Original article

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

*Adrien Foca[1,2], Ammen Dhillon[3], Thomas Lahlali[1,2], Julie Lucifora[1,2], Anna Salvetti[1,2], Michel Rivoire[4], Amy Lee[3], David Durantel[1,2,5]**

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

*Corresponding author e-mail: david.durantel@inserm.fr

Background: 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.

Methods: 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.

Results: A dose as low as 100 ng/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 µM 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.

Conclusions: This study emphasizes that a specific PLK1 inhibition could help in achieving an improved HBsAg loss in CHB patients, likely in combination with other HBsAg-targeting strategies.

Introduction

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 hundreds of millions of 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 with the general population [1]. When patients are successfully and continiously treated with safe nucleoside/nucleotide analogues (NUCs), their risk of developing HCC is 2–5× higher compared with normal individual [2,3]. This remaining risk of HCC under treatment is likely due to the fact that NUCs do not allow the elimination of the HBV replicative intermediate called covalently closed circular DNA (cccDNA), which is responsible for viral persistence and rebound when treatment is stopped [4,5]. In addition to the development of drugs that may target cccDNA directly [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 to be 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; for example, 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 virological and oncological aspects of the disease.

Due to their poor specificity (that is, 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 trialled 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 trialled in patients with advanced HCC (NCT02191878).

In this study, we found that the use of LNP siPLK1 in HBV-infected primary human hepatocytes (PHHs) led 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 hepatitis B surface antigen (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 other reagents

PLK1 inhibitor BI-2536 was purchased from Selleckchem (Munich, Germany). All other chemicals were purchased from Sigma-Aldrich (Lyon, France). Rabbit polyclonal anti-HBc antibodies were purchased from Dako (Agilent Technologies, Les Ulis, France; B0586) or Abcam (Cambridge, UK; 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-S_{137}; rabbit monoclonal anti-PLK1 antibodies from Abcam (ab115095) were used for detection of phospho-PLK1-T_{210} 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]. 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′-AGA**U**CACCC**U**CCU-**U**AAA**U**AUU-3′ and UAUUUAA**G**GAGGGUGA**U**C-UUU-3′, and feature 2′OMe modification at indicated position (bold underlined) as previously described [14]. This dupleix 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 chole-sterol.

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′-CGACCU-UGAGGCAUACUUCUU-3′ and Rv: 5′-GAAGUAGC-CUCAAGGUCGUU-3′ ON-TARGETplus duplex. LNP siRNAs were administrated to cells by direct dilution into the culture medium. Cells were exposed to LNPs for 24 h, 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 hepatitis B e antigen (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, Zhengzhou, China) 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, Düren, Germany) respectively, according to manufacturer's instructions. Reverse

transcription of RNA into cDNA was performed with the SuperScript® III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. All qPCR analyses were performed with LightCycler™ 96 and 480 systems (Roche, Basel, Switzerland), except for cccDNA and pgRNA specific PCRs that were processed under a QuantStudio™ 7 (Applied Biosystems, Waltham, MA, USA), using homemade TaqMan assays [20].

Primary human hepatocyte preparation from chimeric FRG mice and use for antivirals

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), engraphted with PHH and infected with 1.10^9 virus genome equivalent (vge) of HBV genotype D. When HBV viraemia had reached a maximum (that is, around 10e9 vge/ml), the liver was digested by *in situ* collagenase perfusion and recovered hepatocytes seeded in DMEM medium into 24-well plates ($2.7.10^5$ 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 2 days. DNA in the culture supernatants was isolated using QIAamp 96 DNA Blood Kit (Qiagen, Courtaboeuf, France) and quantified by qPCR. HBsAg was quantified using specific CLIA kit (Autobio Diagnostics).

Separation of cytoplasm and nucleoplasm

Extractions of nuclear and cytosolic proteins were performed using NE-PER Nuclear and Cytoplasmic kit (Thermo Scientific, Waltham, MA, USA), 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, Lyon, France) and precipitation with isopropanol. Purity of nuclear versus 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 analysed by agarose electrophoresis in native condition followed by transfer onto an enhanced chemiluminescence membrane (Amersham/GE Healthcare, Velizy, France) 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

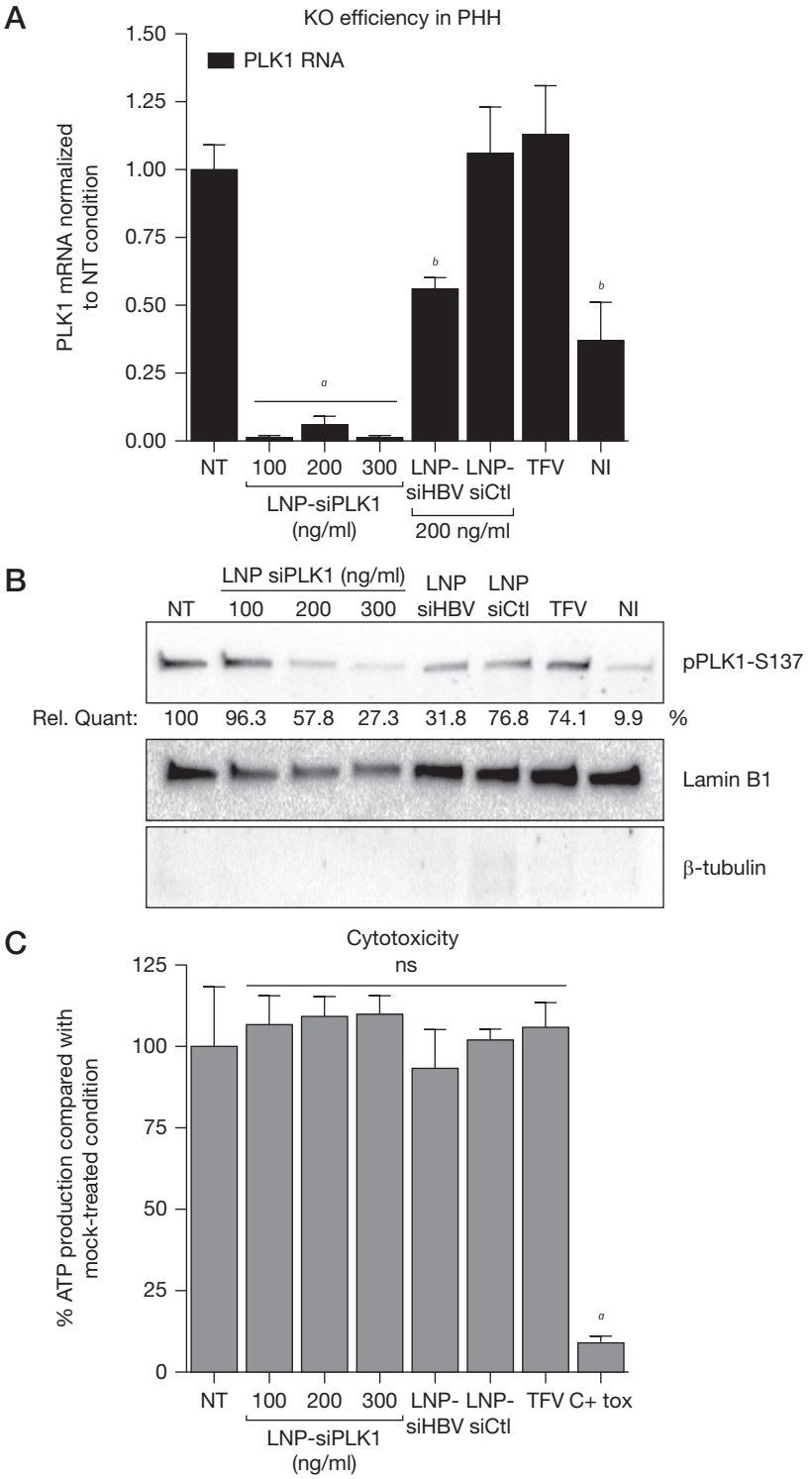
Efficacy and safety of LNP siPLK1 in hepatocytes

Compared with control LNP siRNAs, LNP siPLK1 were efficient at inhibiting PLK1 expression, with a 70% loss of PLK1-mRNA at 13.3 ng/ml without toxicty in proliferating HepG2 cells. As expected, LNP siPLK1 induced a 50% mortality in proliferating HepG2 cells at 133 ng/ml due to active cell division and crucial role of PLK1 in G2/M transition (Additional file 1). Considering these data, as well as the concentration correspondence (that is, 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 concentrations going from 100 to 300 ng/ml. We observed a strong reduction in the levels of PLK1 mRNA in both dHepaRG and PHH (Additional file 2 panel A and Figure 1A) and in the level of PLK1 phosphoprotein in PHH (Figure 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; Figure 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 (three different batches tested) even when LNP siPLK1 was administrated 3 times at 300 ng/ml (Figure 1C). Moreover, PHH remained functional, as measured by the apolipoprotein B (ApoB) secretion (see apoB dosing in Figure 2A), a component of very low density lipoprotein (VLDL) that are made by healthy hepatocytes.

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

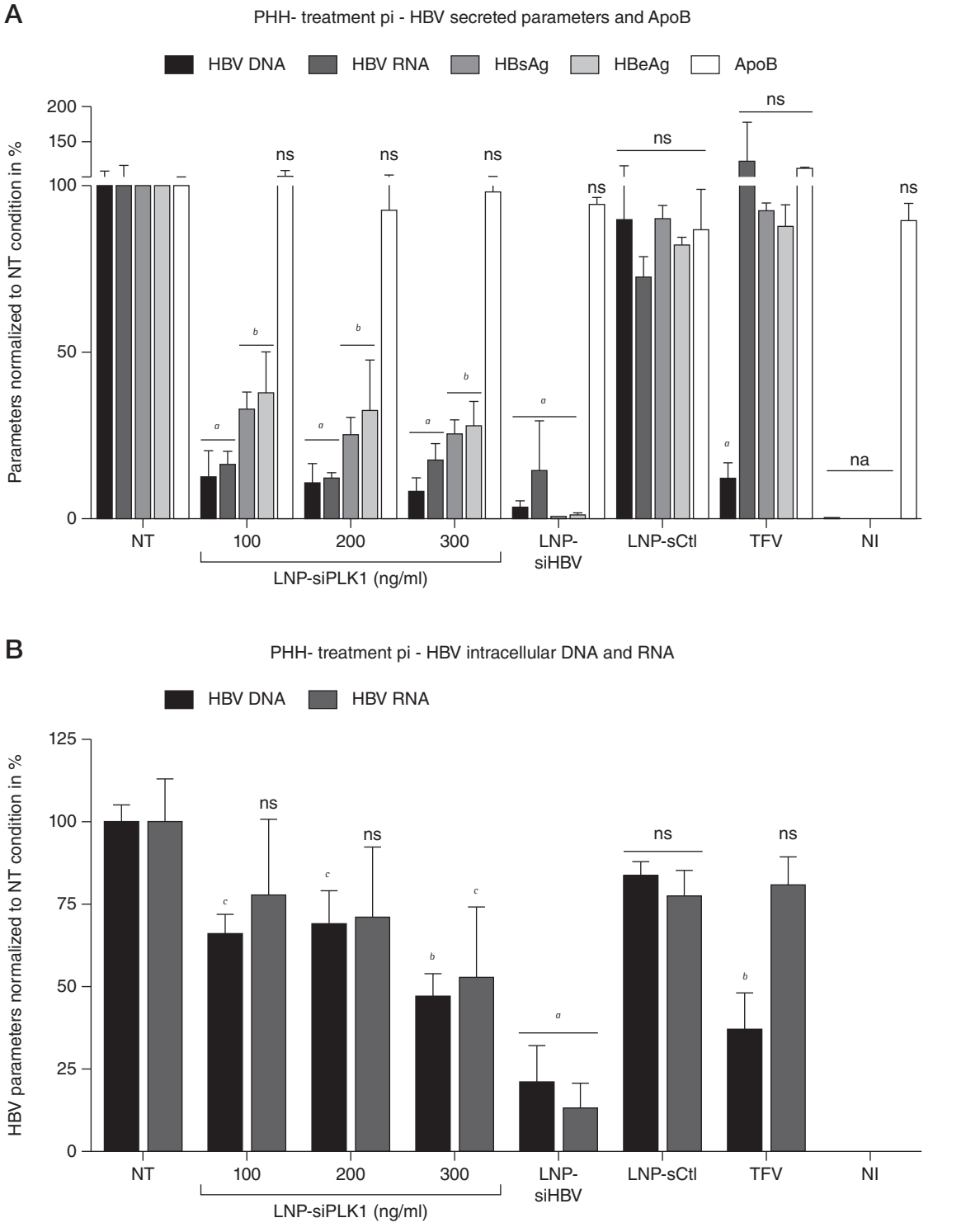
To evaluate the antiviral activity of LNP siPLK1, freshly isolated PHH from three different donors were infected

Figure 1. Evaluation of the efficacy and toxicity of LNP siPLK1 in PHHs



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 viral genome equivalents (vge)/cell. From 4 days post-infection (pi), cells were mock-treated (non-treated control; NT) or treated 3× 3 days apart with increasing LNP siPLK1 concentrations or the indicated control molecules (LNP siHBV and LNP siCtrl) were used at 200 ng/ml and 10 µg/ml of puromycin served as positive death control, tenofovir (TFV) was used at a concentration of 10 µM. Cells and supernatants were harvested at day 13 pi (day 9 post-treatment) for subsequent analyses. **(A)** RNAs were extracted and levels of PLK1 mRNAs (relative to PRNP mRNAs) analysed 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. 30 µg of nuclear fraction for each condition were analysed 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 ±SEM of 3 independent experiments (3 donors of PHH) each performed in biological triplicate. $^{a}P \leq 0.001$ and $^{b}P \leq 0.01$ were considered statistically significant.

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



Primary human hepatocytes (PHH) were left uninfected (non-infected control; NI) or were infected with HBV genotype D at a multiplicity of infection of 200 viral genome equivalents (vge)/cell. From 4 days post-infection (pi), cells were mock-treated (non-treated control; NT) or treated 3× 3 days apart with indicated molecules. LNP siHBV and LNP siCtrl were used at 200 ng/ml and tenofovir (TFV) at 10 μM. **(A)** 3 days after the last treatment (day 13 pi/day 9 post-treatment), secreted viral parameters were measured in the supernatants. RNA and DNA were extracted from viral particles and analysed by HBV-specific RT-qPCR or qPCR, whereas levels of secreted ApoB, hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) were assessed by ELISA. **(B)** Intracellular total RNA and DNA were extracted and analysed by HBV-specific RT-qPCR or qPCR. Results presented in graphs are the mean ±SEM of 3 independent experiments (3 donors of PHH) each performed in biological triplicate and are presented as ratio compared with the non-treated (NT) condition. $^{a}P \leq 0.001$, $^{b}P \leq 0.01$ and $^{c}P \leq 0.05$ were considered statistically significant.

with HBV at a high multiplicity of infection, and at day 4 post-infection (pi), when the average HBsAg production reached around 1,000 IU/ml (which is a level indicating a high replication of HBV), cells were treated 3× every 3 days with increasing LNP siPLK1 doses or control molecules. Secretion of viral DNA (that is, infectious virion) and RNA (that is, RNA-containing particles [24]) were significantly inhibited by LNP siPLK1 (Figure 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 (Figure 1B) was associated with a strong inhibition of virus replication. Interestingly, we also observed a 70% inhibition of viral antigen HBeAg and HBsAg secretion (Figure 2A), without any effect on ApoB secretion, highlighting a specificity of LNP siPLK1 action on those secreted viral proteins. In the meantime, tenofovir (TFV) did not affect HBV antigen 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 (Additional file 2 panel B). 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 (that is, around 1.10^9 copies/ml at day 26 post-infection), mimicking the highly viraemic 'immune tolerant' phase in human. In this model, 300 ng/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 (Additional file 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 insigths on the observed phenotype on secreted HBV antigens, intracellular viral parameters were also analysed. We observed a dose dependent decrease of intracellular HBV DNAs and RNAs in HBV-infected PHH or dHepaRG cells treated with LNP siPLK1 (Figure 2B and Additional file 2 panel C). 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 (Figure 2B and Additional file 2 panel C). 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 with that 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 (analysed by qPCR or Southern blot; Figure 3). These data suggest that the decrease of HBV RNA observed with LNP siPLK1 is a consequence of a transcriptional and/or post-trancriptional event.

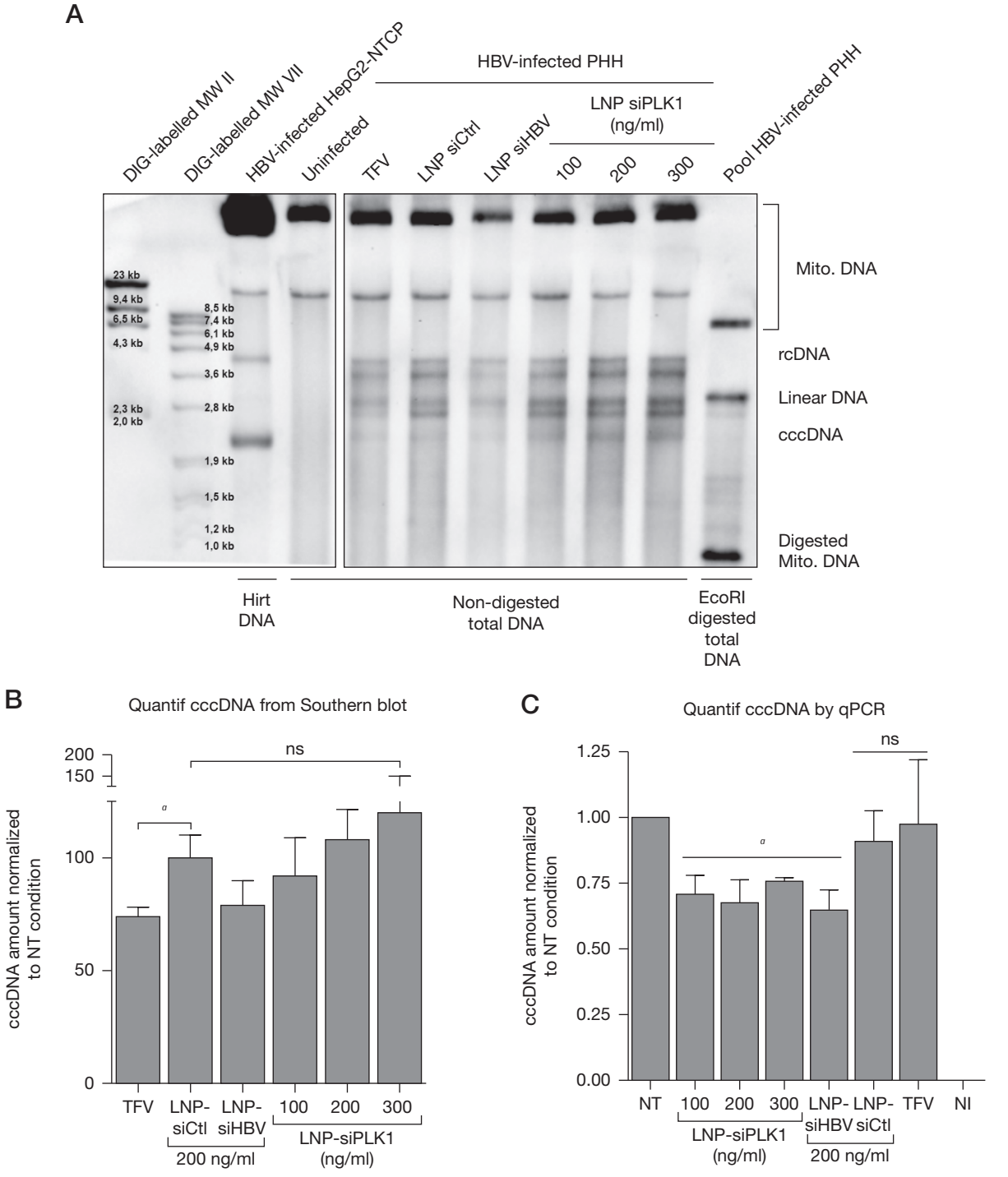
LNP siPLK1 does not inhibit better HBV replication in pre-infection treatment setting

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 5 dpi PLK1 mRNA knockdown was still >75% with 100 and 300 ng/ml of LNP siPLK1 (Figure 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 (that is, PreS1 peptide), which very efficiently prevented HBV infection in this setting (as shown in other panels of Figure 4). Regarding antiviral phenotypes obtained with LNP siPLK1, they were very similar to those obtained in post-infection treatment setting (compare results of Figures 2 and 4). Importantly, cccDNA establishment was not fully blocked by either LNP siPLK1 or LNP siHBV, as compared with that observed with PreS1 peptide, which acts a genuine entry inhibitor. The slight decreased amount of cccDNA observed with LNP siPLK1 and LNP siHBV was poorly significant compared with 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 with 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

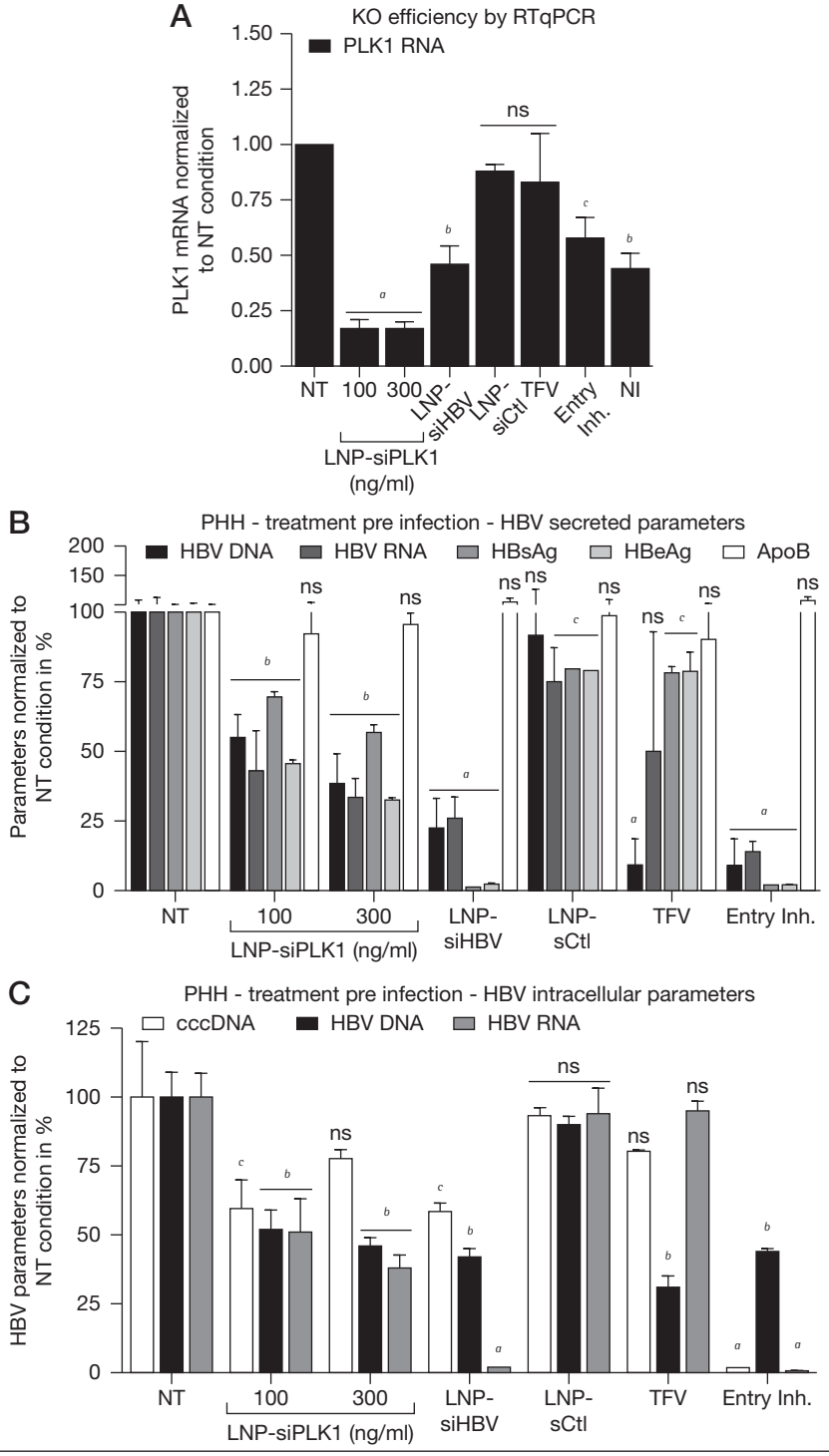
In HBV-infected PHH, activated pPLK1-S_{137} was mainly localized in the nucleus (Additional file 4 and refer also to [6]). As we observed a decrease in HBV

Figure 3. LNP siPLK1 does not affect cccDNA amount



(A) A representative Southern blot performed with pooled total DNA (n=3) from Figure 2 is shown. 2 µg of DNA were loaded per lane and separated into 1% agarose gel. Separated DNA was tranfered onto a nylon membrane and 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 lengh 3.2kb linear DNA). **(B)** Quantification of Southern blot presented in (A) using image Lab software. Mitochondrial DNA was used as loading control and served for normalization. **(C)** Intracellular cccDNA specific Taqman qPCR [20] from primary human hepatocyte (PHH) samples from Figure 2, relative to non-treated (NT) condition. $^{a}P \leq 0.05$ was considered statistically significant. NI, non infected; ns, not significant; TFV, tenofovir.

Figure 4. Pre-infection treatment with LNP siPLK1 in PHHs



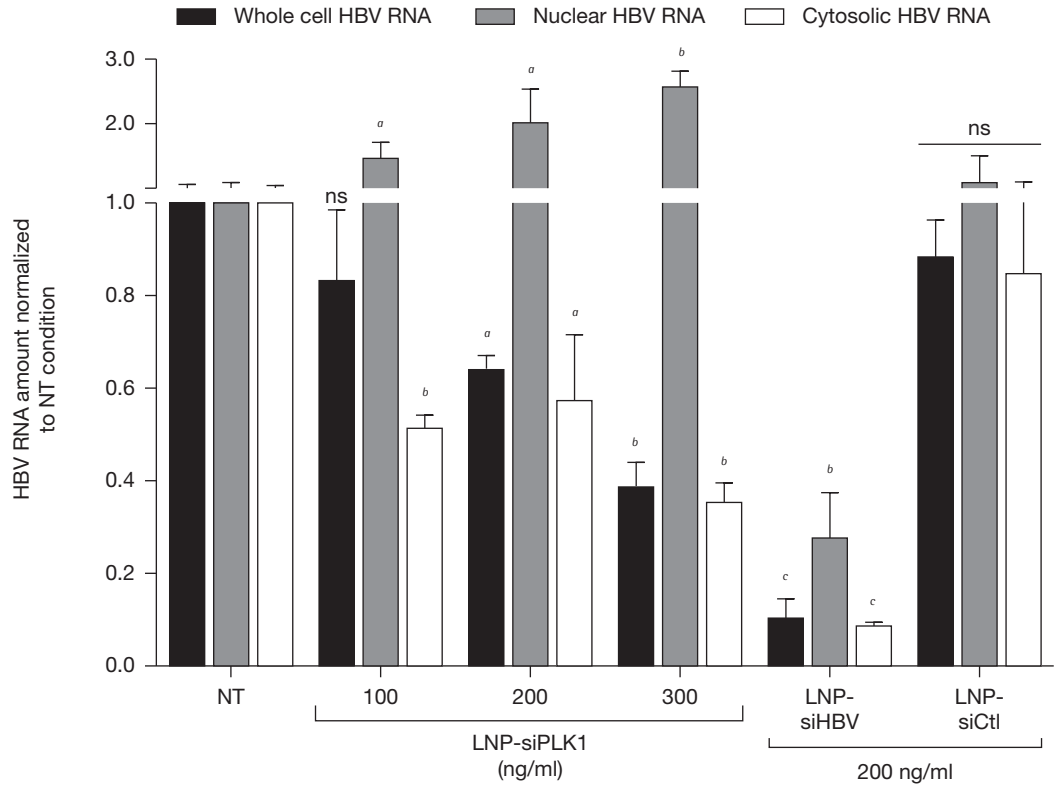
Experiments were conducted on three independent primary human hepatocyte (PHH) batches. Drugs were administrated this time before and during the inoculation with HBV. In total, 3 treatments were performed 3 days apart. The last treatment was concomittant to the inoculation with HBV at 200 viral genome equivalents (vge)/cell. LNP siPLK1 were used at indicated concentrations. LNP siHBV and LNP siCtl were used at 200 ng/ml and tenofovir (TFV) at 10 µM. PreS1 peptide at 100 nM served as an entry inhibitor. NT and NI are respectively infected/non-treated and non-infected/non-treated controls. 3 days after infection (and 3 days after the last treatment), culture medium was changed (no treatement anymore) and cells were maintained for 4 additional days. **(A)** At day 7 post infection (pi), 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 analysed by HBV-specific RT-qPCR or qPCR, whereas levels of secreted ApoB, hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) were assessed by ELISA. **(C)** Intracellular total RNA and DNA extracted from HBV-infected cells were analysed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared with 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. $^{a}P \leq 0.001$, $^{b}P \leq 0.01$ and $^{c}P \leq 0.05$ were considered statistically significant.

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 3 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 RNA 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 RNA levels was observed, similar to that observed with whole cell extracts (Figure 5). By contrast, in the nuclear compartment, a dose-dependent accumulation of HBV RNA was observed under LNP siPLK1 treatment (Figure 5C). As the amount of cytosolic HBV RNA was overall 10× higher than that observed in the nucleus, this accumulation of HBV RNA in the nucleus induced by LNP siPLK1 did not modify the overall observed phenotype (that is, reduction of total RNA accumulation), but certainly accounted for the decrease in viral protein synthesis.

Discussion

HCC ranks second in terms of cancer-induced death worldwide, with an estimated 600,000 deaths/year burden. HCC development is the consequence of the interplay between genetic predispositions, environmental factors (for example, 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

Figure 5. Localization of HBV RNAs under LNP siPLK1 treatment



Differentiated HepaRG cells were infected with HBV at 200 viral genome equivalents (vge)/cell. After 7 days of infection cells were mock-treated (non-treated control; NT) or treated 2× 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 was 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. $^{a}P \leq 0.05$, $^{b}P \leq 0.01$ and $^{c}P \leq 0.001$ were considered statistically significant. ns, not significant.

HCC worlwide. 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 intresting 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, an 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 other kinase functions; this may account for the poor toxicological 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 line with this, 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 purpose 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 more efficiently inhibits HBV replication than naked siRNA or SM-PLK1i (reported in [6]) in the absence of cell toxicity. Indeed 100 to 300 ng/ml of LNP siPLK1 (corresponding to 7.5 to 22.5 nM siPLK1) led to a >75% decrease in HBV viraemia *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 of potently inhibiting 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 previously 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 phetotype of LNP siPLK1 on HBV antigen production is particularly relevant, as the next step for the improvement of current HBV therapy in the clinic is precisely to be capable of reducing antigenaemia 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 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 mechanism of this transcriptional and/or post-transcriptional regulation (that is, 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 C-terminal domain (CTD) could be a determinant for HBc-cccDNA interaction [30] and in our previous 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 the 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 with siRNA targeting HBV itself. Whether siRNA targeting HBV are currently in clinical trials in CHB patients (for example, Phase II for ALN-HBV, NCT02826018; Phase II 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 restoration 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 HCC from HBV aetiology, 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 models 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 (all from CRCL, INSERM, U1052) for PHH isolation as well as the staff from MR'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 programme '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.

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.

Disclosure statement

DD received a research grant from Arbutus Biopharma for this work. AD and AL are Arbutus Biopharma's employees. AS, AF, TL, JL and MR declare no competing interests.

Additional files

Additional file 1: A figure showing efficacy of LNP siPLK1 and toxicity profile in HepG2 cells can be found at https://www.intmedpress.com/uploads/documents/4567_Foca_Addfile1.pdf

Additional file 2: A figure showing antiviral activity of LNP siPLK1 in non-transformed and non-dividing dHepaRG cells can be found at https://www.intmedpress.com/uploads/documents/4567_Foca_Addfile2.pdf

Additional file 3: A figure showing antiviral activity of LNP siPLK1 in PHH isolated from liver humanized mice can be found at https://www.intmedpress.com/uploads/documents/4567_Foca_Addfile3.pdf

Additional file 4: A figure showing pPLK1 is preferentially found in the nucleus of infected PHH can be found at https://www.intmedpress.com/uploads/documents/4567_Foca_Addfile4.pdf

References

1. Chan SL, Wong VWS, Qin S, Chan HLY. Infection and cancer: the case of hepatitis B. *J Clin Oncol* 2016; **34:**83–90.
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:**27.
3. Durantel D, Zoulim F. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. *J Hepatol* 2016; **64:**S117–S131.
4. Bloom K, Maepa MB, Ely A, Arbuthnot P. Gene therapy for chronic HBV-can we eliminate cccDNA? *Genes (Basel)* 2018; **9:**207.
5. Lucifora J, Protzer U. Attacking hepatitis B virus cccDNA--the holy grail to hepatitis B cure. *J Hepatol* 2016; **64:**S41–S48.
6. Diab AM, Foca A, Fusil F, *et al*. Polo-like-kinase 1 is a proviral host-factor for hepatitis B virus replication. *Hepatology* 2017; **66:**1750–1765.
7. Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nat Rev Drug Discov* 2010; **9:**643–660.

8. Studach LL, Menne S, Cairo S, *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). *Semin Cancer Biol* 2011; **21:**4–9.
11. Studach LL, Rakotomalala L, Wang WH, *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. Spänkuch-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. *J Natl Cancer Inst* 2002; **94:**1863–1877.
13. Yao YD, Sun TM, Huang SY, *et al*. Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis. *Sci Transl Med* 2012; **4:**130ra148.
14. Judge AD, Robbins M, Tavakoli I, *et al*. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* 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 Mol Biol* 2010; **640:**261–272.
16. Gripon P, Rumin S, Urban S, *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 Mol Biol* 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. *Pharm Res* 2005; **22:**362–372.
20. Lahlali T, Berke JM, Vergauwen K, *et al*. Novel potent capsid assembly modulators regulate multiple steps of the hepatitis B virus life-cycle. *Antimicrob Agents Chemother* 2018; **62:**e00835-18.
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, *et al*. Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. *Antiviral Res* 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 Mol Biol* 2013; **1030:**151–161.
24. Wang J, Shen T, Huang X, *et al*. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. *J Hepatol* 2016; **65:**700–710.
25. Bissig KD, Wieland SF, Tran P, *et al*. Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *J Clin Invest* 2010; **120:**924–930.
26. Studach L, Wang WH, Weber G, *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. *J Biol Chem* 2010; **285:**30282–30293.
27. Liu Z, Sun Q, Wang X. PLK1, a potential target for cancer therapy. *Transl Oncol* 2017; **10:**22–32.
28. Vitour D, Dabo S, Ahmadi Pour M, *et al*. Polo-like kinase 1 (PLK1) regulates interferon (IFN) induction by MAVS. *J Biol Chem* 2009; **284:**21797–21809.
29. Bock CT, Schwinn S, Locarnini S, *et al*. Structural organization of the hepatitis B virus minichromosome. *J Mol Biol* 2001; **307:**183–196.
30. Chong CK, Cheng CYS, Tsoi SYJ, *et al*. Role of hepatitis B core protein in HBV transcription and recruitment of histone acetyltransferases to cccDNA minichromosome. *Antiviral Res* 2017; **144:**1–7.
31. Audetat KA, Galbraith MD, Odell AT, *et al*. A kinase-independent role for cyclin-dependent kinase 19 in p53 response. *Mol Cell Biol* 2017; **37:**e00626-16.
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. *Biochim Biophys Acta Mol Cell Res* 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-alpha in lipogenesis and viral assembly. *Nat Med* 2013; **19:**722–729.
34. Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. *Nat Rev Cancer* 2014; **14:**598–610.
35. Vivanco I, Chen ZC, Tanos B, *et al*. A kinase-independent function of AKT promotes cancer cell survival. *eLife* 2014; **3:**e03751.
36. Wang Z, Fu M, Wang L, *et al*. p21-activated kinase 1 (PAK1) can promote ERK activation in a kinase-independent manner. *J Biol Chem* 2013; **288:**20093–20099.
37. Dion S, Bourgine 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. *J Virol* 2013; **87:**5554–5563.
38. Martin P, Dubois C, Jacquier E, *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, *et al*. A murine model of hepatitis B-associated hepatocellular carcinoma generated by adeno-associated virus-mediated gene delivery. *Int J Oncol* 2011; **39:**1511–1519.
40. Li TN, Wu YJ, Tsai HW, *et al*. Intrahepatic hepatitis B virus large surface antigen induces hepatocyte hyperploidy via failure of cytokinesis. *J Pathol* 2018; **245:**502–513.

Accepted 25 May 2020; published online 4 June 2020