Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes

Adrien Foca, Ammen Dhillon, Thomas Lahlali, Julie Lucifora, Anna Salvetti, Michel Rivoire, Amy Lee, David Durantel

► To cite this version:

Adrien Foca, Ammen Dhillon, Thomas Lahlali, Julie Lucifora, Anna Salvetti, et al.. Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes. Antiviral Therapy, 2020, 25 (3), pp.151-162. 10.3851/IMP3361 . hal-03315938

HAL Id: hal-03315938

<https://hal.science/hal-03315938>

Submitted on 12 Aug 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes

Adrien Foca^{1,2}, Ammen Dhillon³, Thomas Lahlali^{1,2}, Julie Lucifora^{1,2}, Anna Salvetti^{1,2}, Michel Rivoire⁴, Amy Lee³, and David Durantel^{1,2,5,*}

1. Cancer Research Center of Lyon (CRCL), INSERM U1052, 69008 Lyon, France;
2. University of Lyon, Université Claude-Bernard (UCBL), UMR_S1052, UCBL, 69008 Lyon, France;
3. Arbutus Biopharma Corporation, 100-8900 Glenlyon Parkway, Burnaby, BC, Canada;
4. INSERM U1032, Centre Léon Bérard (CLB), Lyon, France
5. Labex DEVweCAN, 69008 Lyon, France.

* Corresponding author

Correspondence:

David Durantel, PhD, HDR
CRCL - INSERM U1052, 151 cours Albert Thomas, 69424 Lyon Cedex 03, France
Email: david.durantel@inserm.fr
Phone: + 33 472 681 970/+ 33 472 681 959
Fax: +33 4 72 68 19 71

Manuscript information:

Abstract: 247 words
Electronic word count: 3897
Number of table: 0
Number of figures: 5
Number of supplementary figures: 4

Additional Footnotes:

Involvement of authors:

- study concept and design: AF, AD, AL and DD
- acquisition of data: AF, AD, TL, JL and DD
- analysis and interpretation of data: AF, AD, AL, JL and DD
- providing material: MR
- drafting of the manuscript: AF and DD
- critical revision of the manuscript for important intellectual content: AF, AL, JL, AS and DD
- statistical analysis: AF and DD

Conflict of interest:

DD received a research grant from Arbutus Biopharma for this work;
AD and AL are Arbutus Biopharma's employees;
AS, JL and MR: no conflict of interest

List of abbreviations (by alphabetical order):

ApoB, apolipoprotein B; ATP, adenosine triphosphate; cccDNA, covalently-closed-circular DNA; cDNA, complementary DNA; CpAM, core protein allosteric modulator; CHB, chronic hepatitis B; d.p.i., days post infection; DAA, direct acting agent; dHepaRG, differentiated HepaRG cells; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FRG mice, *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-} mice; HBc, HBV core antigen; HBeAg, HBV e antigen; HBsAg, HBV s antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HTA, host targeting agent; IFN- α , interferon-alpha; IU, international unit; LHBS, large HBV s antigen; LNP, lipid nanoparticles; MoA, mode of action; MOI, multiplicity of infection; mRNA, messenger RNA; NUCs, nucleos(t)ide analogs; PHH primary human hepatocytes; PLK1, polo-like-kinase1; pgRNA, pregenomic RNA; qPCR, quantitative polymerase chain reaction; rcDNA, relaxed circular DNA; RT-qPCR, reverse transcriptase coupled quantitative PCR; siRNA, small interfering ribonucleic acid; SM-PLK1i, small molecule PLK1 inhibitor; snRNA, small nuclear RNA; SoC, standard of care; tRNA, transfer RNA; TFV, tenofovir; vge, virus genome equivalent; vRNA, viral RNA; WHO, world health organization.

ABSTRACT

A link between HBV and PLK1 was clearly evidenced in HBV-driven carcinogenesis, and we have also recently shown that PLK1 is a proviral factor in the early phases of HBV infection. Moreover, we have shown that BI-2536, a small molecule PLK1 inhibitor, was very efficient at inhibiting HBV DNA neosynthesis, notably by affecting nucleocapsid assembly as a result of the modulation of HBc phosphorylation. Yet, as small molecule kinase inhibitors often feature poor selectivity, a more specific and safer strategy to target PLK1 would be needed for a potential development against chronic HBV infections. Here, we analysed using both freshly isolated primary human hepatocytes and differentiated HepaRG, the anti-HBV properties of an LNP-encapsulated PLK1-targeting siRNA. Standard assays were used to monitor the effect of LNP siPLK1, or controls (LNP siHBV and LNP siNon-Targeting), on HBV replication and cell viability. A dose as low as 100ng/mL of LNP-siPLK1 resulted in a >75% decrease in secreted HBV DNA (viral particles), which was comparable to that obtained with LNP siHBV or 10 μ M of Tenofovir (TFV), without affecting cell viability. Interestingly, and in contrast to that obtained with TFV, a strong inhibition of viral RNA and HBe/HBsAg secretions was also observed under LNP siPLK1 treatment. This correlated with a significant intracellular decrease of vRNA accumulation, which was independent of any change in cccDNA levels, thus suggesting a transcriptional or post-transcriptional modulation. Such an effect was not obtained with a biochemical approach of PLK1 inhibition, suggesting an enzymatic-independent role of PLK1.

KEYWORDS

Hepatitis B virus; Polo-like-kinase 1; small-interfering RNA, lipid nanoparticle; antiviral; host-targeting agent; hepatocellular carcinoma.

INTRODUCTION

Hepatitis B virus (HBV) infection represents the main cause of hepatocellular carcinoma (HCC), which is the 2nd leading cause of cancer deaths worldwide (WHO data). Out of the hundred million people exposed to HBV, 257 million remain currently chronically infected (CHB) (WHO data; global hepatitis report, 2017). If left untreated, these patients have a 100 fold increased risk of developing HCC compared to the general population [1]. When patients are successfully and continuously treated with safe nucleos(t)ide analogs (NUCs), their risk of developing HCC is 2 to 5 times higher compared to normal individual[2, 3]. This reminiscent risk of HCC under treatment is likely due to the fact that NUCs do not allow the elimination of the HBV replicative intermediate called cccDNA (*i.e.*, *covalently closed circular DNA*), which is responsible for viral persistence and rebound when treatment is stopped [4, 5]. In addition to the development of drugs that may target directly cccDNA [4, 5], the discovery of host-targeting agents (HTA) acting both on HBV replication and HCC initiation and/or progression could increase the efficacy of current standards of care (SoC) for curing patients from the infection and associated liver diseases.

We previously identified the serine/threonine polo-like-kinase 1 (PLK1) as such a host target [6]. PLK1 is known to be overexpressed in many human cancers [7], and was demonstrated an important driver of HBV-induced HCC [8-11]. Moreover, we found that PLK1 is a proviral factor of HBV and that small inhibitory molecules of PLK1 (here after abbreviated SM-PLK1i) (*e.g.*, BI-2536) had a negative impact on HBV replication [6]. The inhibition of PLK1 could therefore be of interest for the treatment of CHB patients with potential effects on both virologic and oncologic aspects of the disease.

Due to their poor specificity (*i.e.*, they target several kinases, not a single one) and overall low safety profile, as demonstrated in the oncology field, SM-PLK1i cannot be reasonably clinically trialed in CHB patients, who require long-term and very safe treatments. Interestingly, small

interfering RNAs directed against PLK1 were also shown to be efficient at targeting PLK1 oncogenic activity [12]. Chemically modified and injectable versions of PLK1 siRNA were also validated in preclinical cancer animal models [13, 14], and one PLK1 siRNA encapsulated in proprietary lipid nanoparticles (LNPs) was clinically trialed in patients with advanced HCC (NCT02191878).

In this study, we found that the use of LNP siPLK1 in HBV-infected primary human hepatocytes (PHHs) lead to a decrease in the amount of intracellular HBV RNAs, that subsequently affect the amount of secreted viral particles and HBe/HBs antigens. Knowing that a loss of HBsAg in patients represents a clinical end-point goal [3], our finding could have relevant therapeutic implications. Moreover, these data point-out a novel PLK1 proviral function, which seems independent of its kinase activity. Indeed besides being involved in HBV reverse transcription through the regulation of capsid assembly [6], our data suggest that PLK1 could also be involved in the regulation of HBV RNA accumulation.

METHODS

Chemicals, antibodies, and others reagents

PLK1 inhibitor BI-2536 was purchased from Selleckchem. All other chemicals were purchased from Sigma-Aldrich. Rabbit polyclonal anti-HBc antibodies were purchased from Dako (B0586) or Abcam (ab115992) and monoclonal anti-HBc antibodies from Abcam (ab8637; Clone C1). Rabbit polyclonal anti-PLK1 antibodies from Abcam (ab21738) were used for detection of phospho-PLK1-S₁₃₇; rabbit monoclonal anti-PLK1 antibodies from Abcam (ab115095) were used for detection of phospho-PLK1-T₂₁₀ and rabbit polyclonal anti-PLK1 antibodies from Abcam (ab109777) for detection of total PLK1.

HepaRG and primary human hepatocyte cultures

Human liver progenitor HepaRG cells [15, 16] were cultured and differentiated (dHepaRG) as previously described [17]. Primary human hepatocytes (PHH) were freshly prepared from human liver resection obtained from the Centre Léon Bérard (Lyon) with French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011) as previously described [18].

Production of siRNA-containing liponanoparticles and use in cell culture

The forward and reverse sequences of siRNA against PLK1 are respectively, 5'-AGAUCACCCUCCUUAAAUAUU-3' and UAUUUAAGGAGGGUGAUCUUU-3', and feature 2'OMe modification at indicated position (bold underlined) as previously described [14]. This duplex siRNA was formulated in liponanoparticles as previously described [19]. The lipid formulation contains 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dilinoleyloxy-N,N-dimethylpropylamine, 3-N-[(ω-methoxypoly(ethyleneglycol)₂₀₀₀)carbamoyl]-1,2-dimyristyloxy-propylamine and cholesterol.

The equivalence between molarity of siPLK1 and amount of LNP siPLK1 in ng/mL is: 1 nM of siPLK1 is equivalent to 13.3 ng/mL of LNP siPLK1. Controls LNP-siRNAs were manufactured the same way. The sequences of siRNA against HBV are Fw: 5'-CGACCUUGAGGCAUACUUCUU-3' and Rv: 5'-GAAGUAGCCUCAAGGUCGUU-3' ON-TARGETplus duplex. LNP siRNAs were administrated to cells by direct dilution into the culture medium. Cells were exposed to LNPs for 24 hours, then washed with culture medium to remove excess of LNPs. Treatment were repeated as indicated in figure legends.

HBV infection and analysis of viral replication

Differentiated HepaRG cells (dHepaRG) and PHHs were infected with HBV genotype D (concentrated from HepAD38 cells supernatants) as described previously [20]. After treatment procedures, levels of intracellular and/or extracellular HBV RNAs and DNAs, HBc, secretion of HBeAg and HBsAg were monitored by quantitative polymerase chain reaction (qPCR), reverse transcription qPCR (RT-qPCR), immunoblot and enzyme-linked immunosorbent assay (ELISA) respectively, as previously described [6, 20]. Briefly, HBeAg and HBsAg were quantified in culture medium using a chemiluminescence immunoassay kit (Autobio Diagnostics) according to manufacturer's instructions. Total intracellular DNAs and RNAs as well as secreted viral DNAs/RNAs were purified from infected cells using NucleoSpin® 96 Tissue kit, NucleoSpin® 96 RNA kit, NucleoSpin® 96 virus kit (Macherey-Nagel) respectively, according to manufacturer's instructions. Reverse-transcription of RNA into cDNA was performed with the SuperScript® III First-Strand Synthesis kit (Invitrogen) according to manufacturer's instructions. All qPCR analyses were performed with LightCycler™ 96 and 480 systems (Roche), except for cccDNA and pgRNA specific PCRs that were processed under a QuantStudio™ 7 (Applied), using homemade TaqMan assays [20].

Primary human hepatocytes preparation from chimeric FRG mice and use for antiviral

This study was approved by the WuXi IACUC (Institutional Animal Care and Use Committee). FRG™ (Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}) mice were purchased from Yecuris (Tualatin, OR, USA), engrafted with PHH and infected with 1.10⁹ virus genome equivalent (vge) of HBV genotype D. When HBV viremia had reached a maximum (i.e., around 10e9 vge/mL), the liver was digested by *in situ* collagenase perfusion and recovered hepatocytes seeded in DMEM medium into 24-well plates (2.7.10⁵ cells/well). LNP treatments were conducted every 4 days starting at day 0 and BI-2536 treatment was started on day 0 and replenished every two days. DNA in the culture supernatants was isolated using QIAamp 96 DNA Blood Kit (Qiagen) and quantified by qPCR. HBsAg was quantified using specific CLIA kit (Autobio).

Separation of cytoplasm and nucleoplasm

Extractions of nuclear and cytosolic proteins were performed using NE-PER Nuclear and Cytoplasmic kit (Thermo Scientific), according to the manufacturer's instructions. Sodium orthovanadate, Complete™ EDTA-free protease inhibitor cocktail (Roche) and sodium fluoride were added freshly to all lysates. Nuclear and cytosolic RNAs were isolated as described in *Weil et al.*, [21], followed by a purification with TRI reagent (Merck) and precipitation with isopropanol. Purity of nuclear vs cytoplasmic extracts, was assessed by RT-qPCR using primers to amplify the nuclear U6 snRNA (Fw: 5'-CTCGCTTCGGCAGCACATATAC-3' / Rv: 5'-GGAACGCTTCACGAATTTGCGTG-3') and the cytoplasmic Human Tyr-tRNA (Fw: 5'-CCTTCGATAGCTCAGCTGGTAGAGCGGAGG-3' / Rv: 5'-CGGAATTGAACCAGCGACCTAAGGATGTCC-3').

Capsid migration assay

The intracellular HBV nucleocapsids were analyzed by agarose electrophoresis in native condition followed by transfer onto an enhanced chemiluminescence membrane (Amersham) and western blot analysis, as previously described [20].

Southern Blotting

Total DNAs extracted with NucleoSpin® 96 Tissue kit (Macherey-Nagel) were pooled, precipitated using 5M NaAc and ethanol and analyzed by Southern blot as previously described [22]. DNAs from HepG2-NTCP cells extracted using a modified Hirt procedure were used as positive controls [23].

Statistical analysis

Statistical analysis was performed using two-way analysis of variance, t tests, or non-parametric Mann-Whitney tests using GraphPad Prism software, version 7.04. For all tests, $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

RESULTS

Efficacy and safety of LNP siPLK1 in hepatocytes

Compared to control LNP siRNAs, LNP siPLK1 were efficient at inhibiting PLK1 expression, with a 70% loss of PLK1-mRNA at 13.3ng/mL without toxicity in proliferating HepG2 cells. As expected, LNP siPLK1 induced a 50% mortality in proliferating HepG2 cells at 133ng/mL due to active cell division and crucial role of PLK1 in G2/M transition (**Fig. Sup. 1**). Considering these data, as well as the concentration correspondence (i.e., 1 nM of naked siPLK1 is equivalent to 13.3 ng/mL of LNP siPLK1) and our previous work done using naked siRNA targeting PLK1 [6], we tested the effect of LNP siPLK1 and controls in HBV-infected primary human hepatocytes, and related dHepaRG cells, with a range of concentration going from 100 to 300 ng/mL. We observed a strong reduction in the levels of PLK1 mRNA in both dHepaRG and PHH (**Fig. Sup. 2A and Fig. 1A**) and in the level of PLK1 phosphoprotein in PHH (**Fig. 1B**). Interestingly, as previously reported, LNP siHBV led also to a reduction of PLK1 mRNA and PLK1 phosphorylation to levels comparable to those observed in uninfected cells (compare lanes LNP-siHBV and NI; **Fig. 1A and 1B**), thus confirming that HBV strongly increases PLK1 activity in PHH. Confirming also our previously published data [6], the inhibition of PLK1 activity was not cytotoxic in PHH (3 different batches tested) even when LNP siPLK1 was administrated 3 times at 300ng/mL (**Fig. 1C**). Moreover PHH remained functional, as measured by the apolipoprotein B (ApoB) secretion (see apoB dosing in **Fig. 2A**), a component of VLDL (very low density lipoprotein) that are made by healthy hepatocytes.

LNP siPLK1 strongly, and specifically, inhibits viral particle and antigen secretion in HBV-infected primary human hepatocytes

To evaluate the antiviral activity of LNP siPLK1, freshly isolated PHH from three different donors were infected with HBV at a high multiplicity of infection, and at day-4 post-infection (p.i.), when the average HBsAg production reached around 1000 IU/mL (which is a level signing a high

replication of HBV), cells were treated three times every three days with increasing LNP siPLK1 doses or control molecules. Secretion of viral DNA (i.e., infectious virion) and RNA (i.e., RNA-containing particles; [24]) were significantly inhibited by LNP siPLK1 (**Fig. 2A**). These inhibitions were comparable to those observed with LNP siHBV in the model. The lack of dose-dependency of the phenotype suggests that even a weak loss of PLK1/phospho-PLK1 (**Fig. 1B**) was associated with a strong inhibition of virus replication. Interestingly, we also observed a 70% inhibition of viral antigen HBeAg and HBsAg secretion (**Fig. 2A**), without any effect on ApoB secretion, highlighting a specificity of LNP siPLK1 action on those secreted viral proteins. In the mean time, tenofovir (TFV) did not affect HBV antigens secretion and inhibition of HBV antigen secretion was stronger with LNP siHBV, as expected due to the direct targeting of HBV RNAs encoding those antigens.

This strong inhibition of HBeAg and HBsAg secretion was also confirmed in the HBV-infected dHepaRG cells with the same experimental setting (**Fig. Sup. 2B**). Moreover, the inhibitory phenotype on virion and HBsAg secretion was also observed by *ex vivo* treatment with LNP siPLK1 of freshly isolated hepatocytes from HBV-infected liver-humanized FRG mice [25], which replicate HBV at high level (i.e., around 1.10^9 copies/mL at day-26 post-infection), mimicking the highly viremic « immune tolerant » phase in human. In this model, 300ng/mL of LNP siPLK1 led to a decrease of 60% in HBV DNA and 65% in HBsAg, whereas treatment with LNP siHBV was very efficient (**Fig. Sup. 3**).

LNP siPLK1 induces a reduction of intracellular viral RNA accumulation without significant modification of cccDNA amount

To further describe the anti-HBV potential of LNP siPLK1 and get insights on the observed phenotype on secreted HBV antigens, intracellular viral parameters were also analyzed. We observed a dose dependent decrease of intracellular HBV DNAs and RNAs in HBV-infected PHH or dHepaRG cells treated with LNP siPLK1 (**Fig. 2B and Fig. Sup. 2C**). The highest dose of LNP siPLK1

resulted in a decrease of 55% (70% in dHepaRG) and 50% (also 50% in dHepaRG) of HBV DNA and RNA respectively (**Fig. 2B and Fig. Sup. 2C**). LNP siHBV treatment resulted in a stronger effect, with 80% and 90% drops, whereas only viral DNA amount decreased with TFV (65% in PHH and 90% in dHepaRG) as expected. The lower inhibition of intracellular DNA amount for all molecules tested, as compared to what observed with secreted DNA is likely due to the detection of remaining input HBV DNA from HBV inoculum as discussed below. Interestingly and contrary to what we described with SM-PLK1i [6], we observed a decrease in the levels of HBV RNAs upon LNP siPLK1 treatment of HBV-infected hepatocytes without observing any significant changes in cccDNA amount (analyzed by qPCR or Southern Blot) (**Fig. 3**). These data suggest that the decrease of HBV RNA observed with LNP siPLK1 is a consequence of a transcriptional and/or post-transcriptional event.

LNP siPLK1 does not inhibit better HBV replication in pre-infection treatment setting

To determine if PLK1 affects HBV infection establishment, we treated PHH twice with LNP siPLK1 or control molecules, prior to HBV infection and a third treatment was done at the time of HBV inoculation in order to maintain PLK1 protein level low from HBV entry to cccDNA formation. Analyses were done at 5d.p.i. PLK1 mRNA knockdown was still >75% with 100 and 300ng/mL of LNP siPLK1 (**Fig. 4A**). As seen before LNP siHBV allowed a PLK1 mRNA decrease, in accordance to the level found in uninfected cells. Interestingly a similar phenotype was observed with an entry inhibitor (*i.e.*, PreS1 peptide), which very efficiently prevented HBV infection in this setting (as shown in other panels of **Fig. 4**). Regarding antiviral phenotypes obtained with LNP siPLK1, they were very similar to that obtained in post-infection treatment setting (compare results of **Fig. 2 and 4**). Importantly, cccDNA establishment was not fully blocked by either LNP siPLK1 or LNP siHBV, as compared to that observed with PreS1 peptide, which acts a genuine entry inhibitor. The slight decrease amount of cccDNA observed with LNP siPLK1 and LNP siHBV was poorly significant compared to controls (LNP si Ctrl and TFV), and could therefore not explain *per se* other observed

inhibitory phenotypes. It is worth mentioning here that PreS1 peptide did not lead to an absence of detection of intracellular HBV DNA in this setting, as compared to other parameters (cccDNA, intracellular RNA, HBeAg, HBsAg), thus confirming that residual HBV DNA from inoculation perturbs analyses, as mentioned earlier. This is the reason why results obtained from supernatant analyses are more reliable, and without ambiguity demonstrate the overall strong effect of LNP siPLK1 on HBV replication.

PLK1 knockdown results in a partial nuclear vRNA sequestration in HBV-infected PHH

In HBV-infected PHH, activated pPLK1-S₁₃₇ was mainly localized in the nucleus (**Fig. Sup. 4** and refer also to [6]). As we observed a decrease in HBV RNAs in absence of change in the level of cccDNA under LNP siPLK1 treatment, we wondered if PLK1 could be involved in the modulation of HBV RNA levels. To address this, we infected PHHs, treated them as above and harvested cells three days after the last treatment. We performed a cytosolic/nuclear fractionation in order to quantify HBV RNAs in both compartments. RNA purity of our extracts was checked using nuclear-specific U6 snRNA and cytosolic-specific Tyr-tRNA. LNP siHBV resulted in a decrease of HBV RNAs levels in both compartments, whereas LNP siPLK1 led to a dual phenotype, depending on subcellular compartments. Indeed, in the cytosol, a 50% decrease of HBV RNAs levels was observed, similar to that observed with whole cell extracts (**Fig. 5**). By contrast, in the nuclear compartment, a dose-dependent accumulation of HBV RNAs was observed under LNP siPLK1 treatment (**Fig. 5C**). As the amount of cytosolic HBV RNAs was overall 10 times higher than that observed in the nucleus, this accumulation of HBV RNAs in the nucleus induced by LNP siPLK1 did not modify the overall observed phenotype (i.e., reduction of total RNA accumulation), but certainly accounted for the decrease in viral protein synthesis.

DISCUSSION

Hepatocellular carcinoma ranks second in term of cancer-induced death worldwide, with an estimated 600,000 death/year burden. HCC development is the consequence of the interplay between genetic predispositions, environmental factors (e.g., exposure to aflatoxins, alcohol, etc...), as well as oncogenic viruses; the latter are involved in 80% of cases, and HBV for itself accounts for 50% of HCC worldwide. As 257 million people are chronically infected with HBV and only a minority of them (<1%) are effectively diagnosed and treated, the HBV-mediated HCC burden is far from being eradicated. If a better diagnostic of infected patients and a more universal use of antiviral therapies are needed, it would be also interesting to identify novel molecules that could target both viral replication and hepatocellular cancer cell growth.

Our previous study demonstrated that PLK1 is activated upon HBV infection and acts as a proviral factor. Consequently, we showed that its inhibition by BI-2536, a SM-PLK1i, leads to an inhibition of HBV replication. In addition, the group of Dr O. Andrisani have shown in several studies that PLK1, which is induced by HBx, is a driver of murine hepatocyte cancerous transformation *in vitro* and that its inhibition by BI-2536 could prevent it [11, 26]. Moreover PLK1 being a validated target in oncology, important research efforts to identify potent SM-PLK1i were made, as reviewed recently [27]. However most of SM-PLK1i are not strictly specific to PLK1 itself and cross-inhibit of other kinase functions; this may account for the poor toxicologic profiles and side effects of these chemical inhibitors. The possibility to use siRNA to more specifically target kinases involved in cancer has opened novel perspectives. In this line, the clinical trial NCT02191878 performed with TKM-080301 in subjects with advanced HCC was instrumental and led us to consider LNP siPLK1 as a good candidate for early inhibition of HBV replication, in the dual perspective of use described above.

Here we confirmed that PLK1 is crucial for HBV replication in non-dividing hepatocytes by using PLK1-targeting-siRNAs encapsulated in lipid nanoparticles (Arbutus Bioparma proprietary composition; the LNP-siPLK1 used in this study was closely related, but different from TKM-080301). In particular, we demonstrated that LNP siPLK1 inhibits more efficiently HBV replication than naked siRNA or SM-PLK1i (reported in [6]) in the absence of cell toxicity. Indeed 100 to 300ng/mL of LNP siPLK1 (corresponding to 7.5 to 22.5nM siPLK1) led to a >75% decrease in HBV viremia *in vitro*. Moreover, in contrast to BI-2536, the SM-PLK1i that was previously profiled in depth *in vitro* and in liver-humanized FRG mice [6], we found that LNP siPLK1 was also capable to potently inhibit the secretion of HBV antigens in the supernatant of treated cells. This indirectly indicates that a kinase-independent function of PLK1 could account for production and secretion of HBV antigens. PLK1 could interact with a viral or host factor independently of its kinase activity and through this interaction contribute to an optimal production of HBV antigens. This protein-protein interaction model to explain activity was precedently exemplified for PLK1 by Vitour and colleagues [28]. In their study, it was shown that the polo-box domain of PLK1 could bind to the mitochondria-bound innate immunity adapter MAVS and inhibit its ability to activate the IRF3 and NF-kappaB pathways, without any requirement of the kinase activity.

Such an unexpected phenotype of LNP siPLK1 on HBV antigens production is particularly relevant, as the next step for the improvement of current HBV therapy in clinic is precisely to be capable to reduced antigenemia in patients, in particular HBsAg, to potentially restore immune responses and control infection [3]. The LNP siPLK1-induced inhibition of HBeAg and HBsAg in supernatant correlated with a reduced accumulation of intracellular HBV RNAs in LNP siPLK1 treated cells, in the absence of any change in cccDNA amount. This observation points out toward a role of PLK1 in transcriptional and/or post-transcriptional regulation. In this study, we limited our work to the analysis of the impact of LNP siPLK1 on the accumulation of viral RNA in nucleoplasm and cytoplasm after fractionation, and found that HBV RNAs tended to accumulate in the nucleus

under LNP siPLK1 treatment, while the total amount of RNA was decreased. Although the precise underlying mechanistic of this transcriptional and/or post-transcriptional regulation (*i.e.*, cccDNA activity, vRNA stability, vRNA trafficking...) is yet to be defined, it is tempting to speculate that the previously described interplay between PLK1 and HBc [6] could also be at work. Indeed, HBV core protein is known to interact with cccDNA [29] and potentially in the regulation of transcription [30]. Moreover, it has been suggested that the HBc CTD could be determinant for HBc-cccDNA interaction [30] and in our precedent study, we have shown that PLK1 targets HBc CTD. Finally, HBc is also known to be an RNA-binding protein physically interacting with vRNAs. Of course, a role of PLK1 independently of HBc should also be considered. In this case PLK1 could interact with other known or unknown host-regulators of both/either cccDNA transcription and/or post-transcriptional events, and regulate their functions in a kinase-independent manner. Whether this is yet to be demonstrated regarding the pro-HBV role of PLK1, there are in literature many examples of kinases, which feature kinase-independent regulatory functions [31-36]. Taken together, this provided a nice hypothetical landscape for further mechanistic studies to document LNP siPLK1 mode of action (MoA).

Of course, it would also be important to evaluate the anti-HBV efficacy of LNP siPLK1 in a liver humanized mouse model to confirm its potential therapeutic interest on the virology side, and to compare it or combine it to siRNA targeting HBV itself. Whether siRNA targeting HBV are currently in clinical trials in CHB patients (*e.g.*, phase-2 for ALN-HBV, NCT02826018; phase-2 for JNJ-3989 ,NCT03982186 and NCT04129554) and remains the most straightforward and relevant RNAi option against HBV replication, the co-administration of siRNAs targeting both HBV and PLK1 could be an original way to further improve such a therapeutic approach.

Regarding the possibility to test the relevance of using LNP siPLK1 as a double bullet compound, which would be able to both contribute to HBV replication inhibition in “add on therapy” with SoC

and prevention of HBV-driven carcinogenesis, we would need a preclinical model able to recapitulate the entire pathogenesis process. A mouse model based on immune-competent mice transduced with AAV-HBV vectors could be of interest, as it features a persistent viral replication [37], in the context of an HBV immune tolerance [38], and was also reported to support HCC development [39]. Alternatively other transgenic mouse models featuring the overexpression of HBV proteins, HCC development and a demonstrated implication of PLK1 in carcinogenesis could be used to this end. Recently, it was shown that genomic integrity of hepatocytes was disrupted by viral LHBS and that PLK1 acted as a major modulator in LHBS-mediated G2/M checkpoint override. In this model, SM-PLK1i resulted in a G2/M checkpoint restauration and suppression of tumorigenesis [40].

To summarize, in this study we have confirmed the proHBV role of PLK1 and reported a potent and specific antiviral activity of LNP siPLK1 in HBV-infected hepatocytes, which includes a strong loss of intracellular vRNA accumulation, leading to reduction of HBV antigens secretion. As it is now rather well accepted that PLK1 is an important HCC driver, its inhibition by RNA interference in patients featuring an HCC from HBV etiology, in which HBV replication would be still active, could result in a combined effect on the virus and cancer cell growth. These results warrant additional investigations in preclinical animal model to further determine the usefulness of such a therapeutic strategy.

ACKNOWLEDGEMENTS

The authors thank Maud Michelet, Océane Floriot, Jenifer Molle, Laura Dimier, Anaëlle Dubois and Judith Fresquet for PHHs isolation as well as the staff from Michel Rivoire's surgery room for providing us with fresh liver resections.

This work was supported by a grant from ANRS (French national agency for research on AIDS and viral hepatitis; CSS4) and by INSERM core grant to DD. This work was also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR) to DD. The work was also supported by a research contract with Arbutus Biopharma.

REFERENCES

- [1] Chan SL, Wong VWS, Qin S, Chan HLY. Infection and Cancer: The Case of Hepatitis B. *Journal of Clinical Oncology* 2015.
- [2] Yoo J, Hann HW, Coben R, Conn M, DiMarino AJ. Update Treatment for HBV Infection and Persistent Risk for Hepatocellular Carcinoma: Prospect for an HBV Cure. *Diseases* 2018;6.
- [3] Durantel D, Zoulim F. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. *Journal of hepatology* 2016;64:S117-S131.
- [4] Bloom K, Maepa MB, Ely A, Arbuthnot P. Gene Therapy for Chronic HBV-Can We Eliminate cccDNA? *Genes* 2018;9.
- [5] Lucifora J, Protzer U. Attacking hepatitis B virus cccDNA--The holy grail to hepatitis B cure. *Journal of hepatology* 2016;64:S41-S48.
- [6] Diab AM, Foca A, Fusil F, Lahlali T, Jalaguier P, Amirache F, et al. Polo-like-kinase 1 is a proviral host-factor for hepatitis B virus replication. *Hepatology* 2017.
- [7] Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nature reviews Drug discovery* 2010;9:643-660.
- [8] Studach LL, Menne S, Cairo S, Buendia MA, Hullinger RL, Lefrancois L, et al. Subset of Suz12/PRC2 target genes is activated during hepatitis B virus replication and liver carcinogenesis associated with HBV X protein. *Hepatology* 2012;56:1240-1251.
- [9] Wang WH, Studach LL, Andrisani OM. Proteins ZNF198 and SUZ12 are down-regulated in hepatitis B virus (HBV) X protein-mediated hepatocyte transformation and in HBV replication. *Hepatology* 2011;53:1137-1147.

- [10] Andrisani OM, Studach L, Merle P. Gene signatures in hepatocellular carcinoma (HCC). *Seminars in cancer biology* 2011;21:4-9.
- [11] Studach LL, Rakotomalala L, Wang WH, Hullinger RL, Cairo S, Buendia MA, et al. Polo-like kinase 1 inhibition suppresses hepatitis B virus X protein-induced transformation in an in vitro model of liver cancer progression. *Hepatology* 2009;50:414-423.
- [12] Spankuch-Schmitt B, Bereiter-Hahn J, Kaufmann M, Strebhardt K. Effect of RNA silencing of polo-like kinase-1 (PLK1) on apoptosis and spindle formation in human cancer cells. *Journal of the National Cancer Institute* 2002;94:1863-1877.
- [13] Yao YD, Sun TM, Huang SY, Dou S, Lin L, Chen JN, et al. Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis. *Science translational medicine* 2012;4:130ra148.
- [14] Judge AD, Robbins M, Tavakoli I, Levi J, Hu L, Fronda A, et al. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *The Journal of clinical investigation* 2009;119:661-673.
- [15] Marion MJ, Hantz O, Durantel D. The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. *Methods in molecular biology* 2010;640:261-272.
- [16] Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Canine I, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* 2002;99:15655-15660.
- [17] Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *J Gen Virol* 2009;90:127-135.
- [18] Lecluyse EL, Alexandre E. Isolation and culture of primary hepatocytes from resected human liver tissue. *Methods in molecular biology* 2010;640:57-82.
- [19] Jeffs LB, Palmer LR, Ambegia EG, Giesbrecht C, Ewanick S, MacLachlan I. A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharmaceutical research* 2005;22:362-372.
- [20] Lahlali T, Berke JM, Vergauwen K, Foca A, Vandyck K, Pauwels F, et al. Novel potent capsid assembly modulators regulate multiple steps of the Hepatitis B virus life-cycle. *Antimicrobial agents and chemotherapy* 2018.
- [21] Weil D, Boutain S, Audibert A, Dautry F. Mature mRNAs accumulated in the nucleus are neither the molecules in transit to the cytoplasm nor constitute a stockpile for gene expression. *Rna* 2000;6:962-975.
- [22] Lucifora J, Salvetti A, Marniquet X, Mailly L, Testoni B, Fusil F, et al. Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. *Antiviral research* 2017;145:14-19.
- [23] Cai D, Nie H, Yan R, Guo JT, Block TM, Guo H. A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. *Methods in molecular biology* 2013;1030:151-161.
- [24] Wang J, Shen T, Huang X, Kumar GR, Chen X, Zeng Z, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. *Journal of hepatology* 2016;65:700-710.
- [25] Bissig KD, Wieland SF, Tran P, Isogawa M, Le TT, Chisari FV, et al. Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *The Journal of clinical investigation* 2010;120:924-930.
- [26] Studach L, Wang WH, Weber G, Tang J, Hullinger RL, Malbrue R, et al. Polo-like kinase 1 activated by the hepatitis B virus X protein attenuates both the DNA damage checkpoint and DNA repair resulting in partial polyploidy. *The Journal of biological chemistry* 2010;285:30282-30293.
- [27] Liu Z, Sun Q, Wang X. PLK1, A Potential Target for Cancer Therapy. *Translational oncology* 2017;10:22-32.
- [28] Vitour D, Dabo S, Ahmadi Pour M, Vilasco M, Vidalain PO, Jacob Y, et al. Polo-like kinase 1 (PLK1) regulates interferon (IFN) induction by MAVS. *The Journal of biological chemistry* 2009;284:21797-21809.
- [29] Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, et al. Structural organization of the hepatitis B virus minichromosome. *Journal of molecular biology* 2001;307:183-196.
- [30] Chong CK, Cheng CYS, Tsoi SYJ, Huang FY, Liu F, Seto WK, et al. Role of hepatitis B core protein in HBV transcription and recruitment of histone acetyltransferases to cccDNA minichromosome. *Antiviral research* 2017;144:1-7.
- [31] Audetat KA, Galbraith MD, Odell AT, Lee T, Pandey A, Espinosa JM, et al. A Kinase-Independent Role for Cyclin-Dependent Kinase 19 in p53 Response. *Molecular and cellular biology* 2017;37.

- [32] Jang KH, Jang T, Son E, Choi S, Kim E. Kinase-independent role of nuclear RIPK1 in regulating parthanatos through physical interaction with PARP1 upon oxidative stress. *Biochimica et biophysica acta Molecular cell research* 2018;1865:132-141.
- [33] Li Q, Pene V, Krishnamurthy S, Cha H, Liang TJ. Hepatitis C virus infection activates an innate pathway involving IKK- α in lipogenesis and viral assembly. *Nature medicine* 2013;19:722-729.
- [34] Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. *Nature reviews Cancer* 2014;14:598-610.
- [35] Vivanco I, Chen ZC, Tanos B, Oldrini B, Hsieh WY, Yannuzzi N, et al. A kinase-independent function of AKT promotes cancer cell survival. *eLife* 2014;3.
- [36] Wang Z, Fu M, Wang L, Liu J, Li Y, Brakebusch C, et al. p21-activated kinase 1 (PAK1) can promote ERK activation in a kinase-independent manner. *The Journal of biological chemistry* 2013;288:20093-20099.
- [37] Dion S, Bourguine M, Godon O, Levillayer F, Michel ML. Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. *Journal of virology* 2013;87:5554-5563.
- [38] Martin P, Dubois C, Jacquier E, Dion S, Mancini-Bourguine M, Godon O, et al. TG1050, an immunotherapeutic to treat chronic hepatitis B, induces robust T cells and exerts an antiviral effect in HBV-persistent mice. *Gut* 2015;64:1961-1971.
- [39] Huang YH, Fang CC, Tsuneyama K, Chou HY, Pan WY, Shih YM, et al. A murine model of hepatitis B-associated hepatocellular carcinoma generated by adeno-associated virus-mediated gene delivery. *International journal of oncology* 2011;39:1511-1519.
- [40] Li TN, Wu YJ, Tsai HW, Sun CP, Wu YH, Wu HL, et al. Intrahepatic hepatitis B virus large surface antigen induces hepatocyte hyperploidy via failure of cytokinesis. *The Journal of pathology* 2018;245:502-513.

FIGURE LEGENDS

Figure 1. Evaluation of the efficacy and toxicity of LNP siPLK1 in primary human hepatocytes.

PHHs were left uninfected (non-infected control; NI) or were infected with HBV genotype D at a multiplicity of infection of 200 vge/cell. From 4 days post-infection (p.i.), cells were mock-treated (non treated control; NT) or treated 3 times 3 days apart with increasing LNP siPLK1 concentrations or the indicated control molecules (LNP siHBV and LNP siCtrl were used at 200ng/mL and 10 μ g/mL of puromycin served as positive death control, Tenofovir (TFV) was used at a concentration of 10 μ M). Cells and supernatants were harvested at day-13 p.i. (day-9 post-treatment) for subsequent analyses. (A) RNAs were extracted and levels of PLK1 mRNAs (relative to PRNP mRNAs) analyzed by RT-qPCR. Results were normalized to the NT condition. (B) Proteins were extracted from cytosol and nuclear compartments with NE-PER Nuclear and Cytoplasmic kit. Thirty μ g of nuclear fraction for each condition were analyzed by western blot. Lamin B1 served as loading control for nuclear proteins while β -Tubulin served as control for cytoplasm contamination (a representative blot is shown). (C) ATP production, reflecting cell viability, was measured using Cell TiterGlo One Solution Assay. Results presented in graphs are the mean $\pm$ SEM (standard error of the mean) of 3 independent experiments (3 donors of PHH) each performed in biological triplicate. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

Figure 2. Antiviral activity of LNP siPLK1 in non-transformed and non-dividing HBV-infected PHH.

PHH were left uninfected (non-infected control; NI) or were infected with HBV genotype D at a multiplicity of infection of 200 vge/cell. From 4 days p.i., cells were mock-treated (non treated control; NT) or treated 3 times 3 days apart with indicated molecules. LNP siHBV and LNP siCtrl were used at 200ng/mL and TFV at 10 μ M. (A) Three days after the last treatment (day-13 post-infection/day-9 post-treatment), secreted viral parameters were measured in the supernatants. RNA and DNA were extracted from viral particles and analyzed by HBV-specific RT-qPCR or qPCR,

whereas levels of secreted ApoB, HBeAg and HBsAg were assessed by ELISA. (B) Intracellular total RNA and DNA were extracted and analyzed by HBV-specific RT-qPCR or qPCR. Results presented in graphs are the mean $\pm$ SEM of 3 independent experiments (3 donors of PHH) each performed in biological triplicate and are presented as ratio compared to the non-treated (NT) condition. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

Figure 3. LNP siPLK1 does not affect cccDNA amount. (A) A representative Southern blot, performed with pooled total DNA ($n=3$) from **Fig. 2** is shown. Two micrograms of DNA were loaded per lane and separated into 1% agarose gel. Separated DNA were transferred onto a nylon membrane and were hybridized with a digoxigenin labelled HBV probe, as described in detail in [22]. EcoRI digestion serves as control as it allows cccDNA linearization of HBV genotype D (shift from coiled 2.2kb band to full length 3.2kb linear DNA). (B) Quantification of Southern blot presented in panel B using image Lab software. Mitochondrial DNA was used as loading control and served for normalization. (C) Intracellular cccDNA specific Taqman qPCR [20] from PHH samples from **Fig. 2**, relative to non-treated (NT) condition. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

Figure 4. Pre-infection treatment with LNP siPLK1 in primary human hepatocytes. Experiments were conducted on three independent PHH batches. Drugs were administered this time before and during the inoculation with HBV. In total 3 treatments were performed 3 days apart. The last treatment was concomitant to the inoculation with HBV at 200vge/cell. LNP siPLK1 were used at indicated concentrations. LNP siHBV and LNP siCtrl were used at 200ng/mL and TFV at 10 μ M. PreS1 peptide at 100nM served as an entry inhibitor. NT and NI are respectively infected/non-treated and non-infected/non-treated controls. Three days after infection (and 3 days after the last treatment), culture medium was changed (no treatment anymore) and cells were maintained

for 4 additional days. (A) At day-7 post infection, RNA were extracted, reverse-transcribed and subjected to specific PLK1 qPCR. Normalization was done on PrP and quantification is relative to PLK1 expression in non-treated (NT) condition. (B) At the end of the experiment, secreted viral parameters were measured in the supernatants. RNA and DNA were extracted from viral particles and analyzed by HBV-specific RT-qPCR or qPCR, whereas levels of secreted ApoB, HBeAg and HBsAg were assessed by ELISA. (C) Intracellular total RNA and DNA extracted from HBV infected cells were analyzed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared to non-treated (NT) condition, whereas intracellular cccDNA was analysed by specific Taqman qPCR [20]. Results presented in graphs are the mean of 3 independent experiments with SEM. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

Figure 5. Localization of HBV RNAs under LNP siPLK1 treatment. Differentiated HepaRG cells were infected with HBV at 200 vge/cell. After 7 days of infection cells were mock-treated (non treated control; NT) or treated 2 times 3 days apart with indicated molecules at indicated concentration. At day-13 post-infection (day-6 post treatment) total RNA were extracted, reverse transcribed and submitted to HBV specific qPCR. Cytosolic and nuclear compartments were recovered using a homemade protocol adapted from *Weil et al.*, [21]. RNA were extracted using trizol and reverse transcribed. Home-designed primers were used to amplify the nuclear specific U6 snRNA and the Human Tyr-tRNA for the cytoplasm compartment and served as purity control as well as housekeeping genes. Normalized on housekeeping gene and relative to non-treated (NT) condition. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

8. SUPPORTING FIGURE LEGENDS

Supporting Figure 1. Efficacy of LNP siPLK1 and toxicity profile in HepG2 cells. (A) HepG2 cells were treated with increasing doses of LNP siPLK1, ranging from 1.3ng/mL to 133ng/mL. RNA were extracted, reverse transcribed and PLK1 mRNA quantified using specific qPCR. Normalized to housekeeping gene and relative to Mock-Treated condition. (B) Cell viability was assessed with CellTiter Blue reagent and plotted among with knockdown of PLK1 RNA under various LNP siPLK1 concentrations.

Supporting Figure 2. Antiviral activity of LNP siPLK1 in non-transformed and non-dividing dHepaRG cells. Differentiated HepaRG were left uninfected (non-infected control; NI) or were infected with HBV genotype D at a multiplicity of infection of 200 vge/cell. From 7 days p.i., cells were mock-treated (non treated control; NT) or treated 2 times 3 days apart with indicated molecules. LNP siHBV and LNP siCtrl were used at 200ng/mL and TFV at 10 μ M. (A) RNAs were extracted and levels of PLK1 mRNAs (relative to PRNP mRNAs) analyzed by RT-qPCR. Results were normalized to the NT condition. (B) Three days after the last treatment (day-13 post-infection/day-6 post-treatment), levels of secreted HBeAg and HBsAg were assessed by ELISA. (C) Intracellular total RNA and DNA were extracted and analyzed by HBV-specific RT-qPCR or qPCR. Results presented in graphs are the mean $\pm$ SEM of 3 independent experiments (3 donors of PHH) each performed in biological triplicate and are presented as ratio compared to the non-treated (NT) condition. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

Supporting Figure 3. Antiviral activity of LNP siPLK1. PHH from liver-humanized mice. Mice were perfused for 34 days to obtain PHH and were infected with 1.10^9 HBV genotype D. Liver was digested by *in situ* perfusion and PHH seeded into 24-well plates. LNP treatments were conducted every 4 days starting at Day 0 and BI-2536 treatment was started on Day 0 and replenished every

tow days. Prior to treatments, the culture supernatants were collected and analysed for secreted HBV DNA and HBsAg levels. Secreted HBV DNA at D16 (Ai) and HBsAg at D16 (Aii).

Supporting Figure 4. pPLK1 is preferentially found in the nucleus of infected PHH. Western Blot of nuclear extracts shown in Figure 1D with above the quantification method used with Image Lab software. The pPLK1-S₁₃₇ / Lamin B1 ratio allows correction of uneven overall protein quantity between each condition. Quantifications are presented relative to Mock-Treated condition, as indicated by the green R. The right panel shows western blot of corresponding cytosolic extracts, 30µg prot./lane. Lamin B1 and β-Tub served as purity control of cyto/nucleus fractionation.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

SUPPLEMENTAL FIGURES

Supplemental Figure 1

A

B

Supplemental Figure 2

A

B

C

Supplemental Figure 3

Ai

Aii

- Mock-Treated
- 100ng/mL LNP siCtrl
- DMSO
- 500nM BI-2536
- ▲ 100ng/mL LNP siPLK1
- ▲ 300ng/mL LNP siPLK1
- ◆ 3ng/mL LNP siHBV

Supplemental Figure 4

Nuclear extract

Cytosolic extract