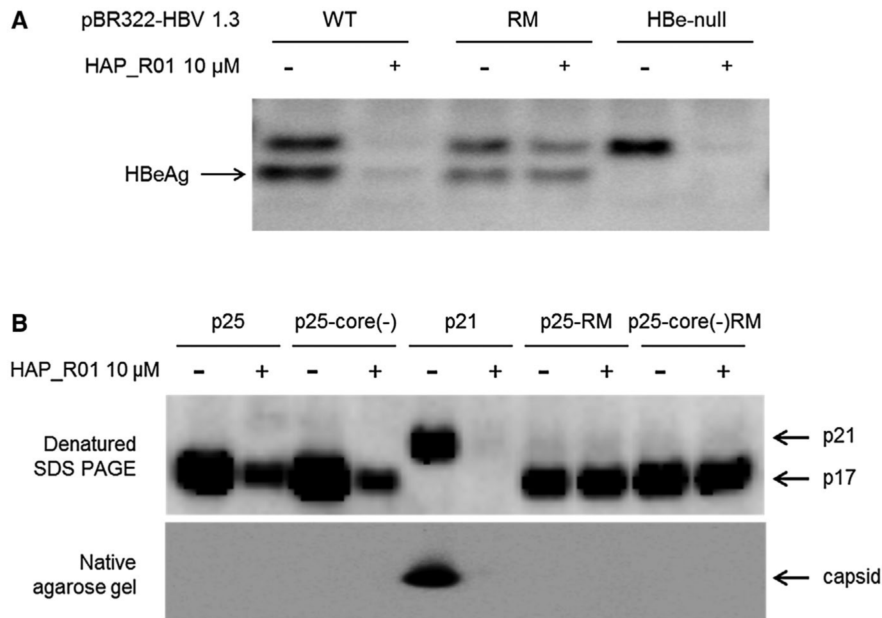

FIG. 6. HBeAg/core mutant abolishes HAP_R01-induced HBeAg reduction. HepG2 cells were transfected for 24 hours with the indicated DNA constructs, which contained (A) a 1.3-unit length of HBV genome in a pBR322 vector or (B) a p25/p21 ORF in a pcDNA3.1 vector, and were treated with 10 μM HAP_R01 or 1% DMSO in 2% FBS media for another 6 days. Supernatant was collected for HBeAg western blot analysis using anti-core pAb (Dako). Abbreviation: pAb, polyclonal antibody.

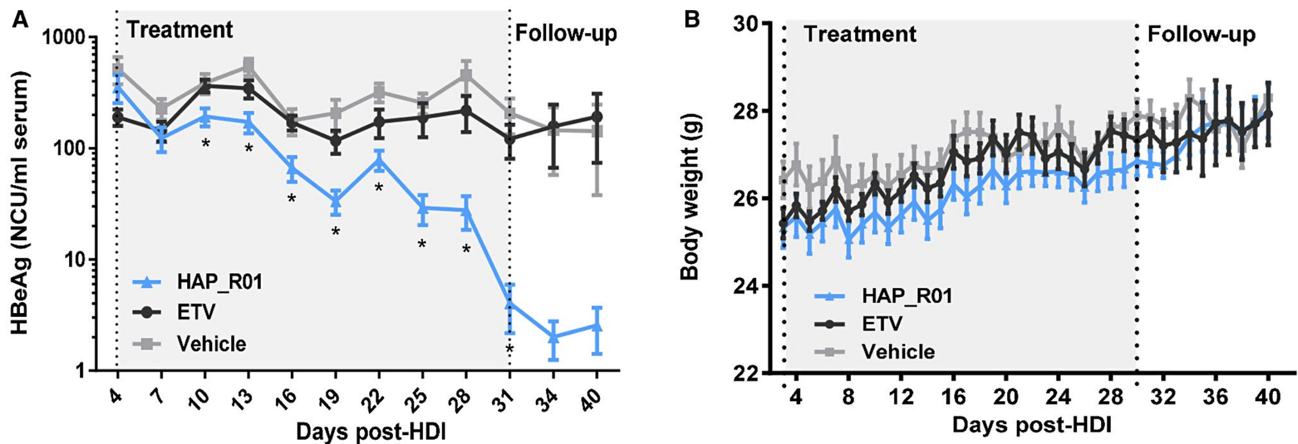

FIG. 7. HAP_R01 reduces HBeAg levels *in vivo.* On day 0, 10 μg of pHBV1.3-B6.2 plasmid was hydrodynamically injected into the tail vein of FVB/N mice. The mice were randomized into three groups based on body weight. On days 4 through 31, the mice were treated with a daily oral dosing of 30 mg/kg HAP_R01 (n = 10), 0.1 mg/kg ETV (n = 9), or vehicle (n = 10). From days 32 to 40, the treatments were stopped, and five mice from the HAP_R01 group, four mice from the ETV group, and five mice from the vehicle group were randomly selected for a 9-day follow-up. (A) Serum samples were collected for HBeAg detection at the indicated time points, and (B) body weight was monitored during the whole assay. Each data point represents the mean ± SEM. *P* values were determined using Student *t* test, $^{*}P < 0.05$.

period. These results are consistent with our *in vitro* observations and further support the development of HAP_R01 as an effective therapeutic option for CHB treatment.

Discussion

HBeAg and its precursor proteins are key viral proteins that regulate HBV replication, persistence,

and host immune responses and are essential for the pathogenesis of CHB.[10] In the present study, we observed that, aside from inhibiting HBV DNA replication, HAP_R01, but not ETV or AT-130, can also directly reduce HBeAg levels in both cell culture and mouse models. Our current results suggest that the precore protein participates in two closely linked pathways: HBeAg secretion and capsid assembly. HAP_R01 promotes the misassembly of precore and core proteins and leads to the reduction of precore protein entering into the HBeAg secretion pathway. Although the misassembled protein complexes can be efficiently removed in the cytoplasm, they form nonuniform aggregates and accumulate stably in the nucleus (Fig. 8).

Compared with its strong inhibitory activity on HBV DNA (EC_{50} <30 nM in HepG2.2.15 cells), the potency of HAP_R01 for HBeAg reduction is much weaker (EC_{50} = 1.12 μM in HepG2.2.15 cells). In this regard, it has been reported that HAP compounds enhance the rate and extent of capsid assembly at low concentrations. At high concentrations, HAP compounds can further induce the assembly of free core protein and the dissociation of already assembled capsids by scavenging core protein and redirecting their assembly into noncapsid polymers.[38,39] This phenomenon suggests that although the DNA inhibitory effect is primarily driven by the impact of HAPs on the thermodynamics and kinetics of the capsid assembly process, the HAP_R01-mediated HBeAg reduction is driven by the depletion of free precore protein and the formation of protein aggregates at high compound concentrations.

HAP_R01's HBeAg inhibition potency, as determined by HBeAg ELISA, varied among different models. The difference is likely caused by the different relative abundance of intracellular precore and core proteins in these systems. It is likely that in the

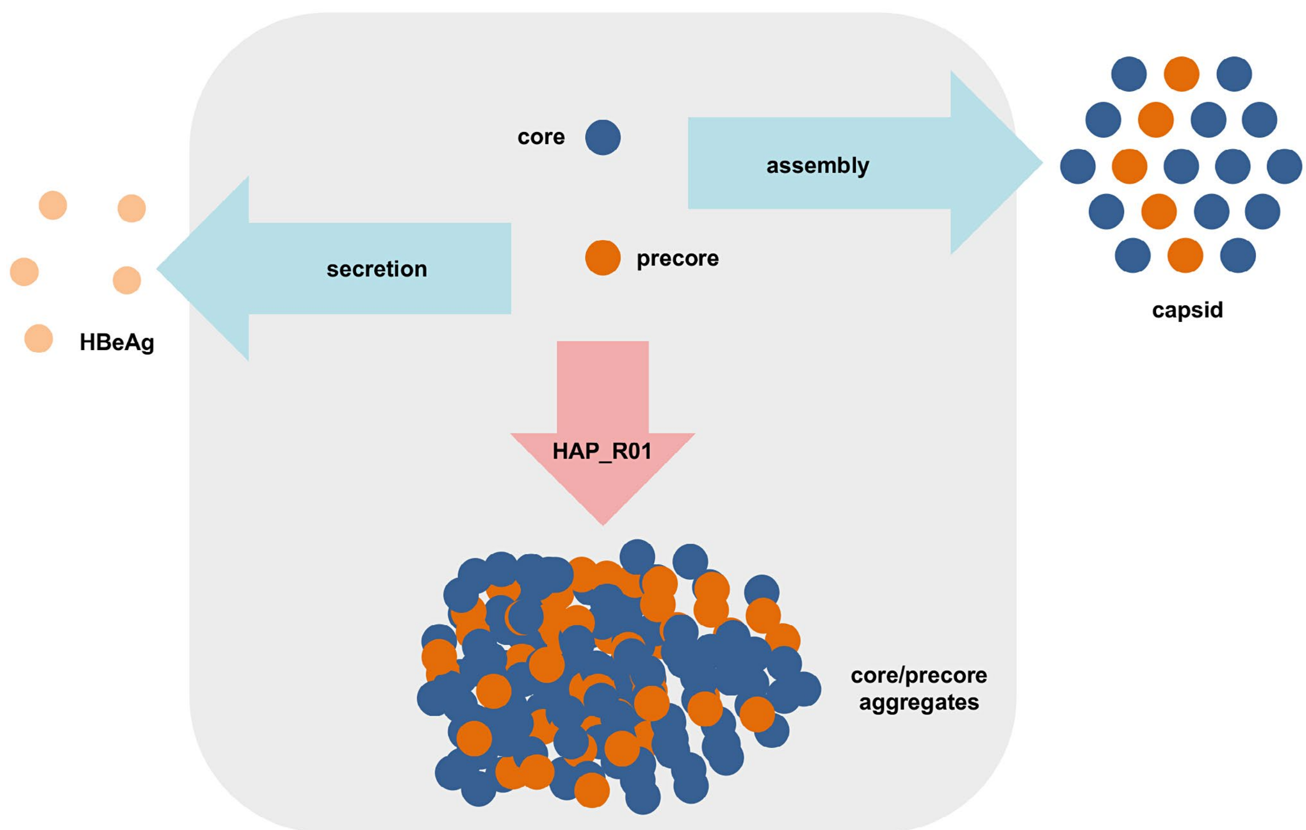

FIG. 8. Working model. In HBV-infected cells, newly generated precore protein participates in two distinct but closely linked pathways: HBeAg secretion and capsid assembly. HAP_R01 promotes the misassembly of precore and core proteins, and leads to the depletion of free precore protein, a reduction of HBeAg secretion, and the formation of protein aggregates in the nucleus.

presence of high amounts of intracellular core protein, precore proteins may be more susceptible to HAP_R01-induced aggregation, leading to increased HBeAg inhibition potency. In addition, the HBeAg ELISA kit cross-reacts with both HBeAg and naked capsid (data not shown), which may further contribute to this effect when capsids significantly contribute to the signals detected by HBeAg ELISA. Nevertheless, on HAP_R01 treatment, significant HBeAg reductions in cell culture supernatant have been further confirmed with the western blot method.

Under reducing conditions, capsid-like structures were detected by EM and SEC-MALS, as reported[24,27] (Fig. 1). In addition, it has been reported that the expression of p22 alone leads to the formation of nucleocapsids in COS and human embryonic kidney 293 cells.[25,40] However, using a more relevant system, we did not observe a detectable capsid signal when p25, the precursor of p22, was expressed alone in HepG2 cells in either cell culture supernatant (Fig. 6B) or cell lysate (data not shown), suggesting that in the absence of core coexpression, p25 preferentially goes into the secretion pathway and transforms into HBeAg.

A previous study suggested that p22 and p21 physically interact and form hybrid nucleocapsid structures devoid of pgRNA.[25] In the HepBHAe82 cell line, when the core is coexpressed, we observed very similar distribution patterns of HA-tagged p22 and core signals in the capsid bands on the native agarose gel, which supports the previous report. However, after pull-down with anti-HA antibody, a significant enrichment of HBV DNA was detected. Although the differences between pure capsid (formed by p21) and hybrid capsid (formed by p21 and p22) are still unclear, our results suggest that p22-containing capsids are still capable of pgRNA encapsidation and DNA synthesis. It is possible that the ratio between p21 and p22 in hybrid capsids may be important in regulating pgRNA encapsidation.

Although the overall levels of intracellular core and precore proteins were reduced on HAP_R01 treatment, their levels were substantially increased in the nucleus, presumably due to the aggregation of misassembled proteins. HAP compounds have been shown to deplete core protein through the proteasome pathway.[22] However, it is not clear why the degradation pathway is less effective in the nucleus. It is also not clear how these aggregates contribute to the sustained HBeAg reduction observed *in vivo*. Further investigations are needed in these areas.

Previous studies have suggested that HBeAg may modulate host innate and adaptive immune systems. HBeAg suppresses innate signal transductions through interactions with major vault protein and toll/interleukin-1 receptor domain–containing adaptor proteins, such as toll-like receptor 2, MyD88 adapter-like, and TRIF-related adaptor molecule.[14,41,42] HBeAg can also suppress the respiratory burst and the mobility of human monocytes and neutrophils.[43] In addition, HBeAg may help deplete HBeAg-specific and hepatitis B core (HBc) antigen–specific T helper type 1 cells and block the anti-HBc antibody response.[11,44,45] Along these lines, it has been demonstrated that HBeAg exerts a negative effect on HBV immune-based therapies, and higher pretreatment HBeAg levels have been closely associated with poor treatment response rates to PEG-IFNα.[46] Therefore, it is possible that HAP_R01-mediated HBeAg reduction may remove the suppression and enhance host innate and adaptive anti-HBV responses, while also increasing the response rate when coadministered with immune-based therapies.

It has to be noted that HBsAg loss, as the optimal treatment endpoint, remains the goal of new therapies. In addition, many patients are HBeAg negative. Thus, HBeAg-targeted agents may have limited therapeutic potential and therefore should not be the major focus and goal for the development of new therapies.

In conclusion, our current results suggest that HAP_R01 reduces HBeAg levels by inducing misassembly of its precursor. As similar HAP compounds, such as GLS4 (Sunshine Lake Pharma, Guangdong, China) and QL-0A6A (Qilu pharmaceutical, Jinan, China), are currently under clinical development, evaluations of whether such an HBeAg-reducing effect could be achieved in patients with CHB are warranted.

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