JVI Accepted Manuscript Posted Online 1 July 2015
J. Virol. doi:10.1128/JVI.00466-15

An aptamer against the matrix binding domain on the hepatitis B virus capsid impairs virion formation

Running title: aptamers binding to HBV capsids

Authors: Ahmed Orabi[1,2], Maria Bieringer[1,3], Arie Geerlof[4], and Volker Bruss[1#]

Institutions: [1]Institute of Virology, Helmholtz Zentrum München, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany
[2]Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Sharkia Governorate, Egypt
[4]Protein Expression and Purification Facility, Institute of Structural Biology, Helmholtz Zentrum München, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany

[#]Corresponding author: Volker Bruss
Institute of Virology
Helmholtz Zentrum München
Ingolstädter Landstraße 1
D-85764 Neuherberg
Germany
Phone: x49 89 3187 3021
Fax: x49 89 3187 3329
email: volker.bruss@helmholtz-muenchen.de

[3]Present address: Institute for Medical Microbiology and Hospital Hygiene, University of Marburg, Hans Meerwein Straße 2, D-35032 Marburg, Germany

Keywords: aptamer, capsid envelopment

Words count in abstract: 250

Words count in text: (excluding ref., tables, Fig. legends) 4204

Abstract

The hepatitis B virus (HBV) particle is an icosahedral nucleocapsid surrounded by a lipid envelope containing viral surface proteins. A small domain (matrix domain, MD) in the large surface protein L and a narrow region (matrix binding domain, MBD) including isoleucine 126 on the capsid surface have been mapped where point mutations like core-I126A specifically blocked nucleocapsid envelopment. Possibly, both domains interact with each other during virion morphogenesis. By the SELEX method we evolved DNA aptamers from an oligonucleotide library binding to purified recombinant capsids but not binding to the corresponding I126A mutant capsids. Aptamers bound to capsids were separated from unbound molecules by filtration. After 13 rounds of selections and amplifications 16 different aptamers were found among 73 clones. The four most frequent aptamers represented more than 50 % of the clones. The main aptamer AO-01 (13 clones, 18 %) showed the lowest dissociation constant (K_d) of 180 +/- 82 nM for capsid binding among the four molecules. Its K_d value for I126A capsids was 1306 +/- 503 nM. Cotransfection of Huh7 cells with AO-01 and an HBV genomic construct resulted in 47 % inhibition of virion production 3 days post transfection but showed no inhibition by cotransfection of an aptamer with random sequence. The half-life of AO-01 in cells was 2 hours which might explain the incomplete inhibition. The results support the importance of the MBD for nucleocapsid envelopment. Inhibiting the MD-MBD interaction by a low molecular weight substance might represent a new approach for an antiviral therapy.

Importance

Approximately 240 million people are persistently infected with HBV. To date, antiviral therapies depend on a single target, the viral reverse transcriptase. Future additional targets could be viral protein-protein interactions. We selected a 55 base long single stranded DNA molecule (aptamer) which binds with relatively high affinity to a region on the HBV capsid interacting with viral envelope proteins during budding. This aptamer inhibits virion formation in cell culture. The result substantiates the current model for HBV morphogenesis and shows that the capsid envelope interaction is a potential antiviral target.

Introduction

The hepatitis B virus (HBV) infected more than 40 % of the living human population and causes 240 million persistent infections (1). The treatment of chronic infections is limited to date to inhibitors of the viral reverse transcriptase and stimulation of the immune system by interferon. Alternative antiviral strategies are desirable.

During HBV replication an RNA molecule (pregenome) is packaged together with the reverse transcriptase by 180 or 240 copies of the viral core protein. The assembled particle is an icosahedron with T=3 or T=4 symmetry and has a diameter of approximately 30 nm. The pregenome serves as a template for the synthesis of the viral DNA genome by reverse transcription occurring in the lumen of the capsid (2). This particle can then be enveloped by the three viral transmembrane surface proteins S, M, and L at an intracellular membrane and the resulting virion is subsequently secreted from the host cell (3). Interestingly, the immature capsid containing the pregenome is not enveloped in contrast to the mature, DNA-containing capsid (4). Apparently, a structural change of the capsid surface is coupled to the synthesis of the viral DNA genome (5).

Heterologous expression of the core protein in eukaryotic cells and even in bacteria leads to capsids almost indistinguishable from authentic capsids with respect to their antigenicity and appearance in the electron microscope. The C-terminal 30 amino acid long region of the core protein is very rich in arginine residues and has nucleic acid binding properties. Deletion of this domain is compatible with capsid formation (6).

The budding of mature capsids is supported by cellular factors involved in multivesicular body formation (7). In addition, budding is dependent on a linear, 22

aa long domain (matrix domain, MD) of the surface protein L exposed at the cytoplasmic side of the cellular membrane and on a region on the capsid surface (matrix binding domain, MBD) comprised of a ring like groove around the base of the spike protruding from the capsid and a small area close to the pores of the capsid shell (8). Numerous single point mutations in either of the two domains block nucleocapsid envelopment (9, 10). It seems conceivable that both domains directly interact with each other and that nucleocapsid envelopment presupposes the interaction of MD and MBD. There is no strong biochemical evidence available for this interplay. However, the phenotype of certain core and L protein mutants supports the model: The F97L mutation of the core protein causes the envelopment of capsids containing pregenomic RNA (11, 12). The point mutation A119F in the MD of the L protein can complement the core F97L change restoring the selective envelopment of mature, DNA-containing capsids (13).

We intended to generate a DNA aptamer that binds to the MBD on the capsid surface. The presence of such a molecule in HBV expressing cells was expected to block nucleocapsid envelopment and therefore virion formation. This would support the current model of HBV budding and could serve as a proof of principle that small molecules binding to the capsid surface could inhibit virion formation.

Materials and Methods

Plasmids. The HBV wild type core gene was amplified by PCR from plasmid pHBV1.5 (14) (HBV genotype A) and the core gene point mutant I126A from plasmid pSVHBV1.1LE-I126A (9). The PCR primers were designed to introduce an NcoI restriction site at the initiation codon and a stop codon at triplett 149 plus a SalI restriction site. The PCR products were cleaved with NcoI and SalI and ligated into the T7 RNA polymerase dependent bacterial expression vector pETM-13 replacing

the actin binding domain (ABD) stuffer gene (vector map at http://www.helmholtz-muenchen.de/en/pepf/materials/vector-database/bacterial-expression-vectors/index.html).

In order to produce HBV virions Huh-7 cells were transiently transfected because of biosafety reasons with three plasmids: (i) pSVHBV1.1LE$^-$ containing a genotype A HBV genome with two stop codons in the surface protein ORF (9), (ii) pSV45-31 (15) harbouring the HBV preS1-preS2-S open reading frame for the expression of all three HBV envelope proteins, the preS1 codons 2 to 30 were deleted in this construct because this region of the L protein is not required for virion formation (15) but has been shown to inhibit virion release in a dose dependent fashion (16, 17), and (iii) pSV24H (18) carrying the gene for the small HBV surface protein to optimize the ratio between the HBV envelope proteins for higher virus production.

Protein expression. *E. coli* BL21 Star (DE3) pRARE2 cells were transformed with the HBV core protein expression plasmids and cultures were grown in 2 x YT broth to an OD_{600} of 0.7-1.0. For induction of core protein expression 200 µM isopropyl-β-D-thiogalactopyranoside was added at 20°C for 16 h before harvesting by centrifugation. Cells were lysed by freeze-thawing three times in lysis buffer (5 mM EDTA, 50 mM Tris HCl pH 8.0, 2 mg/ml lysozyme), using 20 ml of lysis buffer per litre of cell culture. Then 0.1 M $MgCl_2$ and 0.2 mg/ml DNAase (end concentrations) were added and the mixture was incubated at room temperature for 15 minutes before centrifugation at 15,000 rpm for 10 min a 4°C to remove cell debris. Capsids in the supernatant were precipitated by adding ammonium sulphate to an end concentration of 50% (w/v). The precipitate was sedimented by centrifugation at 19,000 rpm for 30 min at 4°C and the pellet was resuspended in 10 ml of Tris-buffered saline (TBS) containing 0.1 % (v/v) NP-40.

Protein purification. The capsids were purified firstly by two subsequent steps of size exclusion chromatography using a HiPrep 26/60 Sephacryl S-500 HR (GE Healthcare) column. Fractions containing capsids were pooled, concentrated and applied again to the column. Fractions containing capsids were detected by Coomassie-staining of gels after SDS-PAGE or by native agarose gel electrophoresis and Western blotting with a polyclonal anti-HBc antibody (H800, kindly provided by H. Schaller, Heidelberg). Concentration of the pooled fractions was done by using a concentrator (Millipore 30000) with a cut off of 30 kDa and centrifugation at 2,700 g for 20 min at 4°C. A further step of purification was performed by sucrose gradient ultracentrifugation at 10°C and 25,000 rpm for 24 h using a SW28 rotor and 10 % to 60 % (w/w) sucrose in TBS. Fractions containing capsids were identified, pooled, and desalted using PD-10 columns (GE Healthcare) and elution with TBS. The final protein concentration in the preparations was 1.4 mg/ml for wild type capsids and 0.12 mg/ml for mutant capsids.

Aptamer library. The library was obtained from PURIMEX (Göttingen, Germany). The oligonucleotides carried a fixed, 15 nt long sequence at both ends flanking a random sequence of 25 nt length (5`GCGGGTCGACGTTTG…N(25)… CACATCCATGGGCGG´3). The positions of the random sequence were synthesized in the presence of an equimolar concentration of all four nucleotides A, G, C, and T. Prior to the *in vitro* selection, the aptamer library (10 nmol) was incubated at 85°C for 15 min then snap cooled on ice for 15 min and finally equilibrated at RT for 15 min to induce folding of the aptamers to their 3 dimensional structures. In addition, an initial step of aptamers pre-selection was done by filtering the pre-snap cooled aptamers through an alkali-pretreated Amicon Ultra-2 mL Centrifugal Filter (100 K) to remove aptamers binding to the matrix of the filter. Pretreatment was done with 0.5 M KOH for 20 minutes at room temperature followed by washing 3 times with distilled water

and equilibration with binding buffer (PBS) at room temperature to reduce unspecific binding of aptamers.

***In vitro* selection with counter selection.** Thirteen rounds of consecutive positive and negative selections were performed. Targets of the positive *in vitro* selection were HBV wild type (WT) capsids while the counter targets for negative selection were the I126A mutant capsids. For binding the aptamers and capsids were mixed in phosphate buffered saline (PBS), pH 7.0 and incubated at RT for variable periods of time in a total volume of 100 µl. The selection of aptamers was performed by filtration using KOH-pretreated Amicon Ultra-2 mL Centrifugal Filters (100 K) in a swinging bucket rotor and spinning at 4,000 g and 25°C for 30 min. The aptamers bound to WT capsids in the positive selections and the unbound aptamers in the negative selections were extracted with phenol/chloroform (1:1) and concentrated by using the QIAEX II kit (Qiagen). To induce selective pressure the concentrations of aptamers, WT capsids, and mutant capsids as well as the incubation times and volumes of PBS for washing steps were adjusted during the selection process (Tab. 1).

Streptavidin induced electrophoretic mobility shift for ssDNA preparation. After each positive and negative selection step the aptamer mixture was amplified by PCR. The PCR reaction volume was 50 µl and contained aptamers, 50 µM each of the forward primer 5'GCGGGTCGACGTTTG'3 and the reverse primer 5'biotinylated-CCGCCCATGGATGTG'3, 1 mM of each desoxynucleoside triphosphate and 5 U Taq DNA polymerase (Promega) in Taq DNA polymerase buffer supplied by the manufacturer. After initial denaturation at 95°C for 5 min 15 amplification cycles followed (denaturation at 95°C for 20 sec, annealing at 51°C for 15 sec, and extension at 72°C for 10 sec). Afterwards single-strand DNA plus-strand molecules were isolated from the double-stranded PCR products by using a streptavidin induced

electrophoretic mobility shift (19). The purified PCR product was suspended in streptavidin buffer and incubated with streptavidin (Thermo Scientific) at RT for 30 min (1:4 molar ratio of biotinylated strands:streptavidin). The binding mixture was then heat-denatured and electrophoresed in a 10 % polyacrylamide, 6 M urea gel. The plus-stranded ssDNA running faster in the gel than the streptavidin-bound biotinylated minus-strands was then purified by passive elution from the crushed acrylamide gel and concentrated with the QIAEX II kit (Qiagen).

K_d determination. The dissociation constants (K_d) of the aptamer-capsid binding were measured using immunoprecipitation. Different concentrations of the pre-snap cooled aptamers (from 5 pM to 1 µM) and a fixed concentration of the HBV WT or mutant capsids (1 nM) were used. The aptamer-capsid complexes were immunoprecipitated using protein G coupled agarose beads (Santa Cruz Biotechnologies) coated with rabbit polyclonal antibodies against the HBV core protein (H800). The bound aptamers were recovered by phenol/chloroform extraction, purified by the QIAEX II Kit, and quantified by qPCR. For this PCR the same setup was used as for the aptamer amplification during the selection process except that unbiotinylated reverse primers were used. The K_d values were estimated using the Sigma Blot 12.0 software program.

Cells transfection and virion immunoprecipitation. Huh7 cells were transiently transfected with Fugene 6/HD/X-treme (Roche) in 6-well plates using in total 1 µg of plasmid DNA per well. When different plasmids were mixed equal molar ratios were used. When aptamers were cotransfected 1 µg was mixed with 1 µg of plasmids. The cell supernatants were collected 3 days post-transfection and centrifuged at 13,000 rpm for 10 min. Virions in the supernatants were immunoprecipitated with sheep polyclonal antibodies against hepatitis B surface antigen (kindly provided by Prof. Dr. W. Gerlich, Gießen, Germany). The remnant of the plasmid DNA used for

transfection was degraded by using DNase (Qiagen). The genomes of secreted virions were recovered by proteinase K digestion followed by phenol/chloroform extraction. To measure genome concentrations an HBV genome specific qPCR was used (9).

Aptamer secondary structure prediction. Secondary structures of the selected aptamers were predicted by the Zuker algorithm (20), using Mfold (version 3.2) with conditions set up to 0.15 M NaCl and 25°C.

Determination of the half-life of aptamer AO-01 in cell culture. Huh7 cells were transiently transfected using Fugene 6/HD/X-treme (Roche) in six 10 cm dishes with 1 µg (3.3 x 10^{13} molecules) of aptamer AO-01 per dish. After 6 hours the cells were washed 5 times with 2 ml pre-warmed PBS to remove non-internalized aptamers. The aptamers were harvested 1, 12, 24, 36, 48, and 60 hours post transfection by lysing the cells with 500 µl of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 0.5 % (v/v) Nonidet P40, pH 7.5) per dish. The aptamers were recovered by phenol/chloroform (1:1) extraction and concentrated by ethanol precipitation. The recovered aptamers were quantified by qPCR.

Results

Selection of aptamers. In order to generate aptamers binding to the matrix binding domain on the HBV capsid we used the technique of Systematic Evolution of Ligands by EXponential enrichment (SELEX) (21) with positive and negative selection. A library consisting of single stranded 55 bases long DNA molecules with 15 fixed nucleotides at each end and 25 central positions containing each of the four nucleotides in equimolar ratio served as the source for aptamers. The complexity of the library was 4^{25} (appr. 10^{15}). Thirty µg of aptamers correspond to approximately

Journal of Virology

238 10^{15} molecules. For positive selection the library was incubated with capsids purified
239 from E coli (Fig. 1A, lane 1). These capsids were formed by an HBV core protein
240 lacking the 35 C-terminal arginine-rich amino acids because this domain is
241 dispensable for particle formation but able to bind unspecifically to nucleic acids (6).
242 We therefore suspected that capsids formed by full-length core proteins would bind
243 any aptamer and would not allow the selection of molecules binding to a specific
244 region of the capsid surface. We refer to these capsids as WT capsids in this work.
245 Aptamers attaching to WT capsids were separated from unbound molecules by
246 filtration. The selected aptamers were extracted and then amplified by PCR using a
247 biotinylated primer for the negative strand. Subsequently, the minus strand of each
248 of the double-stranded oligonucleotides was removed by streptavidin induced
249 electrophoretic mobility shift and the remaining positive strands were used for further
250 rounds of selection. For negative selection the aptamers were mixed with purified
251 recombinant capsids also lacking the C-terminal 35 amino acids and carrying in
252 addition the point mutation I126A (Fig. 1, lane 3). The amino acid residue isoleucine
253 126 is part of the matrix binding domain and externally exposed on the capsid
254 surface (22). The mutation to alanine strongly blocks HBV capsid envelopment (10).
255 The residue is highly conserved, 98 % of 4043 full length HBV genomes retrieved
256 from the NCBI database carried a triplett coding for isoleucine at this position of the
257 core gene. We expected that targeting residue I126 would give a higher chance for
258 the identification of an aptamer inhibiting budding than screening for an aptamer with
259 optimal binding just somewhere on the capsid surface. Aptamers not binding to the
260 I126A mutant capsids were isolated by filtration, amplified by PCR, and converted to
261 single stranded oligomers like in the positive selection process.

262 The selection process was started with 11 nmol of aptamers corresponding to
263 approximately 6.6 x 10^{15} molecules. The concentration of aptamers, wild type and

mutant capsids, the incubation time for the aptamer/capsid binding, and the washing conditions were consecutively changed during the 13 rounds of SELEX in order to increase the selectivity during the selection process (Tab. 1).

At the end of the selection double stranded versions of the aptamers were molecularly cloned and sequenced (Tab. 2). Sixteen different sequences were found among 73 isolates. The most abundant sequence AO-01 was present 13 times (18 %). Ten of the 16 different aptamers contained the sequence CGCCA followed by TGTG or TGNN(N)TG. Secondary structure prediction of AO-01 shows a molecule with an imperfect 11 bp long stem/6 b loop at the 5' end and a 3 bp stem/9 b loop at the 3' end connected by 9 unpaired bases (Fig. 2). The structures of the other aptamers were also predicted. They all show a 3' stem loop and a 5' stem loop connected by a single stranded region. The common motive (shown in bold face and underlined) is always at the 3' end of the 5' stem-loop and in the single stranded linker region.

Affinity of aptamers to the matrix binding domain. The dissociation constants K_d for the aptamer/capsid binding was measured for the four most abundant aptamers AO-01 to AO-04 together representing 50 % of the 73 isolates (Tab. 2). For this purpose a constant amount of wild type or mutant capsids were mixed with different amounts of aptamers, bound aptamers were separated after incubation by filtration from unbound molecules and quantified by qPCR (Fig. 3). The K_d values for the binding to wild type capsids (Fig. 3A) correlated with the abundance of the aptamers within the 73 isolates and drop from 180 +/- 82 nM for AO-01 to 369 +/- 285 nM for AO-04. In case of AO-01 the maximal value of 120 bound aptamers per capsid was observed at aptamer concentrations above 240 nM. C-terminally truncated core proteins form capsids with T=4 icosahedral symmetry which consist of 120 core

protein dimers (23). Apparently, a maximal saturation of one aptamer per dimer could not be exceeded.

The dissociation constants for the binding to I126A mutant capsids were 1306 +/- 503 nM for aptamer AO-01 and 2.7 to 7.3 times higher for the other characterized aptamers (Fig. 3B). This suggests that the binding of all 4 aptamers depended partially on the isoleucine residue at position 126. Aptamer AO-01 also reached a saturation of one aptamer per mutant dimer but only at concentrations above 2 µM.

Inhibition of virus formation by aptamer AO-01. We transiently cotransfected Huh7 cells with an envelope-negative but replication competent genomic HBV construct and expression plasmids for the synthesis of all three viral envelope proteins (9) as a positive control. Three days post transfection the amount of secreted virions corresponded to 1.6 x 10^6 viral DNA genome copies/ml culture supernatant as measured by a PCR-based method (Fig. 4, left column) (9). When the plasmid for envelope protein expression was omitted no virions could be formed (14) but the assay resulted in a signal of 2 x 10^5 viral DNA genomes/ml culture supernatant (lane B). This background is mainly due to residual amounts of the genomic plasmid used for transfection which was amplified by the PCR. Cotransfection of both plasmids together with aptamer AO-01 resulted in a 50 % lower virus concentration in the culture supernatant (lane A-01) compared to the transfection without AO-01 whereas cotransfection of a random aptamer (lane A-N) showed no significant change relative to the positive control. Aptamers AO-02, -03-, and -04 showed weaker effects between 18% and 13% inhibition than aptamer AO-01 (data not shown).

The inhibitory effect of aptamer AO-01 on virion formation is evident but relatively weak. This may be due to a short half-live of the aptamer within transfected cells. We

measured the residual amount of aptamer AO-01 in cell cultures 0 to 60 hours after transfection (Fig. 5). The aptamer was exponentially degraded with a half-life $t_{½}$ of $\log_{10}(2)/0.147\ h \approx 2.05\ h$. Therefore, an inhibitory effect of the aptamer on virus production can only be expected during the early period of the cotransfection.

Discussion

The formation of HBV particles is not well characterized. E.g. models for the structural basis of the maturation signal coupling viral DNA genome synthesis in the lumen of the capsid to the competence of the particle for envelopment (4, 24) have been proposed (5, 25, 26) but have not been verified. Also, controversial data regarding the contact sites between capsid and envelope proteins have been reported. One model proposes that the tips of the spikes protruding from the capsid interact with the envelope proteins (27-29). Another model suggests that a domain (MBD) at the base of the spikes and lateral regions interacts with the large envelope protein (8, 30).

We generated an aptamer (31) that binds to or close to the MBD on HBV capsids expressed in bacteria with a dissociation constant of 180 nM by a positive/negative selection process. The I126A mutant capsids were chosen for negative selection because all tested amino acid substitutions at this position (A, V, L, G, W, F, Y, S, T, C, Q, N) with the exception of methionine and proline allowed capsid formation but strongly blocked capsid envelopment in transfected human hepatoma cells (10). The fact that the aptamer AO-01 bound to I126A mutant capsids with a sevenfold higher dissociation constant relative to WT capsids argues for a binding site including this residue. An alternative explanation is, however, that the I126A mutation induces a change at a different site of the capsid which is then involved in AO-01 binding. To date, we cannot rule out the second possibility. Our measurements revealed that a

maximum of 120 AO-01 aptamers can be bound by one capsid. Apparently, two core proteins contribute to one binding site.

The aptamer AO-01 inhibited HBV secretion in Huh7 cells cotransfected with a genomic HBV construct. Although the half-life of AO-01 was only 2 hours a clear inhibition of approximately 50 % on virus secretion during a 3 day period post transfection could be observed. The half-life of AO-01 was measured in cells not expressing HBV capsids. Potentially, the half-life is extended by binding to capsids. It seems possible that the inhibitory effect of AO-01 was relatively strong during the early phase after the transfection while later on during the 3 day period virus production may be more and more undisturbed. A DNA aptamer against the NSB5 protein of hepatitis C virus has a dissociation constant of 132 nM which is similar to the K_d value of aptamer AO-01 and reduced viral mRNA levels by 90 % in a cell culture system (32). Another possible explanation besides the short half-life for the relatively weak inhibition of budding by aptamer AO-01 is that the MBD of mature HBV capsids competent for budding may have a structure different from the MBD of recombinant capsids lacking the C-terminal domain. To date, we cannot rule out this possibility. However, empty capsids apparently can be enveloped (26, 33) and it seems therefore possible, that the MBD of recombinant capsids from bacteria mimics the MBD on mature capsids.

Which step in the viral life cycle was blocked by the aptamer AO-01 is not clear. The most straight forward model would be that the aptamer binds to or close to the MBD and thereby inhibits the interaction of the capsid with the matrix domain of the L protein. This explanation would support the model that the MD-MBD interaction is crucial for envelopment. It is not clear how many MD-MBD interactions would be necessary for complete envelopment of the capsid. If e.g. close to 120 interactions per particle are required for this morphogenesis step a few bound aptamers might be

able to inhibit virion formation. However, if a few MD-MBD interactions per particle are sufficient then an almost complete coverage of MBD sites by aptamers might be necessary for efficient inhibition. However, alternative models are well possible. For example, it is conceivable that aptamer binding blocks the generation of the maturation signal of the capsid or that it blocks the transport of the capsid to budding sites.

Other attempts have been made to inhibit the generation of HBV particles by interfering with the envelope – capsid interaction using peptides or chemicals (34-36). E.g. cell permeable peptides containing the matrix domain added at low micromolar concentrations to HBV producing cells reduced the production of extracellular HBV DNA by 50 % (34), and a oxazolidine derivative found during an *in vitro* screen for molecules inhibiting the matrix domain – capsid interaction reduced HBV DNA released from transiently transfected cells by a factor of 4 at 20 µM concentration (35). Our study supports the notion that molecules binding to the MBD on HBV capsids can suppress hepatitis B virion formation. Whether an aptamer-derived molecule can be applied in humans is open (37). However, in the future this target may expand therapeutic options for the treatment of chronic hepatitis B.

Acknowledgments

The anti-HBc antibody H800 was provided by Heinz Schaller (Heidelberg, Germany). A.O. was supported by the Deutsche Akademische Austauschdienst (DAAD) in cooperation with the Egyptian Ministry of Higher Education and Scientific Reseach. We thank Gabriele Möller (Institute for Experimental Genetics, Helmholtz-Zentrum München, Germany) for assistance in K_d measurements and Ulrike Protzer for financial support.

References

1. **Ott JJ, Stevens GA, Groeger J, Wiersma ST.** 2012. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine **30:**2212-2219.
2. **Beck J, Nassal M.** 2007. Hepatitis B virus replication. World J Gastroenterol **13:**48-64.
3. **Bruss V.** 2007. Hepatitis B virus morphogenesis. World J Gastroenterol **13:**65-73.
4. **Gerelsaikhan T, Tavis JE, Bruss V.** 1996. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. J Virol **70:**4269-4274.
5. **Roseman AM, Berriman JA, Wynne SA, Butler PJ, Crowther RA.** 2005. A structural model for maturation of the hepatitis B virus core. Proc Natl Acad Sci U S A **102:**15821-15826.
6. **Gallina A, Bonelli F, Zentilin L, Rindi G, Muttini M, Milanesi G.** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids. J Virol **63:**4645-4652.
7. **Stieler JT, Prange R.** 2014. Involvement of ESCRT-II in hepatitis B virus morphogenesis. PLoS One **9:**e91279.
8. **Ponsel D, Bruss V.** 2003. Mapping of amino acid side chains on the surface of hepatitis B virus capsids required for envelopment and virion formation. J Virol **77:**416-422.
9. **Schittl B, Bruss V.** 2014. Mutational profiling of the variability of individual amino acid positions in the hepatitis B virus matrix domain. Virology **458-459:**183-189.
10. **Pairan A, Bruss V.** 2009. Functional surfaces of the hepatitis B virus capsid. J Virol **83:**11616-11623.
11. **Yuan TT, Sahu GK, Whitehead WE, Greenberg R, Shih C.** 1999. The mechanism of an immature secretion phenotype of a highly frequent naturally occurring missense mutation at codon 97 of human hepatitis B virus core antigen. J Virol **73:**5731-5740.
12. **Yuan TT, Tai PC, Shih C.** 1999. Subtype-independent immature secretion and subtype-dependent replication deficiency of a highly frequent, naturally occurring mutation of human hepatitis B virus core antigen. J Virol **73:**10122-10128.
13. **Le Pogam S, Shih C.** 2002. Influence of a putative intermolecular interaction between core and the pre-S1 domain of the large envelope protein on hepatitis B virus secretion. J Virol **76:**6510-6517.
14. **Bruss V, Ganem D.** 1991. The role of envelope proteins in hepatitis B virus assembly. Proc Natl Acad Sci U S A **88:**1059-1063.
15. **Bruss V, Thomssen R.** 1994. Mapping a region of the large envelope protein required for hepatitis B virion maturation. J Virol **68:**1643-1650.
16. **Kuroki K, Russnak R, Ganem D.** 1989. Novel N-terminal amino acid sequence required for retention of a hepatitis B virus glycoprotein in the endoplasmic reticulum. Mol Cell Biol **9:**4459-4466.
17. **Prange R, Clemen A, Streeck RE.** 1991. Myristylation is involved in intracellular retention of hepatitis B virus envelope proteins. J Virol **65:**3919-3923.

18. **Bruss V, Ganem D.** 1991. Mutational analysis of hepatitis B surface antigen particle assembly and secretion. J Virol **65:**3813-3820.
19. **Pagratis NC.** 1996. Rapid preparation of single stranded DNA from PCR products by streptavidin induced electrophoretic mobility shift. Nucleic Acids Res **24:**3645-3646.
20. **Zuker M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res **31:**3406-3415.
21. **Tuerk C, Gold L.** 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science **249:**505-510.
22. **Wynne SA, Crowther RA, Leslie AG.** 1999. The crystal structure of the human hepatitis B virus capsid. Mol Cell **3:**771-780.
23. **Zlotnick A, Cheng N, Conway JF, Booy FP, Steven AC, Stahl SJ, Wingfield PT.** 1996. Dimorphism of hepatitis B virus capsids is strongly influenced by the C-terminus of the capsid protein. Biochemistry **35:**7412-7421.
24. **Summers J, Mason WS.** 1982. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. Cell **29:**403-415.
25. **Dhason MS, Wang JCY, Hagan MF, Zlotnick A.** 2012. Differential assembly of Hepatitis B Virus core protein on single- and double-stranded nucleic acid suggest the dsDNA-filled core is spring-loaded. Virology **430:**20-29.
26. **Ning X, Nguyen D, Mentzer L, Adams C, Lee H, Ashley R, Hafenstein S, Hu J.** 2011. Secretion of genome-free hepatitis B virus--single strand blocking model for virion morphogenesis of para-retrovirus. PLoS Pathog **7:**e1002255.
27. **Tan WS, Dyson MR, Murray K.** 1999. Two distinct segments of the hepatitis B virus surface antigen contribute synergistically to its association with the viral core particles. J Mol Biol **286:**797-808.
28. **Seitz S, Urban S, Antoni C, Bottcher B.** 2007. Cryo-electron microscopy of hepatitis B virions reveals variability in envelope capsid interactions. EMBO J **26:**4160-4167.
29. **Bottcher B, Tsuji N, Takahashi H, Dyson MR, Zhao S, Crowther RA, Murray K.** 1998. Peptides that block hepatitis B virus assembly: analysis by cryomicroscopy, mutagenesis and transfection. EMBO J **17:**6839-6845.
30. **Kluge B, Schlager M, Pairan A, Bruss V.** 2005. Determination of the minimal distance between the matrix and transmembrane domains of the large hepatitis B virus envelope protein. J Virol **79:**7918-7921.
31. **Feng H, Beck J, Nassal M, Hu KH.** 2011. A SELEX-screened aptamer of human hepatitis B virus RNA encapsidation signal suppresses viral replication. PLoS One **6:**e27862.
32. **Bellecave P, Cazenave C, Rumi J, Staedel C, Cosnefroy O, Andreola ML, Ventura M, Tarrago-Litvak L, Astier-Gin T.** 2008. Inhibition of hepatitis C virus (HCV) RNA polymerase by DNA aptamers: mechanism of inhibition of in vitro RNA synthesis and effect on HCV-infected cells. Antimicrob Agents Chemother **52:**2097-2110.
33. **Luckenbaugh L, Kitrinos KM, Delaney WEt, Hu J.** 2014. Genome-free hepatitis B virion levels in patient sera as a potential marker to monitor response to antiviral therapy. J Viral Hepat doi:10.1111/jvh.12361.
34. **Pan XB, Wei L, Han JC, Ma H, Deng K, Cong X.** 2011. Artificial recombinant cell-penetrating peptides interfere with envelopment of hepatitis B virus nucleocapsid and viral production. Antiviral Res **89:**109-114.

35. **Asif-Ullah M, Choi KJ, Choi KI, Jeong YJ, Yu YG.** 2006. Identification of compounds that inhibit the interaction between core and surface protein of hepatitis B virus. Antiviral Res **70:**85-90.

36. **Bottcher B, Tsuji N, Takahashi H, Dyson MR, Zhao S, Crowther RA, Murray K.** 1998. Peptides that block hepatitis B virus assembly: analysis by cryomicroscopy, mutagenesis and transfection. EMBO J **17:**6839-6845.

37. **Bruno JG.** 2015. Predicting the Uncertain Future of Aptamer-Based Diagnostics and Therapeutics. Molecules **20:**6866-6887.

Figure Legends

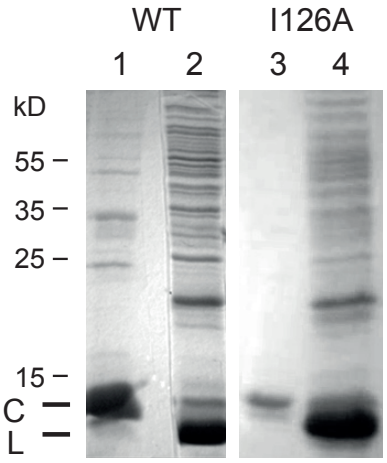
FIG. 1. Purification of recombinant capsids consisting of C-terminal deleted core proteins. Ten µl of purified WT and I126A mutant capsids (lanes 1 and 3, respectively) and 20 µl of cleared lysates (lanes 2 and 4, respectively) were separated by PAGE and stained with Coomassie. Numbers to the left indicate the position of molecular weight marker proteins, C: C-terminal deleted core proteins, L: lysozyme.

FIG. 2. Predicted secondary structure of aptamer AO-01. The secondary structure of AO-01 was predicted by the Zuker algorithm (20). A motif common to 10 out of 16 different aptamers is shown in bold face and underlined. The fixed positions at both ends are shown in lower case letters.

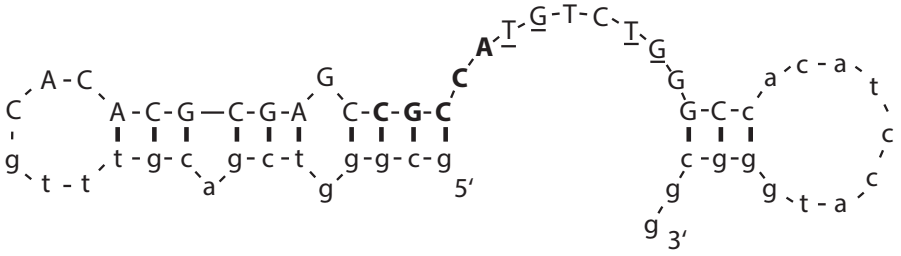
FIG. 3. Determination of K_d values for aptamer binding to WT and mutant capsids. Capsids at a constant concentration of 1 nM were mixed with five different concentrations of the four most abundant aptamers AO-01 to AO-04. Capsid-aptamer complexes were separated and bound aptamers were quantified. (A) The dissociation constants of aptamer binding to WT capsid and the abundance of aptamers as shown in Tab. 2 were negatively correlated. A saturation of AO-01 binding was reached with 120 aptamers per capsid. (B) Binding to I126A mutant capsids. The standard deviation (N = 3) is shown only for aptamer AO-01. The standard deviation for the other aptamers was in the same range (not shown). Designation of the symbols as shown in panel B applies also to panel A.

FIG. 4. Inhibition of virus release by aptamer AO-01. Huh7 cells were transiently transfected with plasmids initiating HBV formation (-) and the concentration of viral genomes in the culture supernatant was measured 3 days post transfection. Leaving out the plasmid for HBV envelope protein expression prevented virion formation (14) and resulted in a background signal (B). Cotransfection of the plasmids with the aptamer AO-01 reduced the amount of virus in the culture supernatant by approximately 50 % (AO-01) whereas cotransfection of an aptamer with random sequence had no effect (A-N).

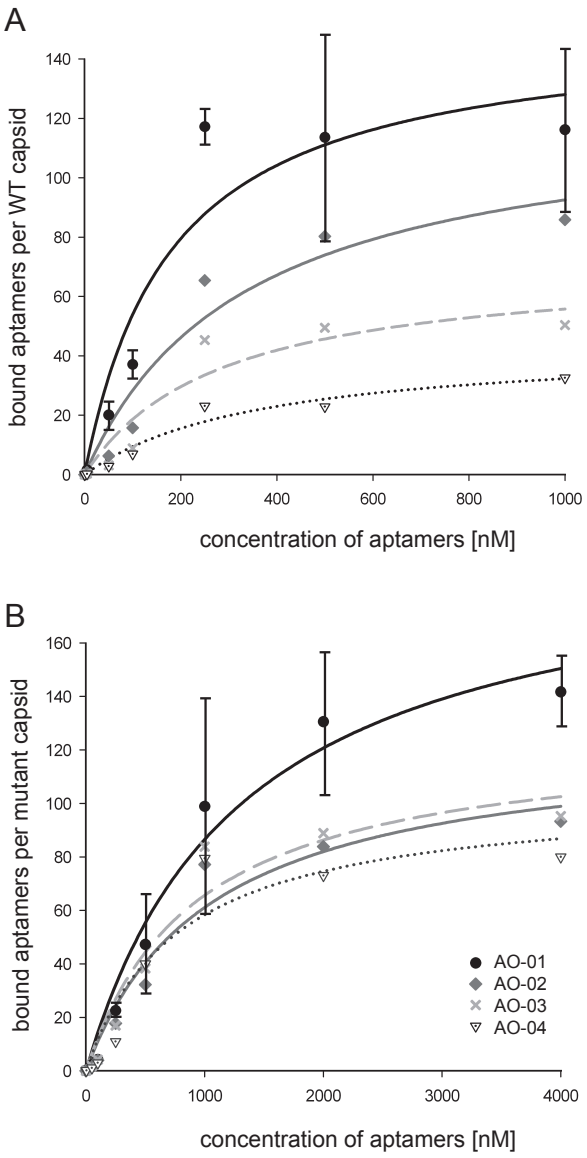
FIG. 5. Determination of the half-life of aptamer AO-01 in Huh7 cells. Huh7 cells were transiently transfected with aptamer AO-01. The aptamer in the culture was quantified every 12 hours by PCR. The amount of aptamers dropped exponentially. The half-life was approximately 2 hours. The experiment was performed twice (squares and circles).



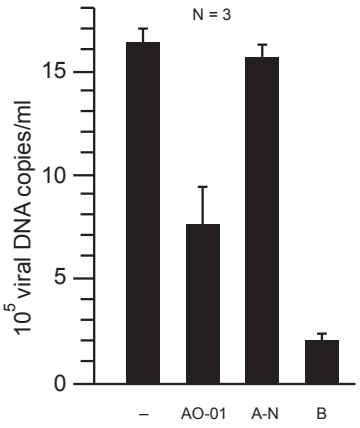
Orabi et al., Fig. 1



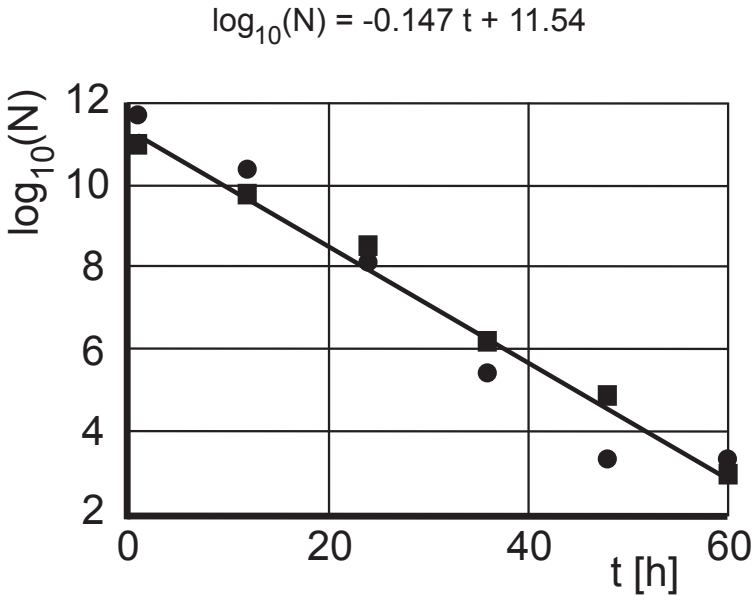
Orabi et al., Fig. 2



Orabi et al., Fig. 3



Orabi et al., Fig. 4



Orabi et al., Fig. 5

Table 1

Conditions during the aptamer selection process.
The volume of the binding reaktion was 100 µl.

round	1 - 3	4 - 6	7 - 9	10 - 13
aptamers[a]	110	110	55	27
WT capsids[b]	14	6	6	0.2
mutant capsids[b]	6	10	10	14
IT[c] WT capsids	60	60	30	15
IT[c] mutant capsids	30	30	60	60
WV[d] positive selection	1	1	2	2
WV[d] negative selection	1	1	0.4	0.4

[a] concentration (µM)

[b] concentration (nM)

[c] incubation time (minutes)

[d] volume of washing buffer (ml)

Journal of Virology

Table 2
Sequence and frequency of selected aptamers

aptamer	sequence[a]	N[b]	f[c] (%)	L[d] (nt)
A-01	CACACGCGAGC**CGCCA**TGTCTGGGC	13	17.8	25
A-02	GGGAC**CG**CAGAAGA**CCA**CATGTGCC	11	15.1	25
A-03	GGGACGGCC**CGCCA**TTCCGTGTGGC	7	9.6	25
A-04	GTCGACG**CGCCCA**TTCCGTGGGGTG	6	8.2	25
A-05	GGCACACAACGT**CGCCA**TGGCTGTG	4	5.5	25
A-06	CCCACGCAACGG**CGCCA**TGGCTGTG	4	5.5	25
A-07	GCGTCGGCGCG**CGCCA**TTGTGGTGC	4	5.5	25
A-08	GGGCAGGGTCGAC**CGCCA**TGGCTGTG	4	5.5	26
A-09	GGCACAAACG**CGCCA**TGGCTGC	4	5.5	22
A-10	GCCAACGACGGGC**CGCCA**TGGTCTG	3	4.1	25
A-11	GGCACAAACG**CG**GG**CCA**TCCATGC	3	4.1	24
A-12	GGCACCCAA**CG**CCC**CCA**TGGGTGTG	2	2.7	25
A-13	GGGCAGGGTCGAC**CGCCA**TGGCTGG	2	2.7	25
A-14	CCGAGGGGCAACGG**CGCCA**TGGCTG	2	2.7	25
A-15	CATAA**CG**TTGCCCC**CCA**TGTGTTG	2	2.7	24
A-16	GGCAGCCT**CG**ACCCC**CCA**TGGC	2	2.7	22

[a] only the sequence of the central variable region is shown

[b] number of appearance of the sequence within 73 isolates

[c] frequence of the aptamer in % (N * 100/73)

[d] length of the variable region of the aptamer