WP3.2. Development of functional DNA motifs that target HBV (Pichova, Curtis, Hocek)

Research aims

- The major aim of this WP is to develop DNA motifs which will covalently link themselves to the HBV core protein and inhibit the biological function of this key player of HBV infection. The specific aims will include:
- Design and synthesis of single-stranded DNA pools to which various small molecule inhibitors of HBV capsid formation are covalently linked
- Cloning of the wt assembly capsid domain (Cp 149), the Cp Y132A mutant, and a series of Cp mutants containing truncated RNA binding domains
- Development of a purification procedure that will enable the isolation of stable proteins for *in vitro* selection experiments
- Identification of DNA motifs that covalently link themselves to the HBV core protein using *in vitro* selection
- Investigating the ability of DNA motifs isolated in our selections to inhibit HBV replication

Research plan and methodology

The starting point for these experiments will be a series of purified variants of the HBV core protein. The predominant form of the HBV capsid consists of 120 homodimers of the core protein arranged with a T-4 icosahedral symmetry[1]. Cp is a 183-residue protein consisting of a 149-residue assembly domain and a 34-residue RNA binding domain at the C-terminus. Cp dimers can spontaneously self-assemble, and this process is accompanied by conformational changes of the homodimer[2]. Empty capsids can also be assembled *in vitro* from the Cp assembly domain (Cp149). The nucleation step in the assembly process is formation of a trimer of homodimers. The individual inter-dimer contacts are weak but globally they result in a stable structure. The extent of assembly can be increased by higher temperature and ionic strength and can be disrupted by the addition of urea or mutating residues important for Cp dimer formation[3]. For example, mutating tyrosine 132 in Cp149 to alanine (Y132A) leads to disruption of capsid assembly[4]. An important characteristic of this mutant is that it can still form trimers of dimers and therefore reflects the basic capsid building block[5]. For this reason, the Y132A mutant is an excellent starting point for *in vitro* selection experiments that target the HBV core protein. Moreover, this mutant is already available in our laboratory. To generate sufficient material for *in vitro* selection we will express CpY132A in *E. coli* and develop an optimized protocol for dimer purification. We will also optimize conditions for the stable storage of Cp dimers. In parallel, we will search for conditions that prevent assembly of the wild type Cp149 dimer into capsids. For example, adding urea or reducing the ionic strength of the buffer are both expected to result in less efficient capsid formation. Dimers with different interface conformations will also be investigated. In addition, we will express and purify variants of the core protein containing the complete assembly domain and different number of residues from the RNA binding domain. Cloning, expression, and purification will be performed by the team of Iva Pichova.

For each *in vitro* selection experiment (Figure 1), a single-stranded DNA pool will be generated in which a random sequence domain is flanked by primer binding sites.

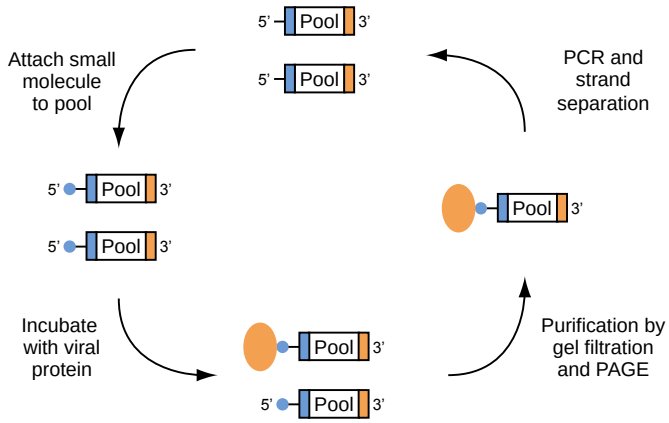

Fig. 1. *Isolation of DNA motifs that covalently link themselves to the HBV core protein.*

In addition to a conventional DNA pool, a series of chemically modified pools will be generated in which each member contains a small molecule inhibitor of HBV capsid formation covalently linked to its 5' terminus by a PEG linker. Possible examples include the fluorescent dye 5,50-bis[8-(phenylamino)-1-naphthalenesulfonate] and certain heteroaryldihydropyrimidine compounds[6]. These pools will be designed by the team of Edward Curtis and synthesized by the team of Michal Hocek. To increase the likelihood of success, two types of selections will be performed. In one case, we will search for DNA motifs that covalent link themselves to core protein homodimers under conditions in which capsids cannot form. This can be accomplished by incubating the wild-type core protein in an appropriate buffer or by using the CpY132A mutant, which can form dimers and trimers of dimers but not capsids. In a second approach, we will search for DNA motifs that covalent link themselves to core protein dimers under conditions in which capsids can form. A significant advantage of this experimental setup is that it selects for pool members that inhibit capsid formation rather than those that simply bind to the core protein. After purifying DNA-dimer complexes by gel filtration, a second purification will be performed under denaturing conditions to recover pool members that are covalently linked to the core protein. Surviving pool members will be amplified by PCR using a conventional all-DNA primer and a second primer that contains a single RNA linkage at its 3' end. After base hydrolysis, full-length molecules will be purified by PAGE to generate material for the next round of selection. Once activity is detected, pool members will be cloned, sequenced, and individually tested for the ability to both covalently link themselves to the target protein and to inhibit capsid formation *in vitro*. Binding will also be characterized by thermal shift assays and electron microscopy.

Promising DNA motifs will then be optimized using a two step approach (Figure 2). First, each example will be randomly mutagenized at a rate of 20% per position, and improved variants isolated using *in vitro* selection. Once activity is detected, ~10^5 pool members will be sequenced using a MiniSeq high-throughput sequencing instrument that will be purchased as part of this proposal. This step will facilitate secondary structure determination by comparative sequence analysis, and will also provide extensive information about mutations correlated with improved catalytic activity. Second, mutations that occur frequently in these improved variants will be synthetically recombined in approximately 10^{14} different combinations using a method that Edward Curtis developed as a graduate student[7], and the most active variants isolated by additional rounds of *in vitro* selection. In a previous experiment that used conventional sequencing to identify mutations correlated with increased activity, ribozymes with catalytic efficiencies up to 1000-fold higher than that of the initial isolate were obtained[7]. Since high-throughput sequencing will provide us with significantly more information, we expect that in this case the optimization experiments will be even more powerful.

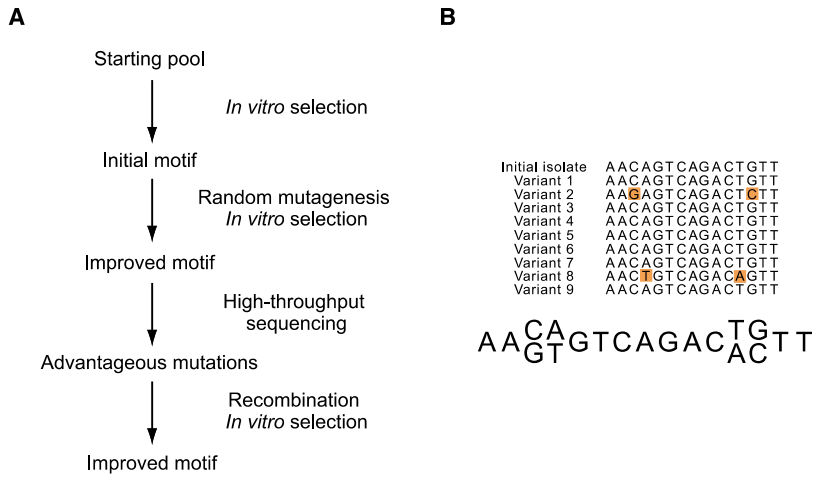

Fig. 2. *Motif optimization.* ***A.*** *Workflow for motif optimization* ***B.*** *Synthetic recombination protocol. Hypothetical sequence alignment of variants generated by random mutagenesis of the initial isolate of a motif followed by in vitro selection. ~10^5 variants will be sequenced from each pool. Below: Example of a synthetically recombined pool designed based on this alignment.*

In the next phase of the project, the effects of these motifs on HBV replication will be determined in collaboration with Jan Weber. To do this, optimized variants of each motif will be transfected into HBV-infected HepG2/NTCP cells and the effects on viral replication will be determined. Control experiments will be performed using different combinations of arbitrary DNA sequences and small molecules. If initial results are promising, these compounds could potentially be used as starting points in clinical trials. In addition to possible uses as drugs, we envision several additional applications for DNA motifs that covalently link themselves to HBV proteins. For example, by labelling DNA motifs with a fluorophore, these compounds could be used as diagnostic tools to monitor HBV infection.

[1] Wynne, S. A.; Crowther, R. A.; Leslie, A. G., The crystal structure of the human hepatitis B virus capsid. *Mol Cell* **1999,** *3* (6), 771-780.

[2] Selzer, L., Katen, S.P., Zlotnick, A. The hepatitis B virus core protein intradimer interface modulates capsid assembly and stability. *Biochemistry*. **2014,** *53*, 5496-5504.

[3] Ceres, P.; Zlotnick, A., Weak protein-protein interactions are sufficient to drive assembly of hepatitis B virus capsids. *Biochemistry* **2002,** *41* (39), 11525-11531.

[4] Bourne, C.R., Katen, S.P., Fulz, M.R., Packianathan, C., Zlotnick, A. A mutant hepatitis B virus core protein mimics inhibitors of icosahedral capsid self-assembly. *Biochemistry.* **2009**,*48*,1736-1742.

[5] Klumpp, K.; Lam, A. M.; Lukacs, C.; Vogel, R.; Ren, S.; Espiritu, C.; Baydo, R.; Atkins, K.; Abendroth, J.; Liao, G.; Efimov, A.; Hartman, G.; Flores, O. A., High-resolution crystal structure of a hepatitis B virus replication inhibitor bound to the viral core protein. *Proceedings of the National Academy of Sciences of the United States of America* **2015,** *112*, 15196-15201.

[6] Wang, Y. J.; Yang, L.; Zuo, J. P., Recent developments in antivirals against hepatitis B virus. *Virus Res.* **2016**, *213*, 205-213.

[7] Curtis, E. A.; Bartel, D. P., Synthetic shuffling and in vitro selection reveal the rugged adaptive fitness landscape of a kinase ribozyme. *RNA* **2013**, *19* (8), 1116-1128

Research schedule

2017- 2019

- Expression and purification of the wild type and Cp Y132A mutant
- Optimization of conditions for the stable purification and storage of Cp dimers.
- Cloning, expression, and purification of C-terminally truncated Cp variants.
- Initial *in vitro* selection experiments.
- Motif optimization by random mutagenesis and *in vitro* selection.
- Identification of mutations that increase activity by high-throughput sequencing.

- Motif optimization by synthetic recombination and *in vitro* selection.
- Enrichment and qPCR analyses

2020-2022

- Investigating the effects of these DNA motifs on capsid formation.
- Determining the effects of these DNA motifs on HBV replication.

Publications and patents

Publications (Jimp)

	Jimp
2017	0
2018	2
2019	3
2020	3
2021	3
2022	4
Total	15

Nucleic Acids Research
Journal of Virology
ChemBioChem
Chemistry & Biology
RNA
RNA Biology
Journal of medicinal Chemistry

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	0
2020	0	
2021	0	1
2022	0	0
Total	0	1

We expect IP protection in the following areas:
New modified aptamers against HBV..

Cooperation with foreign institutions

- Harvard Medical School, bioinformatics analysis of data from high-throughput sequencing of DNA motifs.
- Gilead Sciences, development of anti-HBV DNA motifs