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SRPKIN-1 as an inhibitor against hepatitis B virus blocking the viral particle formation and the early step of the viral infection

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ABSTRACT

New antiviral agents are needed for the treatment of hepatitis B virus (HBV) infection because currently available drugs do not completely eradicate chronic HBV in patients. Phosphorylation dynamics of the HBV core protein (HBc) regulate several processes in the HBV life cycle, including nucleocapsid formation, cell trafficking, and virus uncoating after entry. In this study, the SRPK inhibitors SPHINX31, SRPIN340, and SRPKIN-1 showed concentration-dependent anti-HBV activity. Detailed analysis of the effects of SRPKIN-1, which exhibited the strongest inhibitory activity, on the HBV replication process showed that it inhibits the formation of infectious particles by inhibiting pregenomic RNA packaging into capsids and nucleocapsid envelopment. Mass spectrometry analysis combined with cell-free translation system experiments revealed that hyperphosphorylation of the C-terminal domain of HBc is inhibited by SRPKIN-1. Further, SRPKIN-1 exhibited concentration-dependent inhibition of HBV infection not only in HepG2-hNTCP-C4 cells but also in fresh human hepatocytes (PXB cells) and in the single-round infection system. Treatment with SRPKIN-1 at the time of infection reduced the nuclease sensitivity of HBV DNA in the nuclear fraction. These results suggest that SRPKIN-1 has the potential to not only inhibit the HBV particle formation process but also impair the early stages of viral infection.

1. Introduction

Hepatitis B virus (HBV) is a major causative agent of liver diseases worldwide. Long-term HBV infection increases the risk of progression from hepatic cirrhosis to hepatocellular carcinoma (HCC) (reviewed in Jeng et al., 2023). Antiviral therapy using nucleotide/nucleoside analogues and recombinant interferon (rIFN)-α has significantly improved the prognosis of HBV infection. However, HBV is rarely eliminated completely, and suppression of viral replication below the limit of detection does not eliminate the risk of developing HCC. New therapeutic strategies using HBV inhibitors with mechanisms of action that differ from those of existing drugs are thus needed (reviewed in Dusheiko et al., 2023). Agents acting at different stages of the viral life cycle may exhibit complementary and synergistic antiviral activity.

HBV is a small DNA virus belonging to the Hepadnaviridae family. Infectious HBV particles are spherical structures with a diameter of 42 nm and composed of an icosahedral nucleocapsid and an envelope that consists of three HBV surface proteins and host cell–derived lipids. The nucleocapsid consists of a complex of the HBV core (HBc) protein, the virus-encoded polymerase, and the viral DNA genome. During HBV infection, the viral envelope first fuses with the host cell membrane, and then the nucleocapsid is released into the host cytoplasm and transported to the nuclear membrane pores. Once the nucleocapsid is incorporated into the nucleus and disassembled, the viral genome is converted into a covalently closed circular (ccc)DNA molecule (reviewed in

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Tsukuda and Watashi, 2020). The HBc protein, which consists of 183–185 residues, is divided into two domains, with the N-terminal domain (NTD; residues 1–140) linked via a flexible linker to a basic C-terminal domain (CTD; residues 150–183) that interacts with nucleic acids and is known to self-assemble in the capsid (reviewed in de Rocquigny et al., 2020). The NTD of HBc, known as the assembly domain, is essential for capsid formation; the HBc CTD has been shown to undergo dynamic phosphorylation and dephosphorylation, regulating the electrical homeostasis of HBc, which in turn drives pregenomic (pg) RNA packaging, RT-directed synthesis of the viral DNA, and capsid stability.

In the CTD, S155, 162, and 170 (157, 164, and 172 in the 185–amino acid [aa] HBc variant) are highly conserved among HBV isolates and thought to be the major phosphorylation sites. In addition, seven phosphorylation sites at S/T positions have been identified in the CTD. More recently, two serine residues (S44 and S49) of the 11 S and 12 T residues of the NTD were also proposed as phosphorylation receptors (Luo et al., 2020). Several candidate kinases that phosphorylate HBc protein have been proposed, such as Polo-like kinase 1 (PLK1), cyclin-dependent kinase 2 (CDK2), protein kinase C alpha (PKCα), and serine-arginine protein kinase (SRPK) (Daub et al., 2002; Diab et al., 2017; Ludgate et al., 2012; Wittkop et al., 2010). For example, seven phosphorylation sites, all located in the CTD, were characterized using *Escherichia coli* co-transformed with two plasmids encoding HBc and SRPK1 (Heger-Stevic et al., 2018). SRPKs constitute a subfamily of serine-threonine kinases that specifically phosphorylate serine residues present in repetitive arginine-serine (RS) domains in SR proteins. SRPKs not only play a role in constitutive and alternative mRNA splicing but also affect other cellular activities, such as later steps in mRNA maturation, chromatin reorganization in somatic and sperm cells, cell cycle and p53 regulation, and metabolic signaling (Giannakouros et al., 2011).

Evidence conclusively demonstrates that phosphorylation and dephosphorylation of the HBc protein play important roles in many stages of the HBV life cycle. It is believed that phosphorylation and dephosphorylation reactions must be closely coordinated in space and time during virus replication. In the HBc dimer, the CTD is hyperphosphorylated, and its moderate dephosphorylation occurs simultaneously with pgRNA packaging (Zhao et al., 2018). Partial dephosphorylation might maintain a sufficient level of phosphorylated CTD of HBc for the initial steps of reverse transcription in the RNA-containing capsids. Furthermore, after virus entry into cells and de-envelopment during the infection process, phosphorylation of HBc in the viral capsid could lead to destabilization of the capsid and initiation of the uncoating process (Luo et al., 2020). It has also been shown that phosphorylation and dephosphorylation of HBc regulate the chaperone activity of the CTD (Chen et al., 2011).

Even though hyperphosphorylation may negatively regulate certain steps in the HBV life cycle, phosphorylation of HBc by serine-threonine kinase is undoubtedly an important post-translational modification. Therefore, it is reasonable to consider inhibitors of kinases that might be involved in HBc phosphorylation, such as the four kinase types mentioned above, as potential anti-HBV agents. It has been reported that several inhibitors of PLK1, CDK2, and PKCα also exhibit inhibitory activity against HBV replication or viral infection (Diab et al., 2017; Luo et al., 2020; Wittkop et al., 2010). However, the molecular mechanisms of their inhibitory activity have not been fully elucidated. In addition, no studies analyzing the anti-HBV activity of SRPK inhibitors have been reported to date.

2. Materials and methods

2.1. Chemicals

SRPIN340 was kindly gifted by KinoPharma, Inc. (Tokyo, Japan). SPHINX31 was purchased from Selleck Chemicals (Texas, USA). SRPKIN-1 was purchased from MedChemExpress (New Jersey, USA). The properties of SRPIN340, SPHINX31, and SRPKIN-1, such as target enzyme and inhibitory activity, are summarized in Supplementary Table S1.

2.2. Plasmid construction

To construct pcDNA3.1-HBV1.05-delBGHpA (abbreviated here as pHBV1.05), which drives the expression of an HBV pgRNA under control of a cytomegalovirus (CMV) promoter at the authentic site on the viral DNA, a 1.05-fold unit length HBV DNA fragment (genotype D: GenBank accession no. U95551.1) obtained by PCR from Hep2.2.15 cells was inserted downstream of the CMV promoter of pcDNA3.1, excluding the BGH poly(A) signal sequence beforehand (Blanchet and Sureau, 2006). To generate pSV-HBsAeZeo (abbreviated here as pHBsAe), which expresses the whole open reading frame of HBs proteins (genotype Ae) under the HBV promoter/enhancer, the 2.7-kb fragment obtained from pUC-HBV-Ae (provided by Dr. Mizokami; National Center for Global Health and Medicine, Ichikawa, Japan) (Sugauchi et al., 2004) was inserted into the *Bgl*II site of pSV40/Zeo2 (Thermo Fisher Scientific, Massachusetts, USA). pUC-HB-Ce (abbreviated here as pHBVCe1.24), which contains the 1.24-fold HBV genome derived from a consensus sequence of HBV genotype Ce, was constructed as described elsewhere (Sun et al., 2017). The pUC1.2xHBV/NL and pUC1.2xHBV-D plasmids were provided by Dr. Shimotohno (National Center for Global Health and Medicine, Ichikawa, Japan). Cell-free expression plasmids with an SP6 promoter were generated as follows. The entire coding sequence of HBV core protein obtained by PCR using pHBV1.05 as a template was inserted into pEU-E01 (CellFree Sciences, Matsuyama, Japan) to generate pEU-E01-HBc. Similarly, pEU-E01-FLAG-HBc was constructed by incorporating the cDNA of FLAG-inserted HBc, in which the aa 78–81 region of HBc was replaced with the FLAG sequence (DYKDDDDK) (Wang et al., 2015), into pEU-E01. The open reading frame sequence of SRPK1 (accession no. NM_003137.5) in the pEU-FLAG-GST vector was purchased from Kazusa DNA Research Institute (Kisarazu, Japan). The kinase-dead mutant of SRPK1 (K109A) was prepared using a PrimeSTAR mutagenesis basal kit (Takara Bio, Kusatsu, Japan).

2.3. Cell culture and transfection

Human hepatoma HepG2-hNTCP-C4 (Iwamoto et al., 2014) and Hep38.7-Tet cells (Ogura et al., 2014) were kindly provided by Dr. Koichi Watashi (National Institute of Infectious Diseases). HuH-7, HepG2-hNTCP-C4, and Hep38.7-Tet cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Linear polyethyleneimine, MW 25,000 (Thermo Fisher Scientific), was used for plasmid transfection into HuH-7 cells. PXB cells (PhenixBio, Higashi-Hiroshima, Japan), which are fresh human hepatocytes isolated from chimeric mice with humanized livers (PXB mice), were maintained in 2% DMSO-supplemented hepatocyte clonal growth medium, as described elsewhere (Ishida et al., 2015).

2.4. Viral stock and infection

The culture supernatants of HuH-7 cells transfected with pHBV1.05 or of Hep38.7-Tet cells were collected and precleared for preparation of the inoculum. HepG2-hNTCP-C4 cells or PXB cells were pretreated overnight with 2% DMSO-containing medium and then inoculated with

HBV samples containing 2% DMSO and 5% PEG8000. At 36 h after infection, the culture medium was changed to fresh 2% DMSO/5% PEG8000–containing medium for further culture.

HBV particles consisting of a chimeric HBV virus encoding NanoLuc (HBV/NL) were prepared as described previously (Nishitsuji et al., 2015). After 8 days, the NanoLuc activity of infected cells was measured using a Nano-Glo Luciferase Assay kit (Promega, Wisconsin, USA). The total protein concentration in each cell lysate was measured and used to normalize the luciferase activity.

2.5. RNA extraction and RT-q (quantitative)–PCR

Total RNA was extracted from transfected cells using TRI reagent (Molecular Research Center, Ohio, USA) according to the manufacturer's instructions. Quantification of HBV RNAs and host-derived mRNAs was performed basically as described previously (Ito et al., 2019). Primer sets used for determination of pgRNA, its unspliced form, its major spliced forms (such as Sp1 and Sp9), and total HBV RNA, respectively, were pgRNAF-pgRNAR, unSpF-unSpR, SpF-SpR, and totalF-totalR, as indicated in Supplementary Fig. S1 and Supplementary Table S2.

2.6. Cell viability assay

Cells seeded into wells of a 96-well plate were cultured for 4 days in the presence of various concentrations of test drugs. WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4disulfophenyl]-2H-tetrazolium, monosodium salt) reagent (Cell Counting kit-8, Dojindo Laboratories, Kumamoto, Japan) was then added to each well and incubated for 4 h. The absorbance was measured on a spectrophotometer at 450 nm.

2.7. Subcellular fractionation

Harvested cells were washed with phosphate-buffered saline and resuspended with hypotonic lysis buffer (0.5% NP-40, 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$) by gently tapping the tube for 5 min. The cytoplasm and nuclei were separated by centrifugation at 500 × *g* for 5 min. After removal of nuclear remnants by centrifugation, the supernatants were used as the cytoplasmic fraction. The pellets were washed again using hypotonic lysis buffer and prepared as the nuclear fraction.

2.8. Immunoblotting

Immunoblotting was performed as previously described, with slight modification (Li et al., 2016). Briefly, cell lysates were separated by SDS-PAGE or native agarose gel electrophoresis (NAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated with an antibody against HBc (Li et al., 2016), HBs (Institute of Immunology, Tokyo, Japan), Flag (Sigma-Aldrich, Missouri, USA), or GAPDH (Santa Cruz Biotechnology, Texas, USA) for 1 h. After washing, the membranes were incubated with an HRP-conjugated secondary antibody (Cell Signaling Technology, Massachusetts, USA) for 30 min. Antigen-antibody complexes were detected using a ChemiDoc™ Imaging System (BIO-RAD Laboratories, Tokyo, Japan).

2.9. Quantification of HBV DNA

Quantification of particle-associated HBV DNA was carried out as previously described (Sun et al., 2017), with slight modifications. To completely remove free nucleic acids, pellet samples prepared from culture supernatants of transfected cells were treated with 5 U/μl of DNase I and 20 U/μl of micrococcal nuclease (MNase) for 16 h. To quantify nuclease-resistant HBV DNA in the nucleus, nuclear fractions prepared according to the above method (section 2.7) were suspended in isotonic lysis buffer (50 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% NP40) and treated with 0.3 U/μl of MNase for 16 h. The reaction was then stopped by adding EDTA. The amount of undigested HBV DNA was measured by qPCR. For quantification of nuclease-resistant HBV DNA in the cytoplasm, cytoplasmic fractions were treated with 20 U/μl of MNase with 2.5 mM Ca^{2+} for 1 h. After proteinase K treatment, nuclease-resistant DNA was extracted by phenol/chloroform extraction and subjected to HBV qPCR. To quantify HBV cccDNA followed by the nuclease sensitivity assay (Fig. 4F), DNAs extracted from nuclear fractions with/without MNase treatment were digested using Plasmid-Safe ATP-Dependent DNase (Lucigen, Wisconsin, USA) at 37 °C for 1 h, followed by heating at 70 °C for 1 h for DNase inactivation. The digested products were subjected to qPCR using specific primers (cccDNAF and cccDNAR, Supplementary Fig. S1A and Supplementary Table S2) across two gaps of HBV rcDNA (Liu et al., 2018).

2.10. Capsid-associated HBV RNA extracted from native agarose gels

NAGE was performed using 0.8% agarose gels in 1 × TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) as previously described (Ning et al., 2011), with some modifications. After electrophoresis, gel slices were excised according to the position of the HBc band detected by immunoblotting, transferred to 2-ml clean tubes, and lysed with buffer containing guanidinium thiocyanate. After proteinase K treatment, RNA was extracted using TRI reagent.

2.11. Density fractionation of culture supernatants containing HBV particles

Precleared culture supernatants of cells transfected with the HBV genome were loaded onto a heparin column (Cytiva, Tokyo, Japan) using a peristaltic pump. After washing the column with low-salt buffer (20 mM Tris-HCl [pH 7.5], 140 mM NaCl), the viral particles were eluted with high-salt buffer (20 mM Tris-HCl [pH 7.5], 1 M NaCl). The eluent was concentrated and layered on top of a 12-ml discontinuous 30%–55% sucrose density gradient in Tris buffer. The gradient was centrifuged for 40 h at 34,000 × *g* at 4 °C and fractionated into 0.8-ml fractions from top to bottom. The density of each fraction was calculated according to weight and volume.

2.12. Phos-tag SDS-PAGE

Cell lysates or HBV samples prepared from culture supernatants were mixed with 3 × Laemmli buffer and heated for 3 min at 95 °C before loading onto polyacrylamide-bound Mn^{2+}-Phos-tag gels containing 25 mM PhosTag, 50 mM $MnCl_2$, and 15% acrylamide (Dojindo, Kumamoto, Japan). After SDS-PAGE, separated proteins were transferred onto a PVDF membrane with Towbin buffer containing 0.1% SDS.

2.13. Wheat germ cell-free protein synthesis

RNAs for HBc, FLAG-HBc, SRPK1 wild-type (WT), and SRPK1-K109A were *in vitro*–transcribed from pEU-E01-HBc, -FLAG-HBc, -FLAG-GST-SRPK1, and FLAG-GST-SRPK1-K109A, respectively, in the presence of SP6 RNA polymerase by incubation for 6 h at 37 °C, as described elsewhere (Sawasaki et al., 2002). The synthesized RNAs were subjected to cell-free protein synthesis using a WEPRO 1240 Expression kit (CellFree Sciences) by incubation for 16 h at 26 °C according to the manufacturer's protocol. In the case of *in vitro* phosphorylation of HBc, HBc or FLAG-HBc RNA was mixed with SRPK1-WT or SRPK1-K109A RNA, and the phosphorylation reaction was performed simultaneously with protein synthesis.

2.14. Mass spectrometry analysis (LC-MS/MS) of HBc

Peptides derived from cell-free–synthesized FLAG-HBc together with SRPK1-WT or -K109A in the presence or absence of SRPKIN-1 were prepared for LC-MS/MS analysis as follows. An anti-FLAG antibody was bound to magnetic beads washed once with lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl,1 mM EDTA, 50 mM NaF, 0.5% NP40), followed by addition of HBc-SRPK1 reaction mixture, and the antigen-antibody reaction was carried out for 90 min with rotation at room temperature. After washing 4 times with lysis buffer, immunoprecipitated proteins on magnetic beads were eluted using the DYKDDDDK peptide. The eluted proteins were subjected to ultracentrifugation with Amicon Ultra centrifugal filters to remove the DYKDDDDK peptide, followed by reduction in the presence of 10 mM DTT for 45 min at 56 ˚C and then alkylation with iodoacetamide for 30 min at room temperature. After digestion with proteinase K for 6 h at 56 ˚C, the resulting peptides were purified using SepPAK, followed by concentration to 30–50 μl using a SpeedVac system. The peptides were then diluted 50-fold in 0.1% formic acid and analyzed using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with Xcalibur software (version 4.1.50). Proteome Discoverer software (version 2.2; Thermo Fisher Scientific) was used to generate peak lists from the raw MS data files. To identify peptides, the resulting peak lists were submitted to the SEQUEST search engine (Thermo Fisher Scientific).

2.15. Determination of virus attachment to cells

HepG2-hNTCP-C4 cells were pretreated with the drugs for 16 h and then incubated with HBV for 3 h at 4 ˚C. Drug addition was continued during HBV inoculation. Unbound viruses were then removed by washing three times with cold phosphate-buffered saline. After preparation of total cellular DNA by phenol/chloroform extraction, HBV DNA copies attached to cells were determined using qPCR (Sun et al., 2017).

2.16. Statistical analyses

The statistical significance of differences between two groups was determined using the Student's *t*-test, whereas the significance of differences between three or more groups was determined using Dunnett's test in which $p < 0.05$ was considered significant. R software was used to determine 50% inhibitory concentration (IC50) values.

3. Results

3.1. Anti-HBV activity of serine/threonine kinase inhibitors

First, we evaluated the anti-HBV activity of three SRPK inhibitors, SPHINX31, SRPIN340, and SRPKIN-1, using a cell-based HBV infection model. When pHBV1.05 containing a 1.05-fold unit length of the HBV genome was transiently transfected to produce viral particles, the cells in the presence of each kinase inhibitor were cultured for 4 days. The culture supernatants were collected and added to naïve HepG2-hNTCP-C4 cells and cultured for 8 days, after which HBV total RNA and viral pgRNA were quantified. All three kinase inhibitors showed concentration-dependent anti-HBV activity, with SRPKIN-1 exhibiting the strongest inhibition of pgRNA and total HBV RNA production, with approximately 90% inhibition at 30 μM (Fig. 1A, upper). No cytotoxicity was observed in WST-8 reagent cell viability assays at the drug concentrations used (Fig. 1A, lower). In this experiment, each drug was added at the indicated concentration when HBV-producing cells (pHBV-1.05 transfectants) were cultured, and in the subsequent infection step, a portion of the culture supernatant of the virus-producing cells was taken as the inoculum. Thus, the infective material contained one-half the concentration of the drug in the producer cell culture, and the drug could affect not only the virus replication process but also the time of infection.

To determine the anti-HBV activity of SRPKIN-1 when its effect on the infection process was minimized, another infection experiment was performed with 30-fold–diluted supernatants of HBV-producing cells (Fig. 1B). Although its inhibitory effect was moderately reduced, the concentration-dependent anti-HBV activity of SRPKIN-1 was clearly retained, with an inhibitory activity of approximately 50% at 10 μM at 8 days after infection. From evaluations using a wider concentration range of SRPKIN-1, the IC50 (Figs. 1C) and 50% cytotoxic concentration in the virus-producing cells (Fig. 1D), respectively, were determined as 9.4 μM and >60 μM, respectively. These results suggest that SRPKIN-1 affects both the HBV replication and infection processes.

3.2. SRPKIN-1 inhibited pgRNA encapsidation and nucleocapsid envelopment

To elucidate the inhibitory mechanism of SRPKIN-1 on HBV replication, we first examined steady-state levels of HBc and HBs antigens in HBV-producing cells 5 days after transfection with pHBV1.05. We found that the impact of SRPKIN-1 on HBc production was limited, and levels of HBs (small HBs/SHBs) were moderately reduced (Fig. 2A). In the presence of SRPKIN-1, the number of particle-associated HBV DNA copies in the culture supernatant of virus-producing cells decreased in a drug concentration–dependent manner (Fig. 2B). SRPK is known to regulate pre-mRNA splicing by phosphorylating serine/arginine (SR)-rich splicing factors, and SRPKIN-1 reportedly also affects the splicing patterns of mRNAs (Hatcher et al., 2018). Somewhat unexpectedly, SRPKIN-1 had no significant effect on the levels of total HBV RNA and pgRNA in the unspliced form or on the levels of the spliced forms (Fig. 2C; Supplementary Fig. S2).

To analyze capsid formation and the level of HBV pgRNA in the viral capsids, total RNA was extracted from gel slices containing HBV capsids after NAGE, and HBV pgRNA was quantified. Although the HBc antigen level in the capsid was not changed by SRPKIN-1 treatment, the amount of pgRNA contained therein was markedly reduced in a SRPKIN-1 concentration–dependent manner (Fig. 2D). As the phosphorylation of HBc by serine/threonine protein kinase plays an important role in HBV pgRNA packaging (Ludgate et al., 2016), it is highly likely that SRPKIN-1 exerts an inhibitory effect on HBV packaging. Culture supernatants of HBV-producing cells were concentrated and subjected to NAGE. Western blotting (Fig. 2E) demonstrated that the intensity of bands containing both HBc and HBs, indicated as “virions”, tended to decrease with SRPKIN-1 treatment. In contrast, the level of HBs-free capsids increased following addition of SRPKIN-1. When virion-associated capsid levels (Fig. 2E, closed arrowhead) were standardized according to intracellular capsid levels (Fig. 2D, open arrowhead), with the no treatment control set as 1, SRPKIN-1 treatment at 10 and 30 μM was calculated to be 0.44 and 0.33, respectively.

To further analyze the effect of SRPKIN-1 on capsid-associated nucleic acids, cytoplasmic fractions obtained using the pHBV1.05 transfection system were prepared, and the level of nuclease-resistant total HBV DNA was quantified. Similar to the pattern observed in the encapsidated pgRNA analysis (Fig. 2D), a SRPKIN-1 concentration–dependent suppression of nuclease-resistant HBV DNA in the cytoplasm was observed (Fig. 2F). Inhibition by SRPKIN-1 was also observed when Hep38.7-Tet cells were used as another HBV expression system (Supplementary Fig. S4), although the inhibitory effect in this case was moderate compared to the case of the pHBV1.05 system. To validate the subcellular fractionation method used in this study (Fig. 2F, Supplementary Fig. S4, Fig. 4E and F), Western blotting was used to detect proteins that serve as subcellular cytoplasmic and nuclear localization markers (Supplementary Fig. S3).

To investigate the effect of SRPKIN-1 on the protein expression of large HBs (LHBs), middle HBs (MHBs), and SHBs, HBs levels in culture

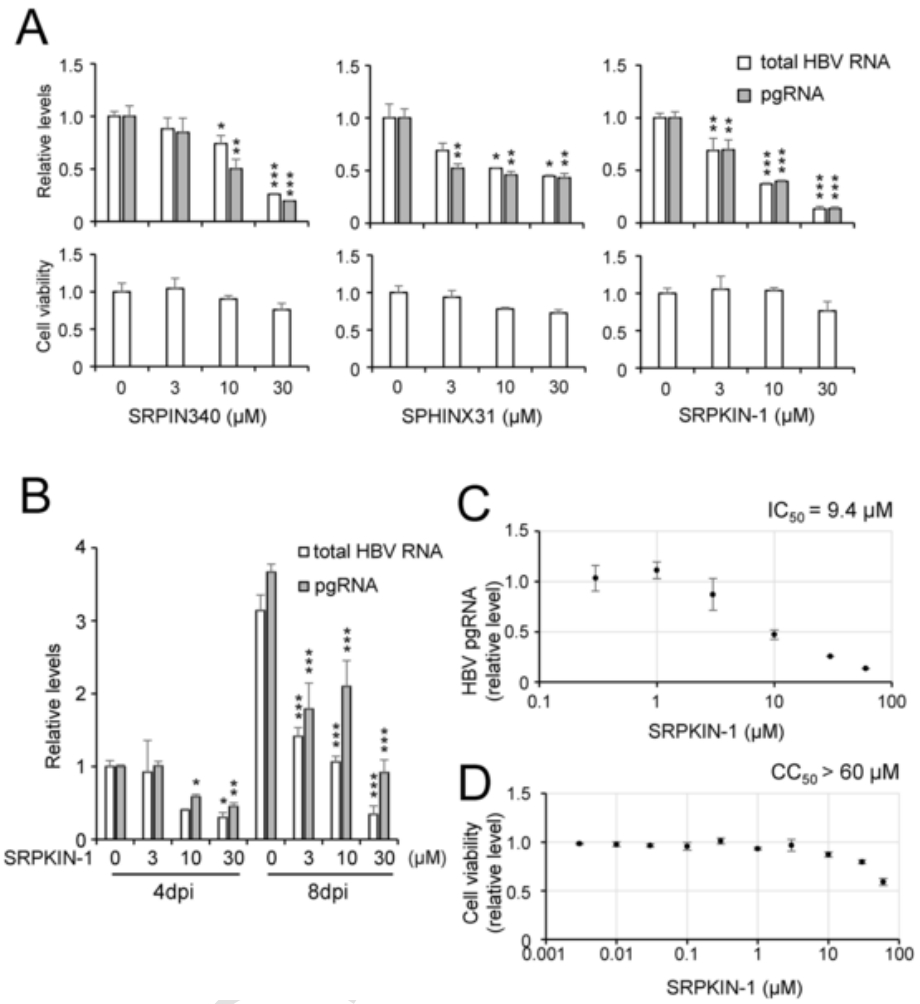

Fig. 1. Anti-HBV activity of serine/threonine kinase inhibitors. HuH-7 cells transfected with pHBV1.05 were treated with each compound at various concentrations (0–30 μM) for 4 days. Cell viability of each culture was determined and values relative to those without drug treatment are indicated (A; lower and D). The culture supernatants of drug-treated HBV producer (transfected) cells were collected, diluted 2-fold with DMSO/PEG solution, and inoculated to HepG2-hNTCP-C4 cells. At 8 days post infection (dpi), HBV total RNA and pgRNA in infected cells were quantified by RT-qPCR (A; upper). Values relative to those without drug treatment are indicated. (B, C) The culture supernatants of the SRPKIN-1-treated producer cells were diluted 30-fold with DMSO/PEG solution, then inoculated to HepG2-hNTCP-C4 cells. The levels of HBV total RNA and pgRNA in infected cells were analyzed at 4 dpi (B) and 8 dpi (B, C). Values relative to those without drug treatment at 4dpi (B) and those at 8 dpi (C) are indicated. Results are expressed as the mean ± standard error of the mean. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

supernatants and intracellularly in cells transfected with two different HBs expression vectors (pHBVCe1.24 and pHBsAe) that enable detection of LHBs, MHBs, and SHBs expression were analyzed by Western blotting. In both expression systems, SRPKIN-1 suppressed the expression of LHBs, MHBs, and SHBs in a concentration-dependent manner (Fig. 2G).

The inhibition of virion formation by SRPKIN-1 was further validated by density gradient centrifugation (Fig. 2H). NAGE analysis of culture supernatants fractionated on a sucrose density gradient revealed that in the supernatant of SRPKIN-1–treated cells, the levels of HBs, HBc, and HBV DNA in virion fractions at densities of 1.13–1.15 g/ml were lower compared to those in the no-treatment control. Thus, these results suggest that SRPKIN-1 suppresses the replication of infectious HBV particles by inhibiting pgRNA packaging into capsids and nucleocapsid envelopment.

3.3. SRPKIN-1 inhibits phosphorylation of HBc in vitro

SRPKIN-1 is a kinase inhibitor specific for SRPK1/2, which was reported as a candidate that phosphorylates the C-terminal domain of HBc (Daub et al., 2002). To determine whether SRPKIN-1 affects the phosphorylation status of HBc, culture supernatants and lysates of cells transfected with pHBV1.05 with or without SRPKIN-1 treatment were subjected to Phos-tag polyacrylamide gel electrophoresis and Western blot analysis. Phos-tag compound, which has specific phosphate-binding activity, has been exploited to separate multiple species of phosphorylated HBc by phospho-affinity gel electrophoresis (Kinoshita et al., 2009), as it can separate phosphorylated isoforms in a variety of phosphorylation states based on the degree of phosphorylation. In addition, bands of non-phosphorylated HBc were estimated using lamda phosphatase treatment of HBc-expressing samples (Supplementary Fig. S5).

Phos-tag gel analysis showed that in culture supernatants of SRPKIN-1–treated cells, the band intensities of hyperphosphorylated- and moderately phosphorylated HBc (indicated by arrows) decreased in a dose-dependent manner, whereas the band intensities of hypophosphorylated (arrowhead) or non-phosphorylated (asterisk) HBc increased (Fig. 3A). It is possible that the top hyperphosphorylated HBc band (upper arrow) represents the dimers of phosphorylated HBc linked by the inter molecular disulfide bond. Intracellularly, SRPKIN-1 treatment resulted in a decrease in the band intensities of moderately phosphorylated (arrow) and hypophosphorylated (arrowhead) HBc. SRPKIN-1 ap-

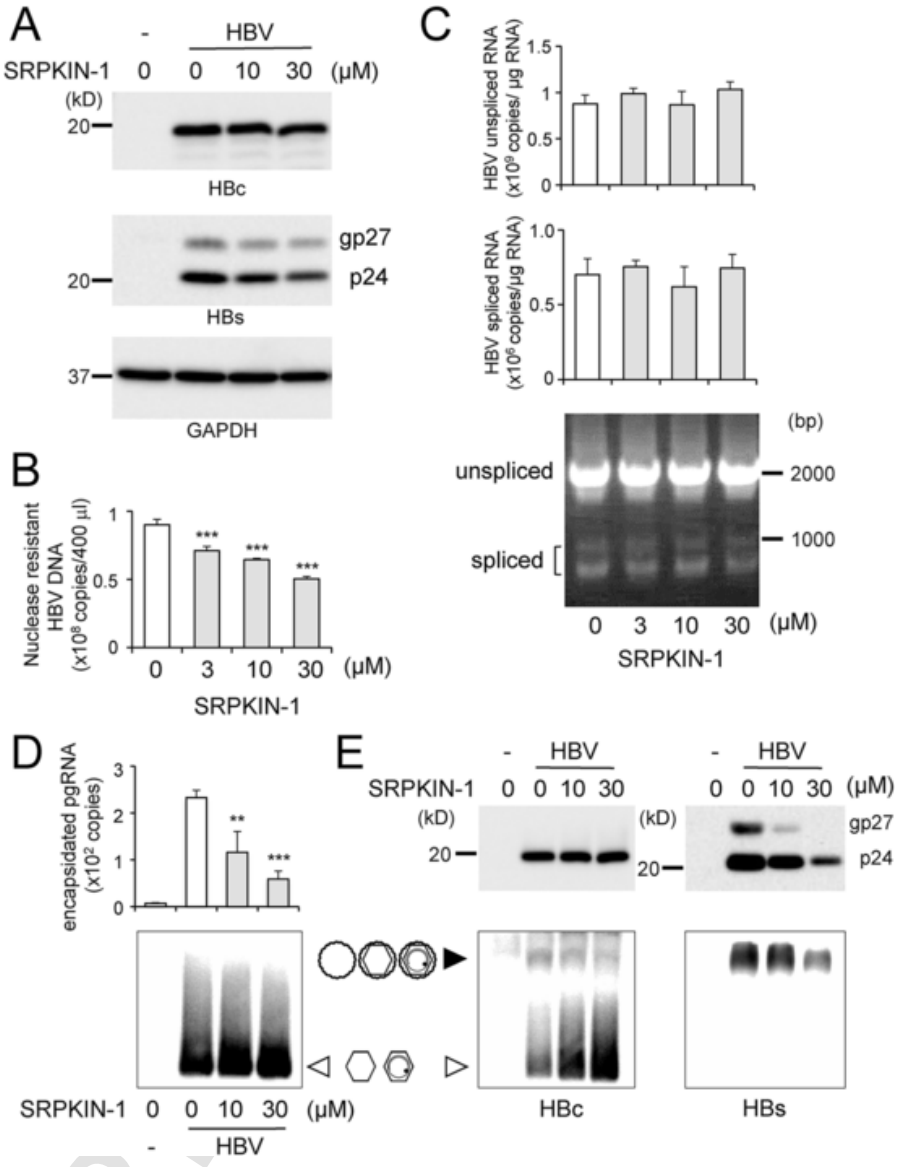

Fig. 2. SRPKIN-1 inhibited pgRNA encapsidation and envelopement of nucleocapsid.
HuH-7 cells transfected with pHBV1.05 were treated with SRPKIN-1 at various concentrations (0–30 μM) for 4 days. (A) Western blot analysis of viral proteins (HBs, HBc) and GAPDH in the cells. (B) Particle-associated HBV DNA levels in the culture supernatants of the transfected cells with or without SRPKIN-1 treatment. (C) RT-qPCR analysis of unspliced- and spliced HBV pgRNAs in the transfected cells with or without SRPKIN-1 treatment. (D) Capsid formation (lower panel) and encapsidated pgRNA (upper panel) in cells. Cell lysates prepared from the transfected cells with or without SRPKIN-1 treatment were separated with native agarose gel electrophoresis (NAGE), followed by Western blotting with an anti-HBc antibody. The gel slices containing the capsids (open triangle) were cut based on Western blot signals. pgRNA levels in the gel slices (capsids) were determined by RT-qPCR. (E) HBV particles in the culture supernatants. The culture supernatants of the pHBV1.05-transfected cells with or without SRPKIN-1 treatment were collected, and HBV particles were partially purified and concentrated via heparin affinity chromatography, subjecting to SDS-PAGE (upper) and NAGE (lower). Western blotting images with anti-HBc (left) and anti-HBs (right) antibodies are shown. In lower panels, the band positions of presumed HBV particles containing HBs and those of non-enveloped capsids are indicated, respectively, as a closed triangle and an open triangle. (F) Nuclease-resistant HBV DNA in cytoplasm of the pHBV1.05-transfected cells with or without SRPKIN-1 treatment was quantified. (G) HuH-7 cells transfected either with pHBVCe1.24 or pHBsAe were treated with SRPKIN-1 at various concentrations (0–30 μM) for 4 days. The culture supernatants concentrated and cells of transfectants were subjected to Western blot analyses of viral proteins; LHBs (gp42, p39), MHBs (gp33) and SHBs (gp27, p24) as well as GAPDH. (H) Fractionation of culture supernatants of HBV producing cells with SRPKIN-1 treatment (30 μM for 4 days)(right) and without treatment (left) by sucrose density gradient centrifugation. Density in each fraction was measured. Fraction samples were analyzed by NAGE, followed by Western blotting with anti-HBc and anti-HBs antibodies. HBV DNA copies in the major fractions positive for HBs and/or HBc (Fr. 2–4, 10–12) were determined by qPCR. Symbols indicating statistical significance are *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

pears to have a minimal impact on intracellular levels of non-phosphorylated HBc (asterisk) compared to levels of the phosphorylated forms (Fig. 3A). We therefore examined whether SRPKIN-1 directly inhibits HBc phosphorylation using a cell-free protein synthesis system (Fig. 3B). When HBc protein was expressed by mixing HBc RNA alone with wheat germ lysate, HBc was phosphorylated at various levels, including hyperphosphorylation, by the intrinsic protein phosphorylation activity within the lysate (lane 2). In contrast, when wild-type SRPK1 (SRPK-WT) RNA was present with HBc RNA, the expressed HBc was mostly in the hyperphosphorylated form, and no low or moderately

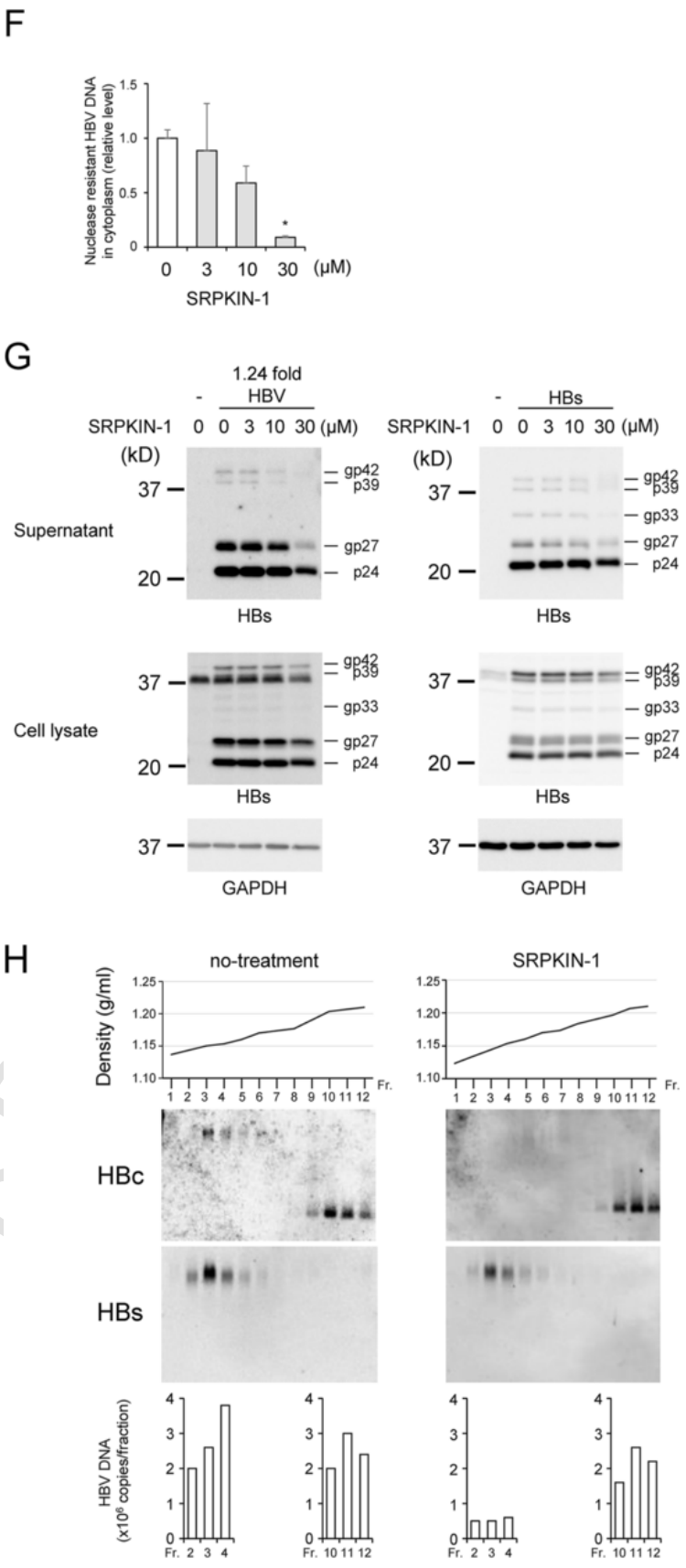

Fig. 2. (*continued*)

phosphorylated HBc was detected (lane 4). When the kinase-dead mutant of SRPK1 (SRPK-Mut) RNA was added instead of SRPK-WT, the moderately phosphorylated form was retained to a considerable extent (lane 3). When SRPKIN-1 was added during HBc synthesis in the presence of SRPK-WT RNA, the pattern of HBc expression was similar to that in the presence of SRPK-Mut RNA (lane 5); that is, compared to when HBc was synthesized with SRPK-WT RNA in the absence of SRPKIN-1, the levels of hyperphosphorylated HBc were lower, and levels of the low and moderately phosphorylated forms were clearly higher.

Furthermore, phosphorylated HBc peptides were analyzed by mass spectrometry to determine whether SRPKIN-1 treatment actually affects the level of hyperphosphorylated HBc (Fig. 3C). HBc produced using the cell-free expression system was digested with proteinase K and subjected to LC-MS/MS analysis. The C-terminal region of HBc is

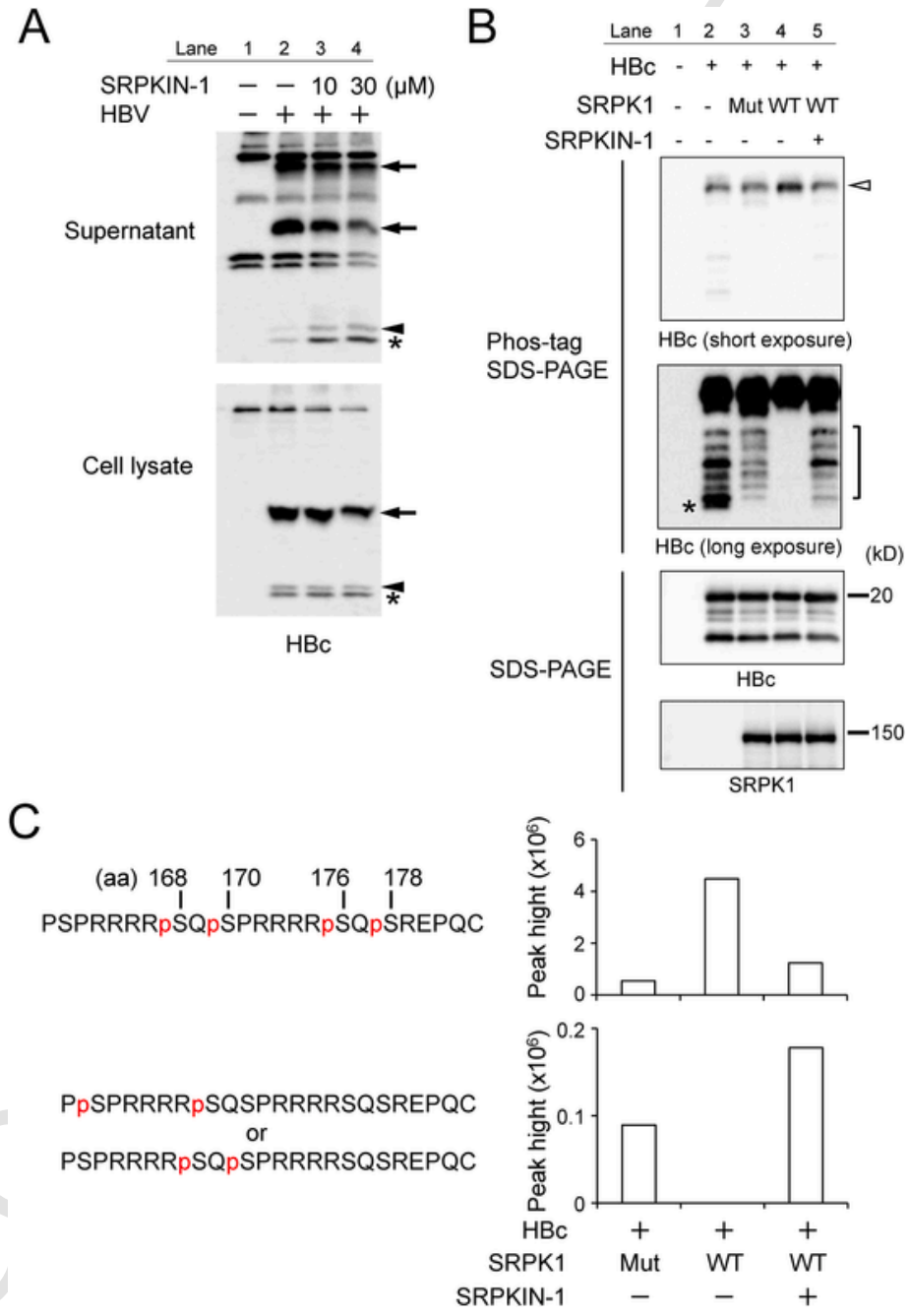

Fig. 3. SRPKIN-1 directly inhibited SPRK-induced HBc phosphorylation *in vitro*. (A) HuH-7 cells transfected with pHBV1.05 were treated with SRPKIN-1 at various concentrations (0–30 μM) for 4 days. The culture supernatants of the transfected cells were collected, and HBV particles were partially purified and concentrated via heparin affinity chromatography, The resulting HBV particle samples (upper panel) and the cell lysates (lower panel) were subjected to Phos-tag SDS-PAGE, followed by Western blotting with the anti-HBc antibody. Arrows; hyper- and moderately phosphorylated HBc. Closed arrowheads; hypophosphorylated HBc. Asterisks; non-phosphorylated HBc. (B, C) HBc protein was co-expressed with wild-type- or mutant SRPK1 protein with/without SRPKIN-1 in wheat germ cell-free protein expression system. (B) Synthesized proteins were subjected to Phos-tag SDS-PAGE or standard SDS-PAGE, followed by Western blotting with the anti-HBc and anti-Flag antibodies. Open arrowhead; hyperphosphorylated HBc. Square bracket; moderately- and hypophosphorylated HBc. Asterisk; non-phosphorylated HBc. (C) Synthesized proteins were subjected to mass spectrometry to identify phosphorylation sites within the C-terminal region of HBc. The HBc aa161-183 peptide with phosphorylation at four Ser residues (aa168, 170, 176,178) (upper panel) and the same region peptide(s) with phosphorylation at two Ser either aa162 and 168 or aa168 and 170 (lower panel) were quantitatively determined.

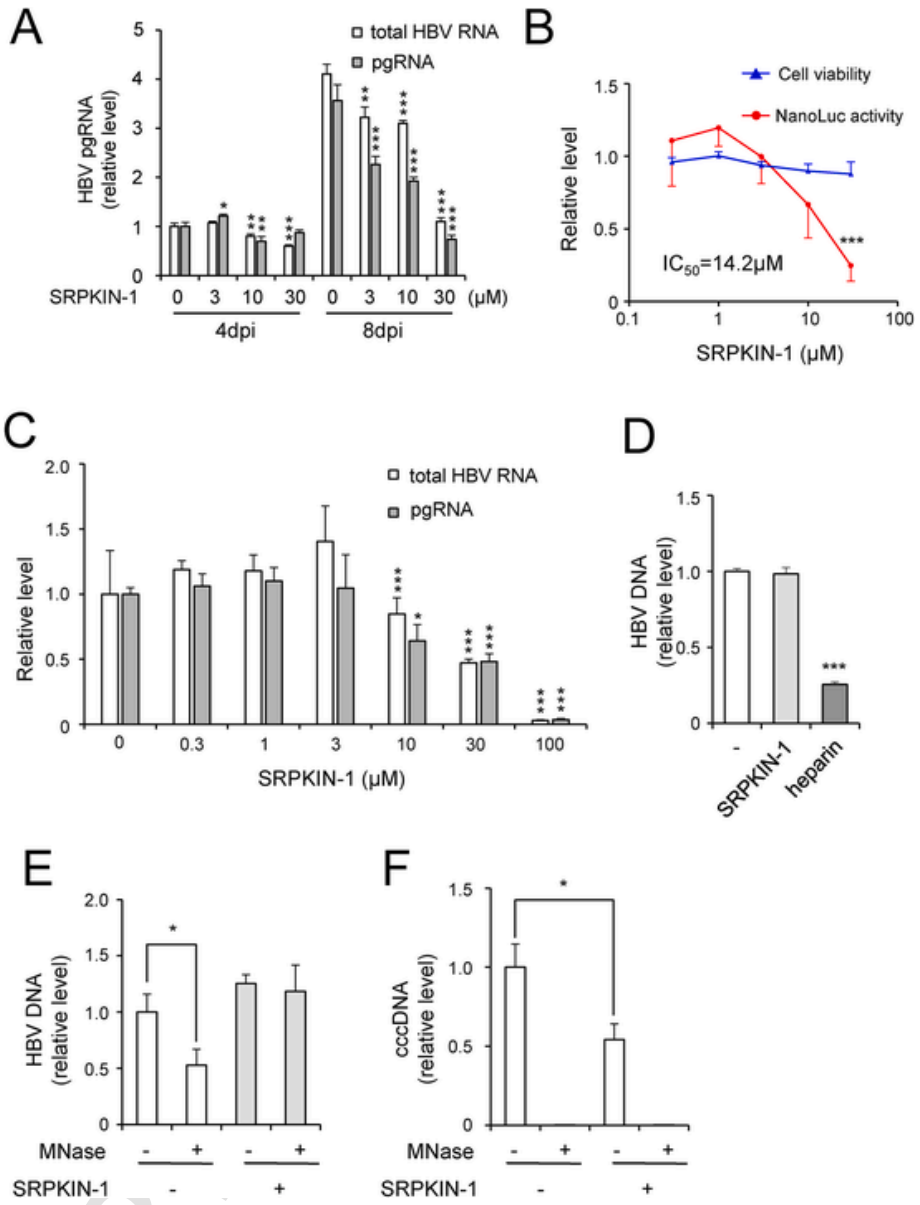

Fig. 4. SRPKIN-1 inhibited the early step of HBV infection. (A) HepG2-hNTCP-C4 cells was inoculated with HBV derived from Hep38.7-Tet simultaneously treated with various concentrations of SRPKIN-1 for 2 days. The infected cells were incubated for an additional 2 or 6 days and collected at 4 and 8 dpi. HBV total RNA and pgRNA were quantified by RT-qPCR. Values relative to those without drug treatment at 4dpi are indicated. (B) HBV/NanoLuc reporter virus was inoculated to HepG2-hNTCP-C4 cells with various concentrations of SRPKIN-1 for 1 day. After an additional 7 days culture without SRPKIN-1, NanoLuc activity in cells was tested (red line). Cell viability was shown by total protein concentrations (blue line). (C) PXB cells were inoculated with HBV produced by Hep38.7-Tet simultaneously treated with various concentrations of SRPKIN-1 for 1 day. After an additional 10 days culture without drug, total RNA was collected from the infected cells. HBV total RNA and pgRNA were quantified by RT-qPCR. (D) HepG2-hNTCP-C4 cells were pretreated with 30 μM SRPKIN-1 or 100 U/ml heparin for 16 h and then inoculated with HBV for 3 h at 4 °C; drug addition was continued during HBV inoculation. The amount of HBV DNA attached to the cells were quantified by qPCR. (E) HepG2-hNTCP-C4 cells were inoculated with HBV simultaneously with or without 30 μM SRPKIN-1 for 72 h. Nucleus fraction of the infected cells was extracted with hypotonic buffer and treatment with or without MNase for 16 h. HBV DNA was then quantified by qPCR. (F) The DNA samples obtained in Fig. 4E were digested with Plasmid-Safe ATP-Dependent DNase, followed by cccDNA-specific qPCR.Symbols indicating statistical significance are *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

known to be phosphorylated at multiple serine residues, and the highest–molecular weight peptides in the aa161-183 region of HBc detected in our analysis were found to be hyperphosphorylated at four serine residues (aa168, 170, 176,178), designated 4P-Ser/161–183. The level of 4P-Ser/161–183 when HBc was synthesized in the presence of SRPK-WT RNA was approximately 10 times higher than when synthesized in the presence of SRPK-Mut RNA. Addition of SRPKIN-1 at 30 μM during HBc synthesis in the presence of SRPK-WT RNA reduced the level of 4P-Ser/161–183 by approximately 80% (upper panel). Conversely, moderately phosphorylated peptides (2P-Ser/161–183), in which two serine residues are phosphorylated, were detected at significant levels when HBc was synthesized with SRPK-WT RNA in the presence of SRPKIN-1, as well as in the case of HBc synthesis with SRPK-Mut RNA. However, 2P-Ser/161–183 was below the detection limit in the case of HBc synthesis with SRPK-WT RNA in the absence of SRPKIN-1 (lower panel).

Thus, mass spectrometry analysis suggested that SRPK is involved in hyperphosphorylation of the C-terminal region of HBc and that SRPKIN-1 inhibits such hyperphosphorylation of HBc.

3.4. SRPKIN-1 inhibits the early step of HBV infection

HBc phosphorylation is potentially important not only in the encapsidation of pgRNA but also in the nuclear transfer of capsids from the de-capsidation of particles during the HBV infection process (Kann et al., 1999; Luo et al., 2020). Therefore, we investigated whether SRPKIN-1 exhibits anti-HBV effects when added only during the infection process (unlike the analysis shown in Fig. 1) and not during the HBV replication process. HBV prepared from culture supernatants of Hep38.7-Tet cells was inoculated into cultures of HepG2-hNTCP-C4 cells simultaneously with various concentrations of SRPKIN-1. The infected cells were incubated for 2 days, washed, and incubated for an additional 2 or 6 days. Cellular RNAs were then isolated, and HBV total RNA and pgRNA were quantified. SRPKIN-1 exhibited a concentration-dependent anti-HBV effect. In the presence of 30 μM SRPKIN-1, the levels of HBV total RNA and pgRNA in the infected cells were reduced to 20–25% of the levels in the vehicle control (Fig. 4A). A moderate decrease in HBV cccDNA level was also observed in infected cells treated with SRPKIN-1 (Supplementary Fig. S6).

The inhibitory effect of SRPKIN-1 on HBV infection was also tested using the HBV/NanoLuc (NL) reporter system, which is useful for analyzing the early steps of the HBV life cycle. In this system, NL functions as an indicator of HBV infection and is expressed by cells infected with HBV particles carrying a chimeric HBV genome in which the HBc gene is replaced with the NL gene. After 1 day of HBV/NL infection in the presence of various concentrations of SRPKIN-1, cells were incubated for another 6 days without SRPKIN-1, and NL activity in the culture was measured. SRPKIN-1 inhibited NL activity in a concentration-dependent manner, with a calculated IC50 value of 14.2 μM (Fig. 4B), suggesting that SRPKIN-1 has the potential to inhibit the early step(s) of the HBV life cycle.

We also tested the inhibitory effect of SRPKIN-1 on HBV infection using an HBV-infection system in which PXB cells (fresh human hepatocytes) were used as host cells instead of HepG2-hNTCP-C4 cells. Concentration-dependent inhibition of HBV infection (based on levels of HBV total RNA and pgRNA) was observed with SRPKIN-1 at concentrations of 10 μM or higher (Fig. 4C). Although higher concentrations of SRPKIN-1 seemed to be required to inhibit infection compared to the analyses shown in Fig. 4A and B, the higher IC50 value (36.7 μM) observed in experimental infection with primary human hepatocytes compared to that observed with HepG2-hNTCP-C4 cells may be due to a case in which the drug metabolic activity was higher in the former cells than in the latter cells (Guo et al., 2011; Yamasaki et al., 2010). In none of the infection experiments in this study did SRPKIN-1 exhibit clear signs of cytotoxicity against host cells (Fig. 4B).

To elucidate the mechanism of SRPKIN-1 inhibition during the early stages of the HBV life cycle, we first determined the effect of SRPKIN-1 on viral attachment to cells. HBV particles were added to cultures of HepG2-hNTCP-C4 cells with or without pre-treatment with 30 μM SRPKIN-1 or 100 U/ml of heparin, followed by a 3-h incubation together with drug treatment at 4 °C. The amount of HBV DNA attached to the cells was significantly reduced with heparin treatment, whereas it was unchanged with SRPKIN-1 treatment (Fig. 4D). The effect of SRPKIN-1 on the process of HBV de-capsidation was evaluated by analyzing the nuclease sensitivity of HBV DNA (Fig. 4E). After HBV entry into cells, a portion of the HBV DNA became sensitive to DNase due to de-capsidation; a decrease in HBV DNA of approximately 50% upon MNase treatment reflected the de-capsidation of HBV particles. In contrast, no such decrease in HBV DNA level following MNase treatment was observed in HBV-infected cells in the presence of SRPKIN-1, suggesting possible inhibition of de-capsidation mediated by SRPKIN-1. Furthermore, HBV cccDNA-specific quantitative PCR was performed on DNA samples obtained in the experiments shown in Fig. 4E after plasmid-safe DNase treatment. As expected, the addition of SRPKIN-1 reduced the amount of HBV cccDNA in the MNase-untreated samples (Fig. 4F). In the MNase-treated samples, no cccDNA was detected with or without SRPKIN-1, which is consistent with the fact that cccDNA should be synthesized after nucleocapsid uncoating.

Collectively, these results suggest that SRPKIN-1 inhibits not only the process of HBV particle formation but also early steps in the viral infection process.

4. Discussion

As HBc phosphorylation by serine-threonine kinase is necessary for HBV propagation, anti-HBV activity has been reported for serine-threonine kinase inhibitors. Although SRPK inhibitors were not examined in previous studies, the effects of various serine-threonine kinase inhibitors on HBc phosphorylation were analyzed, indicating that CDK2 is important for HBc phosphorylation (Ludgate et al., 2012). Indeed, the CDK2 inhibitors K03861 and CDK2 inhibitor III were shown to suppress cccDNA formation during HBV infection (Liu et al., 2021; Luo et al., 2020). Independently, inhibition of capsid formation and related reverse transcription by the PLK1 inhibitor BI-2536 (Diab et al., 2017) and inhibition of extracellular particle release by pseudosubstrate-type inhibitors of protein kinase C (Wittkop et al., 2010) have also been reported. Other studies have shown that serine-threonine kinase inhibitors may suppress HBV replication through inhibition of host factor SAMHD1 phosphorylation (Hu et al., 2018) and modulation of the MAPK signaling pathway (Zhou et al., 2022), not necessarily through inhibition of HBc phosphorylation.

In this study, we showed that the SRPK inhibitors SPHINX31, SRPIN340, and SRPKIN-1 suppress HBV replication in an infection cell system. The inhibitory mechanism was analyzed in detail for SRPKIN-1, which showed the highest anti-HBV activity and is known to function as an irreversible SRPK inhibitor, demonstrating that SRPKIN-1 inhibits not only the HBV particle formation process but also the early steps in the viral infection process. Although several anti-HBV compounds that target the HBc protein, such as GLS4 (Wang et al., 2012) and NVR-3-778 (Lam et al., 2019), have been developed to date, this study is the first to show that multiple stages of the HBV life cycle can be inhibited by altering HBc post-translational modifications.

A previous study using HBc and SRPK expressed in *E. coli* showed that seven serine residues within the C-terminal region of HBc can be phosphorylated by SRPK (Heger-Stevic et al., 2018). In this study, we constructed an HBc phosphorylation system with SRPK using wheat germ cell-free protein synthesis, which retains the protein folding scheme characteristic of eukaryotic cells (Takai et al., 2010). Although HBc was potentially phosphorylated by protein kinase(s) in wheat germ extract present in the *in vitro* translation system, co-expression of SRPK1 increased the amount of hyperphosphorylated HBc, and addition of SRPKIN-1 to this system increased levels of hypophosphorylated HBc (Fig. 3B). LC-MS/MS analysis confirmed that SRPKIN-1 treatment lowers the level of hyperphosphorylated HBc peptide and increases hypophosphorylated HBc levels. Furthermore, this study identified for the first time the serine residues of HBc in which phosphorylation is inhibited by the serine-threonine kinase inhibitor. Nevertheless, given that change in phosphorylation status by SRPKIN-1 was only seen in a fraction of HBc (Fig. 3) and that the anti-HBV activity of SRPKIN-1 is modest (Fig. 1), it may be reasonable to consider that SRPK is only one of many cellular kinases that regulate HBc phosphorylation during the viral life cycle.

Findings from analyses of the status of HBV proteins and genes in virus-producing cells and culture supernatants (Fig. 2D and E) suggested that SRPKIN-1 intervenes and inhibits pgRNA encapsidation and envelopment during HBV infectious particle formation. Phosphorylation of the C-terminal region of HBc is known to contribute to HBV pgRNA encapsidation, and the SRPKIN-1–mediated reduction in encapsidated pgRNA levels confirmed that HBc phosphorylation by SRPK is involved in HBV packaging. Over-expression of SRPK1/2 reportedly in-

hibits the HBV life cycle by possibly suppressing pgRNA packaging (Zheng et al., 2005). Irreversible inhibition of SRPK1 mediated by SRPKIN-1 may lead to stable association of HBc with SRPK, preventing the HBc-pgRNA/polymerase interaction. Although evidence confirming that HBc phosphorylation is involved in the envelopment of HBV particles remains inadequate, it is likely that formation of incomplete capsids due to inhibition of pgRNA packaging by SRPKIN-1 differs from the formation of normal nucleocapsids in their charged state and that this change may affect the envelopment of HBV particles. Analysis of the phosphorylated forms of HBc generated from cells transfected with pHBV1.05 revealed bands of hyperphosphorylated HBc in the culture supernatants but not in cells (Fig. 3A). It is possible that the hyperphosphorylated form of HBc is more suitable for capsid formation than the hypophosphorylated form, or that phosphorylation of HBc occurs during secretion of HBV particles in addition to immediately after translation. SRPKIN-1 was also found to decrease the steady-state levels of HBs proteins (Fig. 2A and G), suggesting that interference with HBs protein expression via a mechanism independent of HBc phosphorylation inhibition may also be involved in the SRPKIN-1–mediated inhibition of HBV particle production.

In addition to its protein kinase activity, SRPK potentially functions as a molecular chaperone in HBV capsid assembly (Chen et al., 2011). It has been proposed that interaction of SRPK with the C-terminal region of HBc triggers HBc phosphorylation and that SRPK is released upon HBc phosphorylation; SRPKIN-1 may reduce the efficiency of correct nucleocapsid formation by suppressing this chaperone activity.

Upon infection of susceptible cells, the HBV genome within the particle is transported to the nucleus, where it is repaired to become cccDNA. HBc phosphorylation during the post–viral entry process is involved in the regulation of uncoating or disassembly of mature nucleocapsids (Kann et al., 1999; Liu et al., 2021; Luo et al., 2020). Although details of the underlying mechanism have not been fully elucidated, phosphorylation of HBc in this process may result in a change in the charge of the capsid, making it more likely to bind to the nuclear membrane. SRPKIN-1 exhibited concentration-dependent inhibition of HBV infection not only in HepG2-hNTCP-C4 cells (Fig. 4A) but also in the infection system using single-round infectious particles (Fig. 4B) and in viral infection of non-cancerous PXB cells (Fig. 4C). Analysis of the mechanism of this inhibitory activity revealed that SRPKIN-1 does not affect HBV attachment to cells (Fig. 4D), whereas MNase sensitivity analysis using nuclear fractions of HBV-infected cells showed that the addition of SRPKIN-1 enhanced the resistance of HBV DNA to MNase (Fig. 4E).

SRPKs specifically phosphorylate serine residues within the RS/SR repeats of SR proteins, which are RNA-binding molecules that play roles in the construction of spliceosomes. Although SRPK may play an important role in the regulation of mRNA splicing in general, the gene selectivity of SRPK-mediated splicing has not been clarified. In experiments in which SRPKIN-1 was added to cells replicating the HBV genome, SRPKIN-1 had no effect on the expression level of HBV mRNAs or on major splicing of pgRNA (Fig. 2C). The possibility that SRPKIN-1 indirectly suppresses HBc phosphorylation as a result of its effect on splicing of cellular gene(s) cannot be completely ruled out, but it is unlikely that intervention in the post-transcriptional processing of HBV is responsible for the anti-HBV activity of SRPKIN-1.

The RS/SR domain is highly conserved in the nucleoprotein or capsid protein of various coronaviruses, including SARS-CoV-2, and phosphorylation of these domains is involved in the assembly and disassembly of coronavirus capsids. Interestingly, SRPK1 and SRPK2 have been identified as the major protein kinases responsible for the phosphorylation of these coronavirus proteins (Nikolakaki and Giannakouros, 2020; Yaron et al., 2022). As SRPK contributes to the regulation of capsid formation not only in DNA viruses such as HBV but also in the life cycle of RNA viruses, SRPK inhibitors may be potentially useful as versatile inhibitors of viral infection.

SRPK1 expression and/or activity reportedly promotes several features of cancer cells, including proliferation, resistance to apoptosis, migration, and angiogenesis (Qiu et al., 2009). Thus, SRPK inhibitors have been actively investigated for development as therapeutic agents, especially for treating several types of cancers (reviewed in Naro et al., 2021). SRPKIN-1 reportedly exhibited strong angiogenic effects and antitumor activity in a mouse model of choroidal neovascularization (Hatcher et al., 2018). SPHINX-31 demonstrated antitumor effects in models of leukemia and solid tumors and did not affect normal hematopoiesis *in vivo*, which is promising with respect to clinical safety (Tzelepis et al., 2018). SRPIN340 suppressed SR protein phosphorylation and inhibited SRSF1-dependent splicing of angiogenesis-promoting VEGF variants in several cell lines (Amin et al., 2011). SRPIN340 also reduced metastasis-related cell traits in triple-negative breast cancer cell lines. The lack of significant splicing changes in these antitumor conditions suggests that SRPK inhibitors would also inhibit SRPK activity unrelated to splicing (Malvi et al., 2020).

More than 350 million people worldwide are chronically infected with HBV, and HBV-positive individuals are at higher risk of developing HCC. Effective chemotherapies for treating HBV-associated HCC have not been established, however. HBV reactivation induced by immunosuppressive agents or cytotoxic chemotherapy is a well-recognized complication that can lead to fatal liver damage in patients with various cancers with pre-existing HBV infection. Therefore, antiviral prophylaxis is now routinely prescribed for HBV surface antigen–positive patients receiving immunosuppressive drugs, but medical treatment for the underlying cancer is also important.

SRPK inhibitors, such as SRPKIN-1, differ from conventional HBc inhibitors in their mechanism of action and are thus novel agents that can inhibit both particle formation and nucleocapsid uncoating, which depend upon HBc phosphorylation. Furthermore, ongoing efforts to develop SRPKIN-1 as an anticancer agent could facilitate the development of new therapies not only for patients with chronic hepatitis B but also for the prevention of HBV reactivation in cancer patients with a history of HBV infection.

Author contributions

K.N. and T.Suzuki designed research; X.L., K.N., M.I., M.M., K.S. and H.T. performed research; X.L., K.N., M.I. and T.C. analyzed the data; X.L., K.N. and T.Suzuki wrote the manuscript; T.K. and T.Sawasaki contributed new reagents/analytic tools. All of the authors discussed the results and commented on the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2023.105756.

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