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**5' Triphosphated Small Interfering RNAs Control Replication of
Hepatitis B Virus and Induce an Interferon Response in Human
Liver Cells and Mice**

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Author involvement:

GE acquired, analysed and interpreted the data; did statistical analysis; prepared figures and text of the paper;

HP designed experiments, prepared reagents;

JL performed experiment for revision, analysed and interpreted data;

NB performed in vivo experiments;

IE performed histological staining and analysis;

KE technical and methodological support; preparation of cells;

GH participated in study concept and design and obtained funding;

UP conceived, designed and supervised the study; obtained funding; drafting and revision of the manuscript;

Abbreviations: 3p-, triphosphate; 3p-RNA, 5'-triphosphated double stranded RNA; cccDNA, covalently closed circular DNA; ctrl-RNA, control-RNA; dsRNA, double-stranded RNA; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IFN, interferon; L-protein, HBV large surface protein; OAS, 2'-5'-oligoadenylate synthetase; PEI, polyethylenimine; pgRNA, pregenomic HBV RNA; poly:IC, polyriboinosinic polyribocytidylic acid; rcDNA, relaxed circular DNA; RIG-I, retinoic acid-inducible protein I; siRNA, small interfering RNA; ssDNA, single stranded DNA; TLR, Toll-like receptors

Abstract:

Background & Aims: Approved therapies for chronic hepatitis B include systemic administration of interferon (IFN)- α and inhibitors of hepatitis B virus (HBV) reverse transcription. Systemic application of IFN- α is limited by side effects. Reverse transcriptase inhibitors effectively control HBV replication, but rarely eliminate the virus and can select drug-resistant variants. We aimed to develop an alternative therapeutic approach that combines gene silencing with induction of IFN in the liver.

Methods: To stimulate an immune response while inhibiting HBV activity, we designed three small inhibitory (si)RNAs that target highly conserved sequences and multiple HBV transcripts of all genotypes. A 5'-triphosphate (3p) was added to the siRNAs turning them into a ligand for the *cytosolic helicase retinoic acid-inducible protein 1 (RIG-I)*, which becomes activated and induces expression of type-1 IFNs. Antiviral activity was investigated in cell lines that replicate HBV, in HBV-infected primary human hepatocytes, and in HBV transgenic mice.

Results: 3p-double-stranded RNA (3p-RNA) activated RIG-I, induced a strong type-I IFN response (expression of IFN- β) in liver cells and showed transient but strong antiviral activity. Bi-functional, HBV-specific, 3p-siRNAs controlled replication of HBV more efficiently and for longer periods of time than 3p-RNAs without silencing capacity or siRNAs that targeted identical sequences but did not contain 3p.

Conclusions: HBV-specific 3p-siRNAs are bi-functional, antiviral molecules that induce production of type-I IFNs in the liver and target HBV RNAs to inhibit viral replication.

Keywords: virology, HBV infection, hepatitis B; gene silencing; interferon

Introduction

About two billion people worldwide have been exposed to HBV, 350 million are chronically infected and 600 000 die each year from consequences of hepatitis B. Approved therapies for chronic hepatitis B include interferon IFN- α and nucleos(t)ide analogues, which inhibit the viral reverse transcriptase and efficiently suppress HBV replication, but rarely lead to elimination of the virus ¹. HBV persists by establishing a nuclear transcription template, the so-called HBV covalently closed circular DNA (cccDNA) in hepatocytes, which is able to initiate a new HBV replication cycle even after serological clearance. Long-term therapy is required in the vast majority of cases bearing the risk of side effects and selection of drug-resistant HBV mutants. Novel therapeutic approaches should at least induce sustained immune control of the virus and terminate chronic hepatitis B. To achieve this, combinatorial strategies targeting HBV from different angles will probably be necessary.

In infected cells, four major classes of mRNAs are transcribed. 3.5 kb pregenomic RNA is reverse transcribed into novel HBV genomes and serves as mRNA for translation of the viral core and polymerase proteins. A minimally longer RNA encodes the secretory HBeAg. 2.4 and 2.1 kb RNAs serve as mRNA for viral envelope proteins L, M and S. From the 0.7 kb RNA the HBV X protein is translated.

Organisms have evolved efficient host-defense mechanisms to sense viruses and block their replication and spread ². RNA interference based on cleavage of dsRNA into small interfering RNA (siRNA) by Dicer helicases is an important antiviral defense mechanism. Several siRNAs and small hairpin RNAs have already been shown to combat HBV ³⁻⁶. Since all HBV RNAs share common 3' sequences, they may be targeted by a single siRNA. In addition, innate and adaptive immune responses are needed to overcome viral infection. Viral nucleic acids are recognized by specialized receptors ². The transmembrane Toll-like receptors 3,7,8 and 9 detect viral RNA or DNA in the endosomal compartment of immune cells, whereas the cytoplasmic helicases RIG-I, melanoma differentiation-associated gene 5 and LGP2 are ubiquitous.

111 RIG-I detects viral RNA based on a 3p- present in panhandle structures of negative strand
112 RNA viruses ^{7, 8}. RIG-I is effectively triggered by *in vitro* transcribed 3p-dsRNA
113 oligonucleotides ⁷, which may be designed to serve as siRNAs with an additional
114 immunostimulatory function ^{9, 10}. Antiviral responses triggered by RIG-I activation include
115 the induction of type-I IFN, inflammasome activation and proapoptotic signaling.
116 RIG-like and Dicer helicase families are phylogenetically related and offer the exciting new
117 therapeutic perspective to tackle two fundamental antiviral pathways for the treatment of
118 viral infection by a single molecule. In this project we developed a combinatorial, short
119 double-stranded RNA-based approach for the treatment of HBV infection, combining HBV
120 sequence specific gene silencing by RNA interference with RIG-I-induced antiviral activities.
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Material and Methods

Generation of 3p-RNA and 3p-siRNA. HBV sequence specific siRNAs were designed to target sequences highly conserved among many HBV genotypes. Two target sequences within the common 3' end of all HBV RNA's were identified (targeted by si1.1 and si1.2) and one sequence within all except the X RNA (targeted by si1.3) (Fig. 3A). si1.1 targets all HBV genotypes A to F, si1.2 all except F and si1.3 targets genotypes A and D. Synthesized DNA oligonucleotide of sense and antisense strands (Table 1) were cloned into a suitable vector. The two 3pRNA strands were *in vitro* transcribed from these templates in separate reactions, purified by phenol:chloroform extraction after DNase-I (Ambion, Huntington, UK) digestion of DNA templates, annealed overnight at 37°C and cleaned by Mini Quick Spin™ Oligo Columns (Roche, Mannheim, Germany) as previously described ^{7, 9}. Chemically synthesized siRNAs and polyA RNA were purchased from Eurofins-MWG (Ebersberg, Germany) (Table 1). HCV-sequence specific siRNA ⁶ served as control.

Cell culture. HepG2.2.15 cells are derived from human hepatoma HepG2 cells containing four copies of a 1.1-fold HBV genome, genotype A (adw). HepG2-H1.3 cells contain a single copy 1.3-fold overlength genotype D (ayw) HBV genome ^{11, 12}. Primary human hepatocytes (PHH) were isolated from surgical liver specimens of patients undergoing partial hepatectomy by a two-step collagenase perfusion and differential centrifugation at 50 xg as described ¹³. Informed consent was obtained from each patient, and the procedure was approved by the local Ethics Committee. From supernatant of the 50xg centrifugation step, non-parenchymal liver cells were sedimented at 300 xg and purified by an OptiPrep™ (Pierce, Rockford, USA) density gradient. Liver macrophages (Kupffer cells) were separated by plastic adherence step and cultured in RPMI medium (Invitrogen) containing 5% donor serum. Liver sinusoidal endothelial cells were purified by CD31 magnetic beads (Miltenyi, Bergisch Gladbach, Germany) and HEPES-buffered Williams Medium E (Invitrogen) containing 5% donor serum. Three days after plating, PHH were infected with genotype A HBV concentrated from supernatant of HepG2.2.15 cells at a multiplicity of infection of 100 enveloped, DNA containing particles / cell.

Transfection of RNA. HepG2-H1.3 cells were transfected in suspension before seeding in 12-well or 6-well plates. PHH were transfected adherent to 12-well plates at day 4 after HBV infection in triplicates. To remove the HBV inoculum, medium was exchanged at day 1 after transfection or infection, respectively. 0.5 µg 3p-RNA, siRNA or control-RNA, respectively, or 0.05 µg of polyriboinosinic polyribocytidylic acid (poly (I:C)) mixed with 6 µl Hiperfect (Qiagen, Hilden, Germany) according to manufacturer's instructions were added per ml cell culture medium. As mock control, we used Hiperfect without RNA. To analyze cell viability after transfection, 25µl XTT labeling mixture (XTT Cell Proliferation Kit II, Roche) was added to 10⁴ cells, incubated for 2 h and absorbance was quantified using a scanning multi-well spectrophotometer.

Treatment of HBV transgenic mice. HBV transgenic mice (~20g) of mouse strain HBV1.3 xfs (¹⁴, HBV genotype D, kindly provided by H. Schaller) were grouped by serum HBeAg levels. Each mouse was i.v. injected twice three days apart into the tail vein with 25 µg RNA complexed with *in vivo*-JetPEI^{TM} (Polyplus, New York, USA) according to the manufacturer's instructions in a total volume of 200 µl 5% glucose. As mock control we used polyethylenimine (PEI) without RNA. Injection was repeated after three days and mice were sacrificed six to 15 days after first injection. To determine serum IFN levels, mice were bled retroorbitally, and IFN-α was measured by ELISA (Biosource, Solingen, Germany),

HBV assays. Cell culture medium was collected at indicated time points and analyzed for HBsAg and HBeAg by commercially available immunoassays (AxSYM^{TM}; Abbott, Wiesbaden, Germany). HBV progeny levels were determined in 200 µl of cell culture medium or 50 µl mouse serum by real time PCR using primer HBV1745 and HBV1844 (¹⁵, Supplementary Table). Intracellular HBV replicative intermediates were analyzed by Southern blot analysis of total *HindIII* digested DNA extracted by phenol-chloroform from mouse liver tissue (75 µg) or HepG2-H1.3 cells (20 µg), respectively, after separation through a 0.85% agarose gel. HBV RNA subspecies were analysed by Northern blotting of 25 µg total RNA from HepG2 H1.3 cells separated through a 1% agarose gel containing 37% formaldehyd. For detection, ³²P labeled HBV DNA probes were used.

Analysis of gene expression and HBV pgRNA by RT-PCR. Total RNA from 10^6 HepG2-H1.3 cells was extracted using the RNeasy® total RNA extraction kit (Qiagen). Total RNA from 2×10^6 PHH and 20mg mouse liver were extracted using Trizol (Invitrogen). 1 µg RNA was reverse transcribed into cDNA (Superscript III cDNA synthesis kit, Invitrogen). For gene expression analysis, exon-exon spanning primer pairs were selected (Supplementary Table). Differential gene expression was quantified by real-time PCR relative to GAPDH expression and normalized to a dilution series of calibrator cDNA using the DNA Amplification SYBR Green Kit on a LightCycler™ 480 II machine applying the Relative Quantification Software (Roche).

5' ligase-mediated-RACE PCR. Total RNA isolated from 10^6 HepG2-H1.3 cells was dissolved in 40 µl distilled water, heated to 65°C for 5 minutes and rapidly cooled on ice before ligation to 250 ng Gene Racer™ RNA adaptor (Tab. 2) at 37°C for 1 hour in 50 µl 10mM ATP, 80 U RNaseOut (Invitrogen), and 10 U RNA ligase (Ambion). After purification using Amicon 100k filters (Millipore, Billerica, MA, USA), 10 µl of the RNA ligation reaction was reverse transcribed at 55°C using SuperScript III™ reverse transcriptase (Invitrogen) and HBV-specific primer HBV 3081 rev hybridizing 3' of the predicted si-1.2-mediated mRNA cutting site. After inactivation at 70°C for 15 minutes RACE-PCR was performed using forward primer GR5 fw binding the GeneRacer adaptor and reverse primer HBV 3075 rev (4' 95°C; 32 cycles: 30" 95°C, 20" 60°C, 6" 72°C; 10' at 72°C). PCR products were separated through a 2% TBE Agarose gel and visualized by ethidium bromide.

Western blotting. Lysates from HepG2-H1.3 cells or PHH (15µg protein / lane) were separated by 12.5% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and detected using anti-RIG-I monoclonal antibody (Alexis Biochemicals, Axxora, Lörrach, Germany), anti-HBV-core antiserum H800 (kindly provided by H. Schaller) or anti-HBV-S monoclonal antibody (Murex HBsAg Version 3 ELISA Kit, Abbott).

Histological analysis. Mouse livers slices were fixed in 10% buffered formalin and embedded in paraffin. 8µm tissue sections were stained with HBV core protein specific antibody (Diagnostic Biosystems, Pleasanton, CA, USA) or haematoxylin and eosin (H&E). Semiquantitative analysis of stained sections was performed by counting localization,

intensity, distribution and percentage of positive cell staining (HBV-core) or the number of focal lesions (hepatocyte necroses with inflammatory infiltrates, H&E) throughout the whole tissue specimen.

Statistical analysis. For statistical analysis, data were subjected to unpaired, two-tailed Student's *t*-test and statistical significance was determined setting confidence intervals of 95% using Prism 5.0a software.

Results

5'-triphosphated RNA suppresses HBV replication.

To test the antiviral potential of 3p-RNA in HBV infection, we transfected 3p-RNA (0.5 µg / ml) into HBV-infected PHH cultures (Fig. 1A). After transfection with 3p-RNA, but not with the control polyA RNA, progeny HBV (Fig. 1A, left) as well as viral antigens HBeAg and HBsAg (Fig. 1A, middle) secreted into the cell culture medium were reduced by more than 90% and comparable to the reduction observed after poly(I:C) treatment. Intracellular HBV 3.5 kb RNA levels, decreased significantly (Fig. 1A, right). These effects were confirmed in HepG2-H1.3 hepatoma cells (data not shown), an IFN-sensitive, stable cell line replicating HBV¹¹.

To test the antiviral effect of 3p-RNA *in vivo*, HBV transgenic mice were intravenously (i.v.) injected with 3p-RNA or polyA RNA complexed with linear PEI or PEI only as control. HBV transgenic mice replicate HBV from an integrated 1.3-fold overlength genome and secrete HBV into the bloodstream^{14, 16}. HBV viremia in 3p-RNA treated mice decreased about 1.6 log 6 days after injection (Fig. 1B, left). In addition, serum HBeAg and HBsAg levels (Fig. 1B, middle) and intrahepatic HBV pgRNA (Fig. 1B, right) significantly dropped.

These results demonstrate that 3p-RNA exhibits antiviral activity against HBV *in vitro* and *in vivo*.

3p-RNA induces an interferon response in hepatocytes via activation of RIG-I.

3p-RNA is well known to induce type-I IFN^{7, 8, 17}, but may also induce apoptosis in tumor cells⁹. To exclude apoptosis induction, a non-toxic concentration of 3p-RNA (0.5 µg / ml) - as determined by cell viability test (Fig. 2A) - was used throughout our study.

To determine how 3p-RNA elicits its antiviral effects against HBV, we first checked whether it induces type-I IFN and IFN-induced 2'-5'-oligoadenylate synthetase (OAS). We transfected 3p-RNA into HepG2-H1.3 cells (Fig. 2B, left), HBV infected PHH (Fig. 2B, middle) and i.v. injected 3p-RNA into HBV tg mice (Fig. 2B, right). 3p-RNA treatment resulted in a rapid and potent, but transient type-I IFN response *in vitro* and *in vivo*. IFN-α level peaked after several hours in the serum of treated mice. IFN-induced 2'-5'-OAS expression was detected until day six after treatment (Fig. 2B)

Fig. 2C shows, that hepatoma HepG2-H1.3 cells as well as PHH constitutively express RIG-I. Stimulating IFN-response pathways by addition of recombinant IFN-α or by transfection with 3p-RNA upregulated RIG-I expression. To confirm that 3p-RNA activates IFN pathways via RIG-I in hepatocytes, we knocked down RIG-I expression by transfecting RIG-I specific siRNA resulting in a reduction of RIG-I until day 6 after transfection (Fig. 2D). Since the RIG-I knock-down was incomplete, it significantly, but incompletely antagonized IFN-β induction after 3p-RNA stimulation in HepG2-H1.3 cells (Fig. 2E) and PHH (data not shown). As a consequence (and because RIG-I siRNA per se already had an effect), knocking down RIG-I only incompletely rescued HBV antigen secretion (Fig. 2F). This indicates that low levels of RIG-I are sufficient to induce the antiviral effect, but cannot completely exclude additional mediators.

HBV specific 3p-siRNAs induce a potent interferon response and control HBV replication in hepatocytes.

To enhance the therapeutic efficiency against HBV we intended to combine the immune stimulatory, antiviral effect of RIG-I stimulation with RNA interference mediated gene-silencing effect. Therefore, we designed three HBV-sequence specific siRNAs (Table 1) each matching a panel of different HBV genotypes (for details see methods). siRNAs si-1.1

and si-1.2 target all HBV RNAs, si-1.3 targets all except the X RNA (Fig. 3A). siRNAs furnished with 5'-triphosphate groups by *in vitro* transcription were termed 3p-1.1, 3p-1.2 and 3p-1.3.

To determine whether 3p-siRNA is able to induce an antiviral IFN response, HepG2-H1.3 cells were transfected with HBV-specific 3p-siRNAs and corresponding non-phosphated siRNAs. All three 5'-triphosphated HBV siRNAs designed (3p-1.1, 3p-1.2 and 3p-1.3) led to comparable induction of IFN- β gene expression (Supplementary Fig.1). Using primary human liver cells, IFN- β was induced by 3p-1.2 in PHH as well as in purified primary human liver sinusoidal endothelial cells and liver macrophages, so called Kupffer cells (Supplementary Fig. 2). As exemplarily shown for si1.2, 3p-siRNA but not non-phosphated siRNA induced expression of IFN- β (Fig. 3B). In addition, si-1.2 as well as 3p-1.2 clearly functioned as siRNAs and induced cleavage of HBV RNAs – in contrast to control siRNA, poly(I:C) and 3p-RNA (Fig. 3C). After transfection of 3p-1.1, 3p-1.2 or 3p-1.3 we observed a significant reduction in HBV progeny as well as HBeAg and HBsAg levels released from HBV replicating stable cell line HepG2-H1.3 (Fig. 3D). A comparable, strong antiviral effect was achieved in HBV-infected PHH (Fig. 3E). Northern blot analysis revealed a comparable effect of all three 3p-siRNAs on pre- and subgenomic HBV RNAs (Fig. 3F).

These data suggest that HBV specific 3p-siRNAs stimulate RIG-I, induce an IFN response and control HBV replication and antigen secretion.

Superior anti-HBV activity of 3p-siRNAs combining RIG-I activation and gene silencing.

To analyze whether bi-functional 3p-siRNAs have a superior antiviral effect, we compared the primarily used RIG-I ligand 3p-RNA to our newly designed HBV-sequence specific 3p-siRNAs. Effects on HBV replication in HepG2-H1.3 cells were analyzed at day four and eight after transfection by HBV DNA Southern blot (Fig. 4A). All HBV DNA replicative intermediates were strongly reduced at day four after treatment with the original 3p-RNA and the HBV specific 3p-siRNAs. Intriguingly, 3p-siRNAs displayed a more pronounced and longer-lasting effect on HBV replication at day eight after transfection (Fig. 4A).

Corresponding to their gene silencing ability, all three 3p-siRNAs reduced expression levels of the HBV envelope protein L emerging in a non-glycosylated and a glycosylated form and the capsid protein core – with 3p-1.2 showing the strongest gene silencing effect. Non-silencing 3p-RNA, in contrast had only a very minor effect at early time points after transfection, which rapidly persisted (Fig. 4B). Due to the higher protein turn-over, the effect on L protein was more pronounced.

We next compared non-silencing 3p-RNA with the 3p-siRNAs and the respective non-phosphated siRNAs. At day 8 post transfection, all three 3p-siRNAs proved at least as efficient in reducing HBV progeny release (Fig. 5A), HBeAg secretion (Fig. 5B), intracellular core protein expression (Fig. 5C) and HBV replication (Fig. 5D) as the respective siRNA and superior to non-silencing 3p-RNA.

Because the IFN-mediated antiviral effect appeared to be transient, we studied the antiviral effect of si-1.2 and 3p-1.2 with non-silencing 3p-RNA and poly(I:C) in parallel over a time-course of 16 days. Since HepG2-H1.3 cells start to transcribe HBV RNA only when they are confluent¹⁸, HBV DNA levels reflecting progeny virus release (Fig. 5E) as well as HBeAg levels (Fig. 5F) were low at day 0.5 and increased constantly until day 10 and 16, respectively, in mock treated cells. 3p-RNA, si-1.2 and 3p-1.2 showed a pronounced antiviral activity at early time points after transfection. The antiviral activity of 3p-RNA and poly (I:C), both eliciting their effect via transient activation of an IFN response, ceased first and was comparable to mock treated cells again between day 8 and 10 (Fig. 5E,F). When IFN levels were waning, a rebound effect was observed and HBV progeny production even outnumbered that of mock controls (Fig. 5E). Only triphosphated siRNA 3p-1.2 and its non-phosphated counterpart si-1.2 reduced progeny HBV DNA over a longer time period, but 3p-1.2 was significantly more active from day 10 onwards in reducing HBV progeny release (Fig. 5E) as well as HBeAg secretion (Fig. 5F).

Taken together, bi-functional 3p-siRNAs, which combine HBV gene silencing with immunostimulatory properties, display a superior antiviral activity on HBV and are able to repress virus replication and antigen expression *in vitro* for more than two weeks.

HBV specific 3p-siRNAs have a long lasting antiviral effect *in vivo* in HBV replicating mice.

Finally, we wanted to compare 3p-RNA, 3p-siRNAs and corresponding siRNAs with respect to their antiviral activity in HBV transgenic mice *in vivo*. Complexed with PEI, 3p-RNA and 3p-siRNA serving as RIG-I ligands, but not non-phosphated siRNAs, triggered a rapid IFN- α response 6 hours after intravenous injection (Fig. 6A).

To test the antiviral efficacy, HBV transgenic mice were grouped according to sex and HBeAg levels. Groups of five HBV transgenic mice each were treated with either 3p-RNA, HCV-specific control siRNA or HBV sequence specific siRNAs si-1.1 and si-1.2 or 3p-siRNAs 3p-1.1 or 3p-1.2. To compare the effect on HBV replication, Southern blot analysis of HBV replicative forms in pooled liver DNA from each group of animals was performed (Fig. 6B). Consistent with our *in vitro* data, mice treated with 3p-RNA, siRNA and 3p-siRNA showed a clear reduction of intrahepatic HBV replication (Fig. 6B,C) and HBV pgRNA levels (Fig. 6D, left panel) in comparison to the control group at day 6 after treatment. Hereby, RIG-I ligand 3p-RNA and 3p-siRNAs proved superior to non-phosphated siRNAs. HBV core staining of liver tissue sections revealed a slight reduction in the number of core expressing mouse hepatocytes in si-1.2 treated and a marked reduction in 3p-RNA and 3p-siRNA treated animals (Fig. 6E), but also a reduction of cytoplasmic core staining indicating active virus replication. At day 9 after treatment, however, only the bi-functional 3p-siRNA still showed significant antiviral activity whereas the effects of 3p-RNA and siRNA were not significant anymore (Fig. 6D. right panel).

A mild to moderate inflammatory activity was observed in livers of mice treated with 3p-RNA and 3p-1.2, but not with siRNA indicating that IFN causes the inflammatory reaction (Fig. 6E).

These *in vivo* results confirmed our conclusion from the *in vitro* experiments, that the bi-functional HBV-specific 3p-siRNA is a potent and promising antiviral superior to its mono-functional analogues.

Discussion

This study aimed at creating novel antivirals for the treatment of HBV infection by combining immune stimulatory and gene silencing potentials of RNA-oligonucleotides in a single molecule. We first showed that 3p-RNA, a triphosphated dsRNA serving as RIG-I ligand, induces an antiviral type-I IFN response in HBV replicating hepatocytes. 3p-RNA potently, but transiently controlled HBV. To enhance and prolong the antiviral effect, we designed siRNAs targeting the 3'-end of all HBV RNAs and investigated whether they can be furnished with a 5' triphosphate and act as RIG-I ligands - adding immunostimulatory potential to the siRNA. HBV specific 3p-siRNAs induced a type-I IFN response comparable to 3p-RNA and induced cleavage of HBV RNAs comparable to corresponding siRNA. When used for antiviral treatment, bi-functional 3p-siRNAs proved superior to siRNA and 3p-RNA. They more potently and longer lasting controlled HBV replication *in vitro* in human hepatocyte cultures and *in vivo* in livers of HBV transgenic mice.

HBV-specific siRNAs are powerful tools to target viral genes and inhibit HBV replication *in vitro*¹⁹ and *in vivo*^{3, 4, 6, 20}. Viruses usually occur in several genotypes and in addition show a high sequence variation restricting siRNA targeting to highly conserved regions within the virus genome. We carefully selected three novel siRNAs matching highly conserved genomic sequences. Notably, si1.1 targets all known HBV genotypes. Since HBV RNAs terminate at a common polyadenylation signal directly downstream of the X gene²¹, our siRNAs target multiple HBV RNAs: si-1.1 and si-1.2 target all HBV RNAs, si-1.3 targets all except the X RNA (Fig. 3A). In our experiments we confirmed that the siRNAs are active against HBV genotype A, and D. Targeting of highly conserved HBV sequences makes the selection of escape mutations, which could hamper siRNA binding, very unlikely. Indeed, we found no HBV sequence variation in 3p-1.2 or si-1.2 treated hepatocytes (data not shown).

To treat HBV infection, we wanted to combine gene silencing with the immunostimulatory capacity of RNA. Toll-like receptors 7 and 8 may recognize synthetic ssRNA and dsRNA depending on sequence motives^{22, 23}. However, this is restricted to cells of the innate

immune system. Furthermore, it restricts the selection of siRNA target sequences and makes it very difficult to match highly conserved viral sequences.

We therefore decided to target the ubiquitously expressed cytosolic helicase RIG-I. RIG-I is expressed in HBV infected hepatocytes. Stimulation of cells with IFN further increased its expression²⁸. RIG-I activation requires the presence of a triphosphate group at the 5'-end of an RNA^{7, 8, 24-27}. The optimal ligand is short, double-stranded 5'-triphosphate RNA. This allows converting siRNAs into RIG-I ligands⁹ and explains why all three HBV-specific 3p-siRNAs in this study induced a type-I IFN response dependent on RIG-I expression. Our experiments, however, cannot exclude that 3p-RNA and 3p-siRNA exerts additional antiviral effects besides triggering and IFN response via RIG-I.

In vivo, hepatocytes and non-hepatocytes such as liver macrophages and liver sinusoidal endothelial cells took up fluorescently labeled 3p-RNA (data not shown). Since they secrete IFN upon 3p-RNA uptake (Supplementary Fig. 2), non-parenchymal liver cells contribute to the antiviral activity of RIG-I ligands. Type-I IFN is long known to control HBV infection by the activation of IFN induced pathways, which inhibits the transcription of HBV RNAs at transcriptional and posttranscriptional levels²⁹, prevents the formation of replication-competent HBV capsids³⁰ and activates effector T cell responses³¹. Notably, treatment with exogenous IFN α 2a (even at doses corresponding to 50.000 IU per human liver) was less effective in reducing HBV replication than 3p-RNA or 3p-siRNA and addition of lamivudine or other nucleos(t)ide analogues didn't show any additive effect (Supplementary Fig. 3).

3p-siRNA induced cleavage of HBV RNAs similar to siRNA proving that 3p-siRNAs are truly bi-functional. However, it is unlikely that the same 3p-siRNA molecule binds and activates RIG-I and induces RNA cleavage. Since we observed that RNA interference activity of 3p-1.2 was lower than that of si-1.2 (Fig. 3C), we rather suggest that Dicer and RIG-I compete for the binding of 3p-RNA.

Taken together, our newly designed 3p-siRNA proved superior in controlling HBV replication over sequence-identical siRNA and 3p-RNA. Thus, 3p-siRNAs are promising, bi-

functional antiviral molecules targeting HBV by combining immunostimulation and gene silencing.

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Tables

Table 1: Sequences of *in vitro* transcribed 3p-RNAs and chemically synthesized siRNAs

Name	sense strand 5'- 3'	antisense strand 5'-3'
3p-RNA	3p- GCAUGCGACCUCUGUUUGA	3p-UCAAACAGAGGUCGCAUGC
3p-1.1	3p-UUUCACCUCUGCCUAAUCA	3p-UGAUUAGGCAGAGGUGAAA
3p-1.2	3p-CGACCUUGAGGCAUACUUC	3p-GAAGUAUGCCUCAAGGUCG
3p-1.3	3p-CUAUUAACAGGCCUAUUGA	3p-UCAAUAGGCCUGUUAUAG
si-1.1*	UUUCACCUCUGCCUAAUCA	UGAUUAGGCAGAGGUGAAA
si-1.2*	CGACCUUGAGGCAUACUUC	GAAGUAUGCCUCAAGGUCG
si-1.3*	CUAUUAACAGGCCUAUUGA	UCAAUAGGCCUGUUAUAG
Ctrl siRNA (HCV)*	CUGAUAGGGUGCUUGCGAG	CUCGCAAGCACCCUAUCAG
RIG-I siRNA*	AAACAGCGAUUAGGCACUA	UAGUGCCUAAUCGCUGUUU
Ctrl-RNA (Poly A)	AAAAAAAAAAAAAAAAAAAAA	

* to all chemically synthesized siRNAs a –dTdT was added at the 3' end to increase stability

Figure Legends

Figure 1: Antiviral effect of 3p-RNA. HBV infected primary human hepatocytes **(A)** and HBV transgenic mice **(B)** were treated with 3p-RNA, negative control polyA-RNA (Ctrl-RNA) or nucleic acid delivery reagents only (mock). Primary hepatocytes were additionally treated with poly(I:C) as positive control. Progeny HBV DNA levels (left panels) were determined by quantitative real time PCR, HBeAg and HBsAg levels (middle panels) by immunoassays in hepatocyte culture medium or mouse serum day 6 after treatment. Intrahepatic pregenomic HBV RNA (pgRNA) levels (right panels) were quantified relative to GAPDH by real-time RT-PCR. Mean values from mock treated cells or mice, respectively, were set to 100%. Data are shown as mean $\pm$ SD, n=3 (**p<0.01, ***p<0.001, t-test).

Figure 2: 3p-RNA induces IFN- β production through RIG-I in HBV replicating liver cells. **(A)** Hepatoma HepG2-H1.3 cells were transfected with increasing concentrations of 3p-RNA and cell viability was monitored from d 1 to 16 post transfection (p.tr.). **(B)** HepG2-H1.3 cells (left panel), HBV infected primary human hepatocytes (PHH) (middle panel) and HBV transgenic mice (right panel) were treated with 3p-RNA, polyA-RNA (Ctrl-RNA) as negative and poly(I:C) as positive control. Nucleic acid delivery reagents only served as mock control. IFN- α levels in mouse sera were determined by cytokine bead assay at indicated time points post injection (p. inj). Hepatocellular IFN- β (upper row) and 2'-5'-OAS (lower row) mRNA levels were analyzed by quantitative RT-PCR relative to GAPDH and normalized to mock treatment (set to 1). **(C)** HepG2-H1.3 cells and PHH were cultured with (+) or without (-) exogenous IFN- α (1000 U/ml) or transfected with 3p-RNA. After 36h RIG-I expression was determined by Western blot analysis using β -actin as loading control. **(D)** HepG2-H1.3 cells were transfected with siRNA against RIG-I, lysed at indicated time points post transfection (p. tr.) and analyzed for RIG-I expression by Western blot analysis; β -actin served as loading control. **(E)** Knock-down of RIG-I in HepG2-H1.3 cells before 3p-RNA

treatment and measuring IFN- β expression at day 1; mean values of cells treated with control siRNA was set to 100%. **(F)** HepG2 H1.3 cells were transfected with siRNA and transfected again with 3p-RNA 72h later. 4 days later HBeAg and HBsAg secreted into the cell culture medium was quantified relative to control siRNA. Data are given as mean $\pm$ SD, n=3; *p<0.05 (t-test).

Figure 3: HBV specific 3p-siRNAs are bifunctional. **(A)** HBV specific siRNAs (indicated by arrows) were designed to target the 3' end common to all HBV RNAs. From 3.5 kb RNA precore/core and polymerase proteins, from 2.4 kb RNA the large surface protein (L, consisting of preS1, preS2 and S domains), from 2.1 kb RNA middle (M, consisting of preS2 and S) and small (S) surface proteins and from 0.7 kb RNA the X protein are translated. **(B)** HepG2-H1.3 cells were transfected with siRNA (si-1.2) or 3p-siRNA (3p-1.2). Cells were analyzed for IFN- β expression normalized to GAPDH by real-time RT-PCR after 12h. Mock treated cells were set to 1. **(C)** 5'-RACE PCR analysis of RNA extracted from HepG2-H1.3 cells treated with si-1.2 and 3p-1.2 or HCV sequence specific siRNA (si-Ctrl), poly(I:C) or 3p-RNA as control at d4 p. tr.. The arrow marks the 136 bp amplification product expected after si-1.2 induced cleavage. **(D)** HepG2-H1.3 cells and **(E)** HBV infected primary human hepatocytes were treated with indicated 3p-siRNA. HBV DNA (left panels), HBeAg and HBsAg levels (right panels) secreted into the cell culture medium at d8 are given. **(F)** Northern blot analysis at d8 p.tr. shows HBV RNA levels in HepG2-H1.3 cells transfected with 3p-siRNA. Pregenomic (3.5 kb) and subgenomic (2.4 and 2.1 kb) HBV RNAs are indicated. GAPDH serves as a loading control. In B, D and E mean $\pm$ SD from three, in C one representative experiment are shown; *p<0.05, **p<0.01, ***p<0.001, t-test.

Figure 4: The antiviral effect of 3p-siRNAs is superior to that of 3p-RNA. HepG2-H1.3 cells were transfected with 3p-RNA or HBV specific 3p-siRNAs (3p-1.1, 3p-1.2, 3p-1.3) and analyzed at indicated time points post transfection (p. tr.). HCV specific siRNA served as control (si-Ctrl). **(A)** Southern blot analysis of intracellular HBV replication. Circular (rc), linear, single stranded (ss) HBV DNA and the HBV integrate serving as loading control are

indicated. **(B)** Western blot analysis for HBV L- and core protein and β -actin as loading control.

Figure 5: The antiviral effect of 3p-RNA, HBV-specific siRNA and 3p-siRNA. HepG2-H1.3 cells were transfected with 3p-RNA, HBV specific siRNAs (si-1.1, si-1.2, si1.3) or HBV specific 3p-siRNAs (3p-1.1, 3p-1.2, 3p-1.3). HCV specific siRNA served as control (si-Ctrl). **(A)** HBV progeny DNA and **(B)** HBsAg secreted into the cell culture medium at d4 and d8 were measured. **(C)** Western blot analysis of intracellular HBV core protein (β -actin serving as loading control) and **(D)** Southern blot analysis of intracellular HBV replication at d 8 p.tr.. Circular (rc), linear, single stranded (ss) HBV DNA and the HBV integrate serving as loading control are indicated. **(E)** Over a culture period of 16 days, progeny HBV DNA levels were determined by real-time PCR and **(F)** secreted HBeAg levels were measured by ELISA. Mean $\pm$ SD from triplicate wells are given; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, t-test.

Figure 6: Antiviral effect of 3p-siRNA *in vivo* in HBV transgenic mice. HBV tg mice were i.v. injected with 3p-RNA, siRNA (si-1.1, si-1.2) and 3p-siRNA (3p-1.1, 3p-1.2). HCV specific siRNA served as control (si-Ctrl). **(A)** After 6 h, IFN- α was measured in serum by ELISA. **(B)** Intrahepatic HBV DNA was detected by Southern blot analysis using a ^{32}P labeled HBV-DNA probe and 60 μg total liver DNA pooled from 5 HBVtg mice per lane at day 6. DNA from HepG2-H1.3 cells indicates the size of circular (rc), linear and single stranded (ss) HBV DNA. **(C)** Relative quantification of HBV-DNA replicative intermediates relative to the HBV integrates released by *HindIII* digestion using a phosphoimager. **(D)** In total liver RNA, HBV pgRNA levels were determined at d6 and d9 by real-time RT-PCR relative to GAPDH and normalized to si-Ctrl treated mice. Mean values from si-Ctrl treated mice were set to 100%. In A and D, data from four mice each are shown as mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, t-test. **(E)** Immunohistochemistry staining for HBV core protein and **(F)** haematoxylin / eosin staining at day 9 in liver sections of from representative animals treated with si-Ctrl, si-1.2, 3p-RNA and 3p-siRNA 3p-1.2. Scale bars, 100 μm .

Figure 1
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A HBV infected primary human hepatocytes

B HBV transgenic mice

Figure 2
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Figure 3
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Figure 4
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A

B

Figure 5
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Figure 6
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Supplementary Table: Oligonucleotides used for PCR

Name	Target	Sequence 5' - 3'
rcDNA1745 fw	HBV	GGAGGGATACATAGAGGTTTCCTTGA
rcDNA1844 rev	HBV	GTTGCCCGTTTGTCTCTAATTC
pgRNA 383 fw	HBV	CTCCTCCAGCTTATAGACC
pgRNA 705 rev	HBV	GTGAGTGGGCCTACAAA
cccDNA 92 fw	HBV	GCCTATTGATTGGAAAGTATGT
cccDNA 2251 rev	HBV	AGCTGAGGCGGTATCTA
HBV 3081 rev	HBV	GACCAATTTATGCCTACAGC
HBV 3075 rev	HBV	TTTATGCCTACAGCCTCCTA
mitoDNA 8686 fw	human / mouse	CCCTCTCGGCCCTCCTAATAACCT
mitoDNA 8796 rev	human / mouse	GCCTTCTCGTATAACATCGCGTCA
IFN-β fw	human	GCCGCATTGACCATCT
IFN-β rev	human	AGTTTCGGAGGTAACCTG
2`-5`-OAS fw	human	CAGTTAAATCGCCGGG
2`-5`-OAS rev	human	AGGTTATAGCCGCCAG
GAPDH fw	human	GGTATCGTGGAAGGACT
GAPDH rev	human	GGGTGTCGCTGTTGAA
IFN-β fw	mouse	TTACACTGCCTTTGCCA
IFN-β rev	mouse	GCGTAGCTGTTGTA CTTC
2`-5`-OAS fw	mouse	CAGTGGATTGGACACTCT
2`-5`-OAS rev	mouse	AGCACTTGAATGTT CACC
GAPDH fw	mouse	ACCAACTGCTTAGCCC
GAPDH rev	mouse	CCACGACGGACACATT
GR5 fw	—	CGACTGGAGCACGAGGACACTGA
GeneRacer™ adaptor	—	CGACUGGAGCACGAGGACACUGACAUGGA CUGAAGGAGUAGAAA

Supplementary Figure 1: Interferon induction in HepG2-H1.3 cells. HepG2 H1.3-cells were treated with 3p-siRNAs and poly(I:C) as positive control. IFN-β mRNA levels were analyzed at d 1 by quantitative RT-PCR relative to GAPDH. Mock treatment was set to 1. Data represent one out of three experiments. Data are given as mean ± SD, n=2.

Supplementary Figure 2: IFN-induction of 3p-siRNA after uptake in human parenchymal and non-parenchymal liver cells *ex vivo*. HBV specific siRNA (si-1.2) or triphosphated 3p-siRNAs 3p-1.2 was complexed with PEI and added into the supernatant of (A) isolated primary human hepatocytes, (B) liver sinusoidal endothelial cells and (C) liver macrophages. Cells were analyzed for IFN-β expression relative to GAPDH by real-time RT-PCR 20h after treatment, respectively. Mock treated cells were set to 1. Data are given as mean ± SD, n=3.

A HepG2-H1.3

B Primary human hepatocytes

Supplementary Figure 3: Antiviral effect of 3p-siRNA compared to exogenous interferon and lamivudine treatment. Hepatoma HepG2-H1.3 cells (A) and primary human hepatocytes (B) were treated with lamivudine (LAM, 0.1µm, corresponding to the inhibitory concentration 50 (IC50) in HepG2-H1.3 cells), 3p-siRNA 3p-1.2 (0.5µg/ml), IFN-α (Roferon-A®, Hoffmann-La Roche Ag, Basel, Switzerland; 1000U/ml), a combination of lamivudine and 3p-1.2 or a combination of lamivudine and IFN-α. HBV DNA levels (left panels) and HBeAg (right panels) secreted into the cell culture medium are given. Mean values of DNA copies from mock treated cells were set to 100%. Data are shown as mean ± SD, n=3.

Supplementary Figure 1

A HepG2-H1.3

B Primary human hepatocytes