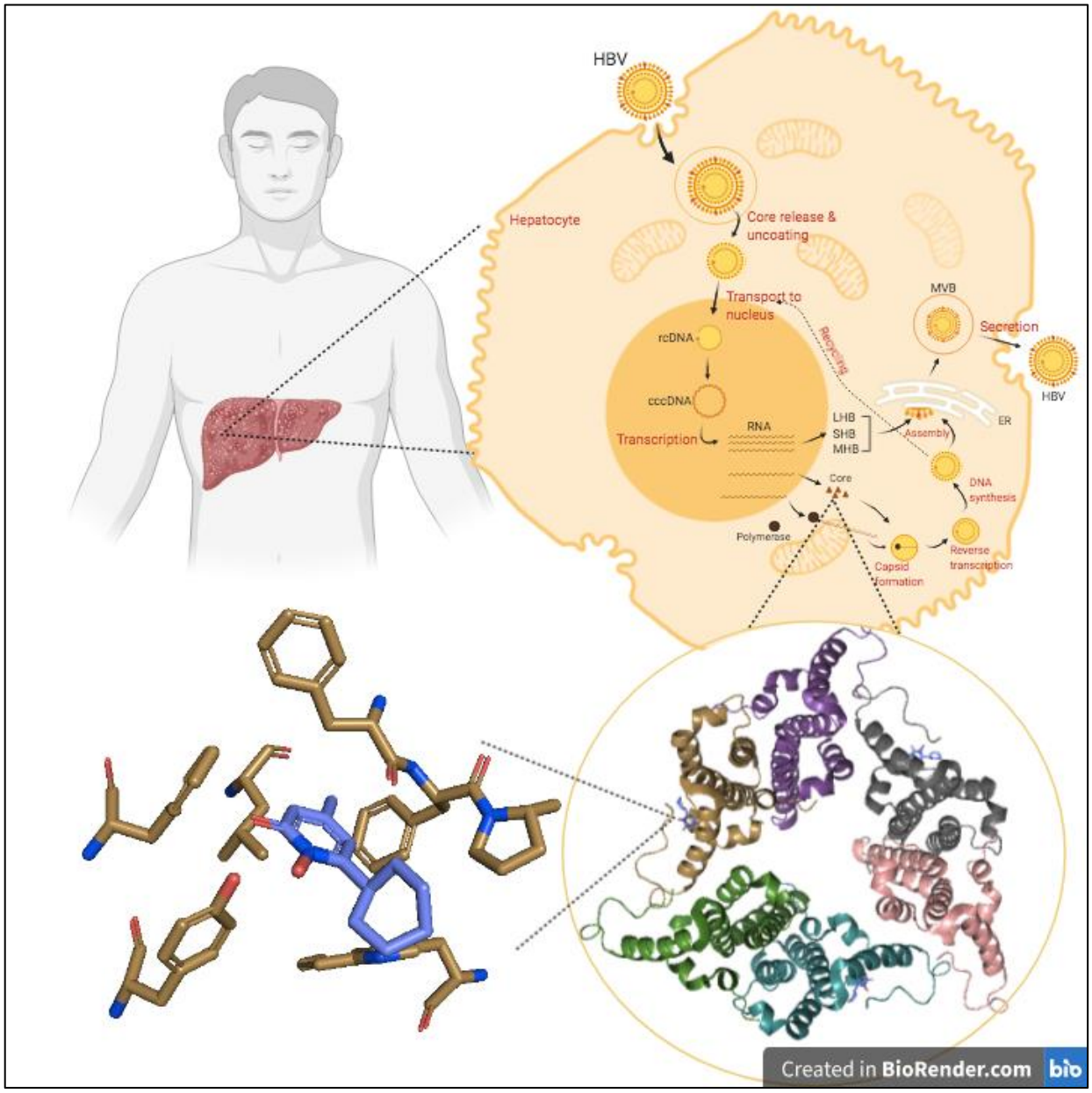
The research of new drug design for the treatment of hepatitis B virus, by looking at the life cycle of the virus

Using structural biology will provide insight in understanding drug efficacy mechanisms and allows for structure-guided design to create drug-like properties and antiviral potency



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Abstract

Worldwide there are about 350 million people with chronic hepatitis B virus (chronic HBV), which can lead to hepatocellular carcinoma and cirrhosis. This can cause 780.000 deaths each year from HBV. Besides, the quality adjusted life year (QALY) is 75.000 dollar per QALY, which is not cost effective. [(1)] Infected patients can be treated but not cured. One of the methods to obtain high quality drugs is structural biology, which will play an important role in discovering new drugs. Structural biology will provide insight in understanding drug efficacy mechanisms and it allows for structure-guided design to create drug-like properties and antiviral potency. [(2)] To improve the drugs of hepatitis B, research has been done on the life cycle of the hepatitis B virus. The drug targets discussed in this review, will give insight into new alternative drug targets for HBV treatment.

One of the effective ways to reduce HBV activity is inhibiting capsid formation by binding of the drug target into the hydrophobic pocket. Normally, core proteins bind into the pocket and forms a capsid, but now inhibitors will bind and block capsid formation. A second method to increase the anti-HBV activity is using capsid formation effectors. These drug targets increase the rate of capsid assembly, which cause a distortion and destabilization of the capsid assembly and changes in tertiary structure of the core protein. In addition, it can block RNA packaging, leading to a capsid without RNA genome. Another essential method is using antibodies against hepatitis B surface antigen (HBsAg), core antigen (HBcAg) and e-antigen (HBeAg), which cause a reduction in viral DNA. The last subject reviewed is about polymerase inhibitors, which inhibit DNA polymerase, using nucleotides reverse transcriptase inhibitors (NRTIs). This part examines the cause of resistance to NRTIs, which will help to design new drug targets for NRTIs conquer drug resistance.

Keywords

Structural biology, hepatitis B virus, life cycle, DNA, core protein, capsid protein, hydrophobic pocket, binding site, antigen, polymerase, resistance.

Abbreviations

3TC	Lamivudine
3TC-TP	3TC-triphosphate
AZT	Azidothymidine triphosphate
bNAbs	Broadly neutralizing antibodies
cccDNA	covalently closed circular DNA
DBT	Dibenzothiazepine
dNTP	deoxynucleotide triphosphate
ETV	Entecavir
ETV-TP	ETV-triphosphate
Fab	Fragment antigen binding
HAP	Heteroaryldihydropyrimidine
HBcAg	Hepatitis B core antigen
HBcAgd	Hepatitis B core antigen dimer
HBeAg	Hepatitis B e-antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus

HCDR	Heavy chain complementarity-determining-region
HIV-1	Human immunodeficiency virus 1
huE6F6-1	Humanized E6F6-1 antibody
LCDR	Light chain complementarity-determining-region
LHB	Large hepatitis B surface protein
MHB	Middle hepatitis B surface protein
MVB	Multi vesicular bodies
NTCP	Sodium Taurocholate co-transporting polypeptide
PPA	Phenylpropenamide
rHBeAgd	recombinant hepatitis B e-antigen dimer
scFv	single chain variable fragment
SHB	Small hepatitis B surface protein
TNV	Tenofovir diphosphate

AMINOACIDS:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
Y132A	Tyrosine 132 A

Introduction

Hepatitis B

Worldwide 350 million people suffer from chronic hepatitis B virus (chronic HBV). Moreover, there are two types of chronic hepatitis B, inactive and active form. The inactive form of the virus can be present without any symptoms and complaints, but sometimes the same complaints occur as the complaints of acute hepatitis B. However, the active form of hepatitis B is more severe, which can lead to liver fibrosis. In long term, it results in liver cirrhosis, where the large amount of scar tissue can lead to liver cell death. Furthermore, liver cirrhosis increases the risk of developing liver cancer. [3]

Life cycle hepatitis B

HBV has a small double stranded DNA that is not covalently closed, nonetheless it has a variable gap in one strand and a nick in the other strand. This makes HBV different from other viruses like papillomavirus. After the attachment of the HBV to the hepatocytes, endosomes will be formed and the viral envelope will be removed (figure 1). Subsequently, the core proteins will be removed and then the virus will be transported to the nuclear pore, which will uncoat the genome and continued with releasing into the nucleus. The uncoated DNA is repaired to covalently closed circular (ccc) DNA by cellular DNA repair factors. This cccDNA

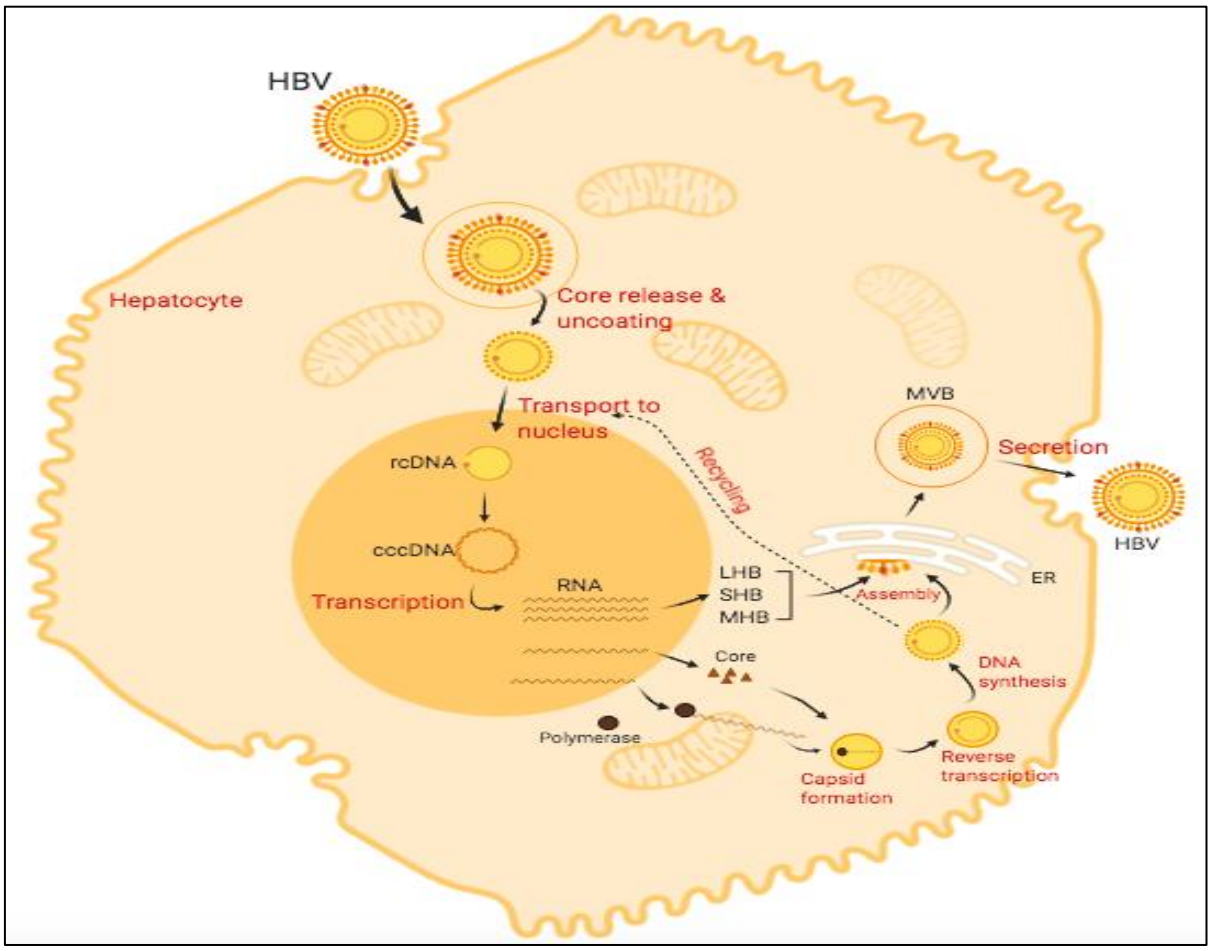

Figure 1. Life cycle of HBV. Attachment and entry of the HBV and fusion with endocytosis. Then core release and uncoating of the virus. After that the virus will go into the nucleus and be repaired to cccDNA, which will transcript into RNAs. Then capsid formation, reverse transcription, DNA synthesis and assembly happens followed by secretion of the virus. LHB, SHB, MHB = large, small and middle hepatitis B surface protein. ER=Endoplasmic reticulum. MVB= multi vesicular body. [4] Figure made with www.biorender.com.

will bind to liver specific cellular RNA polymerase II transcription factors to develop pre-genomic RNAs and sub genomic mRNAs. The translation of the large, small and middle hepatitis B surface protein (LHB, SHB and MHB) from the sub genomic mRNA happens at the endoplasmic reticulum and is secreted by the Golgi system. In the cytoplasm, pre-genomic RNAs are translated into HBV core proteins and RNA polymerase is initiated. Core proteins are important regulatory components in the HBV life cycle and considered as a good antiviral target. [(11)] The core proteins form a capsid around the viral polymerase, which contains RNA copy of the genome. This is followed by reverse transcription and DNA synthesis. Subsequently, this will assemble with the surface proteins in the endoplasmic reticulum. Using multi vesicular bodies (MVB) they will be migrated to the membrane, where exocytosis of the HBV happens. Another possibility, when the capsid is distorted or in the absence of DNA, the mature core particles can be transported to the nucleus again for recycling. [(4)]

This review identifies several novel drug targets for the treatment of HBV. The drug targets have been briefly summarized and the corresponding structures (with protein databank code, PDB) are created, using Pymol (DeLano Scientific). The drug targets are divided into different groups depending on the mode of action of these targets. These are respectively capsid formation inhibitors, capsid formation accelerators, antibodies and the resistance to polymerase inhibitors.

Structures

Capsid formation inhibitors

Ciclopirox (PDB: 6J10)

Ciclopirox could be a good alternative for a new drug in the treatment of HBV, because it inhibits capsid formation of HBV, by binding to the hydrophobic pockets (figure 2), where normally assembly modulators bind for capsid formation. The binding of ciclopirox can be explained by the atomic structure of the ciclopirox. The HBV core protein contains six binding sites. Ciclopirox binds to three hydrophobic pockets and not to the other three binding sites, which are hydrophobic pockets at the dimer-dimer interfaces. The reason why ciclopirox

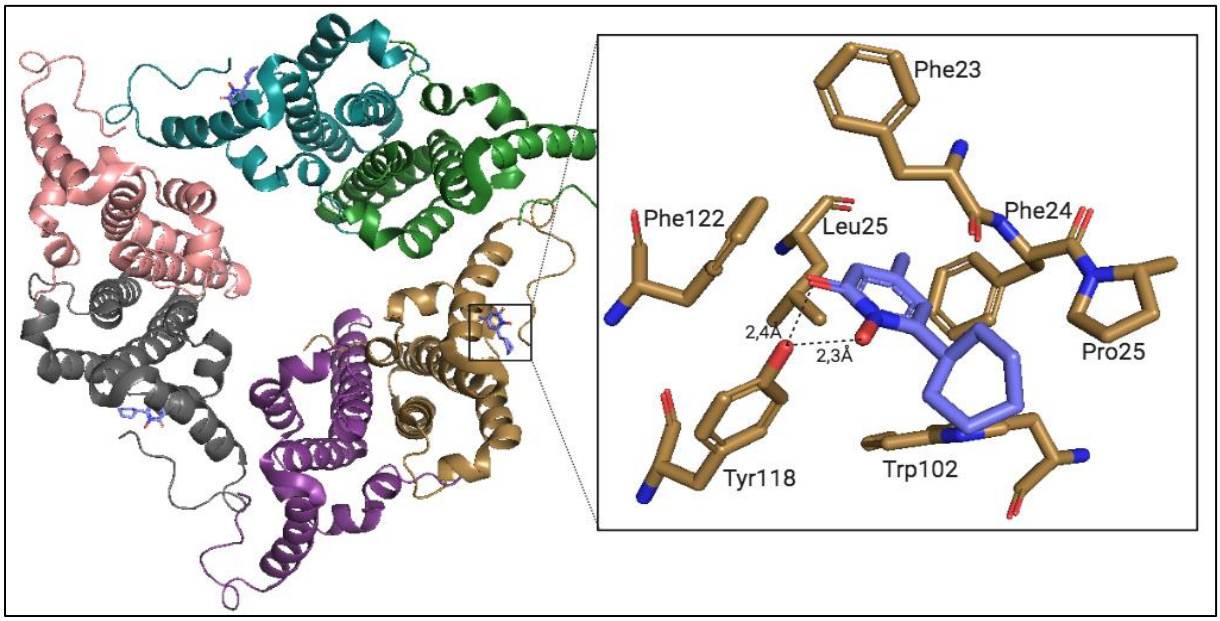

Figure 2. Ciclopirox with the core protein hexamer. Amino acid residues of the hydrophobic pocket are colored dark gold and ciclopirox has a blue color. Made with Pymol (DeLano Scientific), PDB code: 6J10.

disallows binding to these binding sites is the presence of Phe23, which has a stronger affinity with these pockets at the dimer-dimer interface than ciclopirox. Phe23 rotates 142 degrees, so the phenyl ring of Phe23 will directly occupy the free binding site, due to its similar structure as ciclopirox, and will form hydrophobic bonds with neighboring proteins. This leads to a decline of the dimer-dimer interface pocket and blocking the binding of ciclopirox. [5]
However, when ciclopirox will bind to the three hydrophobic pockets, the phenyl ring of Phe23 rotates upwards and makes the pocket free. Afterwards ciclopirox is allowed to bind to the pocket and clamps deep into the HBV core protein. This deep binding generates hydrophobic interactions and prevents ciclopirox from releasing. [5]

Moreover, Tyr118 forms strong salt bridges with a nitrogen, carbonyl oxygen and hydroxyl group of the pyridine ring of ciclopirox. It also forms strong hydrogen bonds with the ciclopirox. This leads to anchor the ciclopirox in the binding pocket and stabilizes the conformation of ciclopirox in the pocket. [5]

(2S,4S)-4,4-difluoroproline substituted analogue 34a (PDB: 5GMZ)

The zwitter ionic target (2S,4S)-4,4-difluoroproline substituted analogue, 34a, will provide good oral bioavailability research in mice, therefore it becomes a drug candidate in the treatment of HBV. This analogue 34a is obtained from 4-methyl heteroaryldihydropyrimidine (HAP). A clear structure of capsid protein Y132A mutant in complex with 34a and the binding site was obtained (figure 3). (2S,4S)-4,4-difluoroproline group of 34a plays an essential role in protein-protein interaction. [6]
The 4-methyl HAP group of 34a fits well into the binding pocket located in the B-chain and C-chain. The fluorphenyl group and 4-methyl group make van der Waals interactions with amino acids Thr128, Val124 and Arg127 from the C-chain, followed by Pro25, Thr33, Trp102, Ser106, Leu30 and Ile105 from the B-chain. The 4-methyl group will provoke steric collision with Thr128. (2S,4S)-4,4-difluoroproline group interact with Trp125, Arg133, Pro134 and Thr128 residues of the hydrophobic pocket. Moreover, the carboxylic acid group of 34a shares a hydrogen bond with Ser141, which will induce a higher binding affinity of the drug target 34a to the hydrophobic binding pocket and contribute to a higher anti-HBV activity. [6]

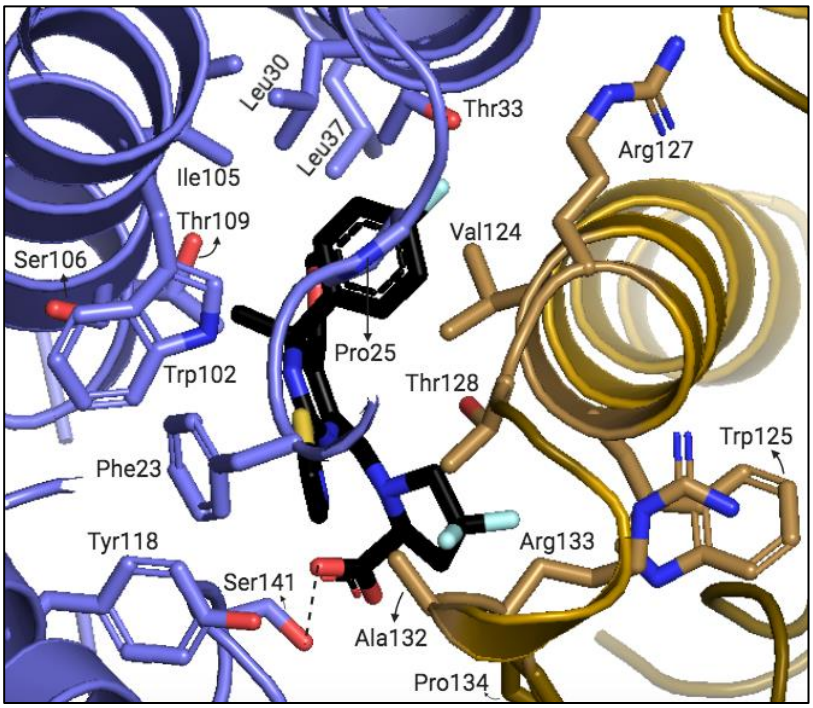

Figure 3. Hydrophobic binding pocket from the B-chain (blue) and C-chain (gold). Black colored compound is the (2S,4S)-4,4-difluoroproline substituted analogue 34a. There is one hydrogen bond between the analogue 34a and residue Ser141. Made with Pymol (DeLano Scientific), PDB code: 5GMZ.

Capsid formation accelerators

HAP1 (PDB: 2G34)

Heteroaryldihydropyrimidine 1 (HAP1) is a potent capsid misdirector and assembly activator of HBV. Capsid proteins have no human homolog and no cellular analogs, which makes them a promising therapeutic target. The structural knowledge of the target will profit the development of capsid drug target strategies. A binding site is identified for the HAP1, which provides changing the structure and misdirection of the capsid assembly. Moreover, saturation with HAP1 will lead to misdirection of assembly, due to the fact that it will promote changing the three- and six-fold icosahedral structures, which will contribute destabilization. The binding site of the HAP1 from the C chain contains the hydrophobic residues Phe23, Pro25, Leu30, Tyr38, Trp102, Ile105, Ser106, Tyr118 (figure 4). Normally, these residues will bind to each other and will stabilize the structure. Nevertheless, when HAP1 is present, it localizes in this pocket and blocks a part of a groove. This groove is normally used to interact with a nearby dimer. This happens so quickly that it misdirects the assembly. Besides, HAP1 strongly changes only the quaternary (and not tertiary) structure of the chains, leading to structural rearrangement. Moving the AB dimer upwards leads to a protrusion of the A chain. Changing of the CD dimer generate a flattening of the six-fold top. Rotation of the CD dimer causes bigger opening of the three-fold top. Summarized, the HAP1 drug target destabilizes the capsid assembly, leading to disruption of the capsid, which makes it a promising target for antiviral new generation therapeutics. [7]

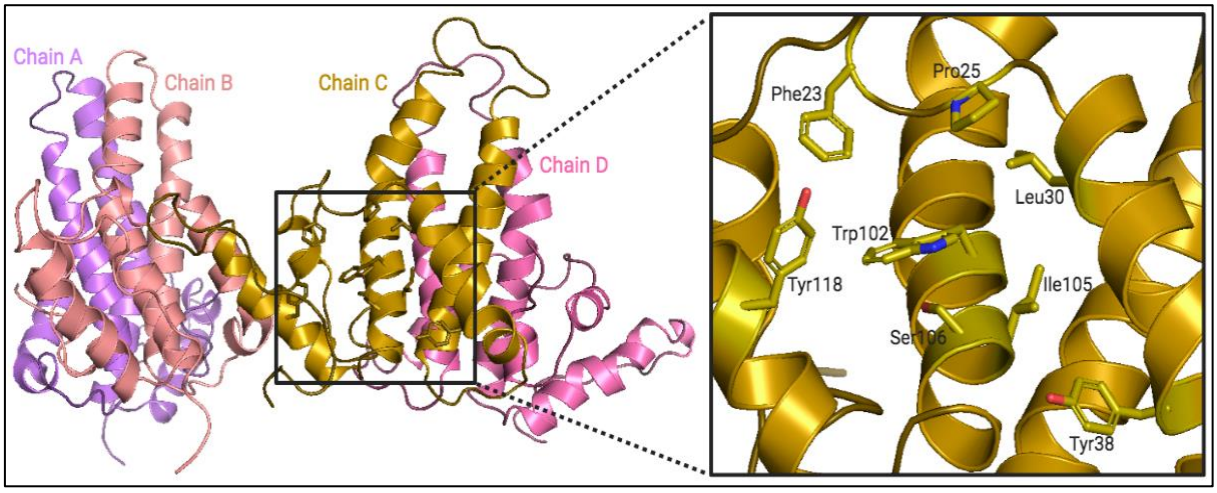

Figure 4. Capsid protein with the HAP1 binding site. Showing AB dimer (purple and salmon color) and CD dimer (gold and pink color) with the binding site of the HAP1 from the C-chain. Made with Pymol (DeLano Scientific), PBD code: 2G34.

AT-130 (PDB: 4G93)

AT-130 is one of the structures which belongs to the phenylpropenamide (PPA) family, a family of assembly effectors. This structure doesn't disrupt the capsid structure, but it changes the tertiary and quaternary structure, by binding on the capsid surface. Several studies suggested that AT-130 blocks RNA packaging, which leads to forming a capsid without the viral genome. In addition, there is shown the quaternary structure of the capsid, which contains two dimers, AB and CD dimers (figure 5). It's known from the literature that the dimer AB causes an upwards pivoting motion when AT-130 is bound. The CD dimer shows changes in the tertiary structure. Moreover, there was an upwards shift of the peak point atoms and a peak broadening.

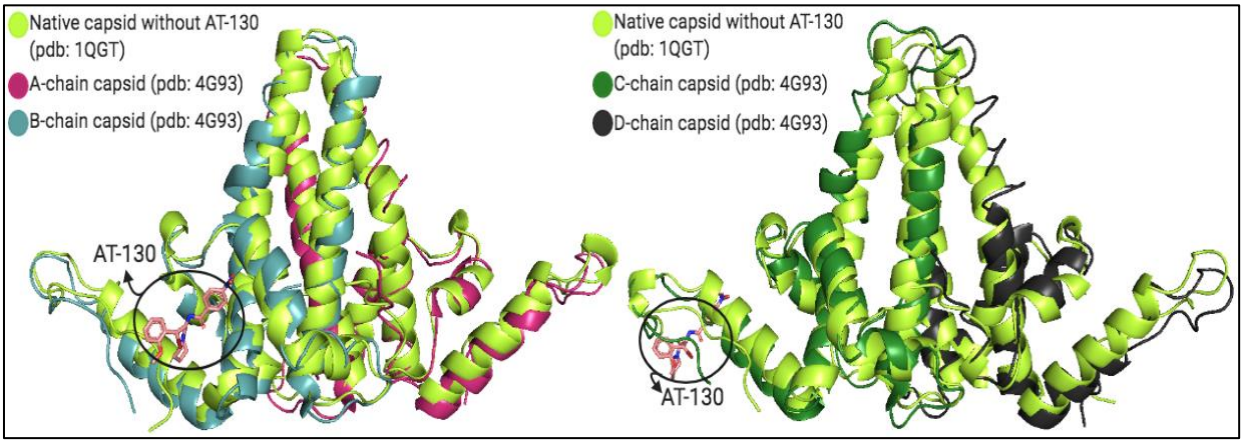

Figure 5. AT-130 dimers shown. In the first picture is shown the AB dimer and the native capsid without AT-130. In the second picture is shown the CD dimer and the native capsid without AT-130 to show the structural changes that is made by AT-130. Made with Pymol (DeLano Scientific), PDB code: 4G93 (AT130) and 1QGT (native capsid).

Although, it can still assemble a capsid. AT-130 has similarities with the HAP1 capsid structure, but shows differences in equivalent binding choices. The AT-130 prefers to bind strongly to the B-chain and has significant changes in tertiary and quaternary structure. Nevertheless, HAP1 has the strongest density binding predilection in the C-chain binding pocket and has only quaternary structural changes. [8]

One of the similarities is that the hydrophobic pocket they bind to is the same. AT-130 as well as HAP1 affect the quaternary capsid structure. So, AT-130 is a promising drug for HBV drug development, due to the fact that it decreases the HBV production well. [8] [9]

HAP-18 (PDB: 5D7Y)

HAP18 is a promising antiviral drug target. HAP18 speeds up capsid assembly and stabilization of the protein-protein interaction by more than 100-fold, causing misdirection of assembly and leading to incompetent capsid formation. HAP18 is a capsid formation inhibitor that belongs to the HAP family, which binds to pockets in the B and C monomers, leading to 120 molecules of HAP18 per capsid. Besides, the hydrophobic pocket binding site for HAP18 has a C-shaped form containing 5 helices (figure 6). HAP18 interacts with the amino acid residues of the chains, using hydrogen bonds as well as hydrophobic interactions. [9]

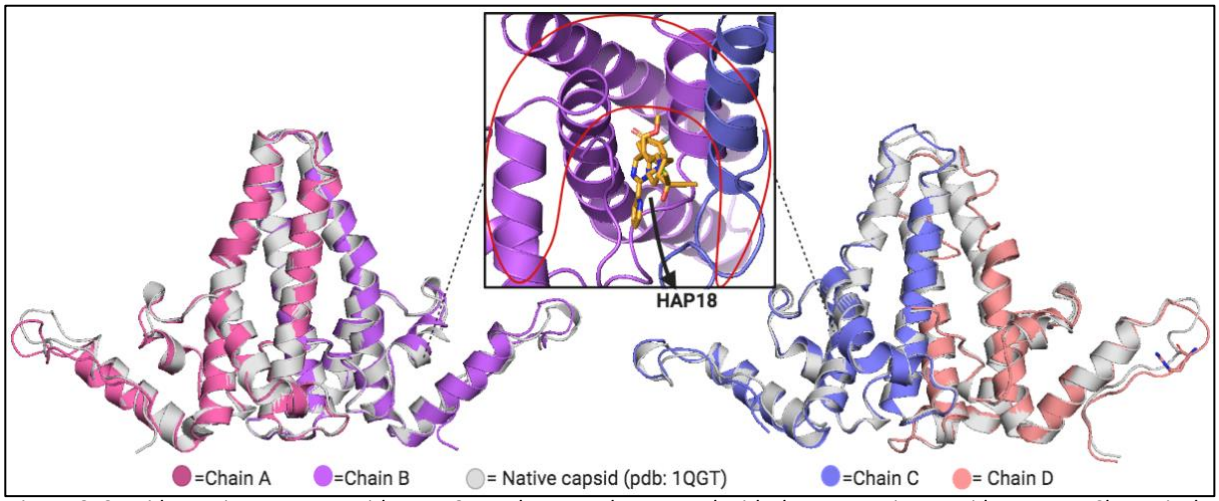

Figure 6. Capsid protein structures with HAP18 are shown and compared with the grey native capsid structure. Shown is the AB dimer on the left and CD dimer on the right picture. In the middle is shown the C-shaped (red line) hydrophobic pocket of the HAP18 from the B and C monomer. Made with Pymol (DeLano Scientific), PDB code: 5D7Y (HAP18) and 1QGT (native capsid).

Including residues Thr33 from the B-chain and Thr128 from the C-chain share a hydrogen bond with the pyrimidine ring of HAP18. [9] [10]
Moreover, very small quaternary structural differences are shown between HAP18 bound capsid and the native capsid. However, HAP1 and AT-130 have more quaternary differences with the native capsid compared to HAP18. When HAP18 is compared with the native capsid structure (grey color) (PDB code: 1QGT), tertiary structural changes are seen (figure 6). The AB dimer has a small shift of 0.5Å, whereas the CD dimer has a large structural displacement of 1.4Å. A shift of the D-chain leads to an interaction of Arg133 of the D-chain with HAP18. Conformational alterations of the C-chain subdomain are related to changes in helices structure of the dimer interface. [9]
When HAP18 is absent, a protein-protein hydrophobic interactions become weaker and the hydrophobic pocket becomes less stable. That's why HAP18 ensures that the inter-dimer interaction is stabilized and has a higher effect on the assembly rate, resulting in misdirection of assembly and incapable capsid formation. This will lead to a reduction of HBV replication. [9]

HAP_R01 (PDB: 5WRE)

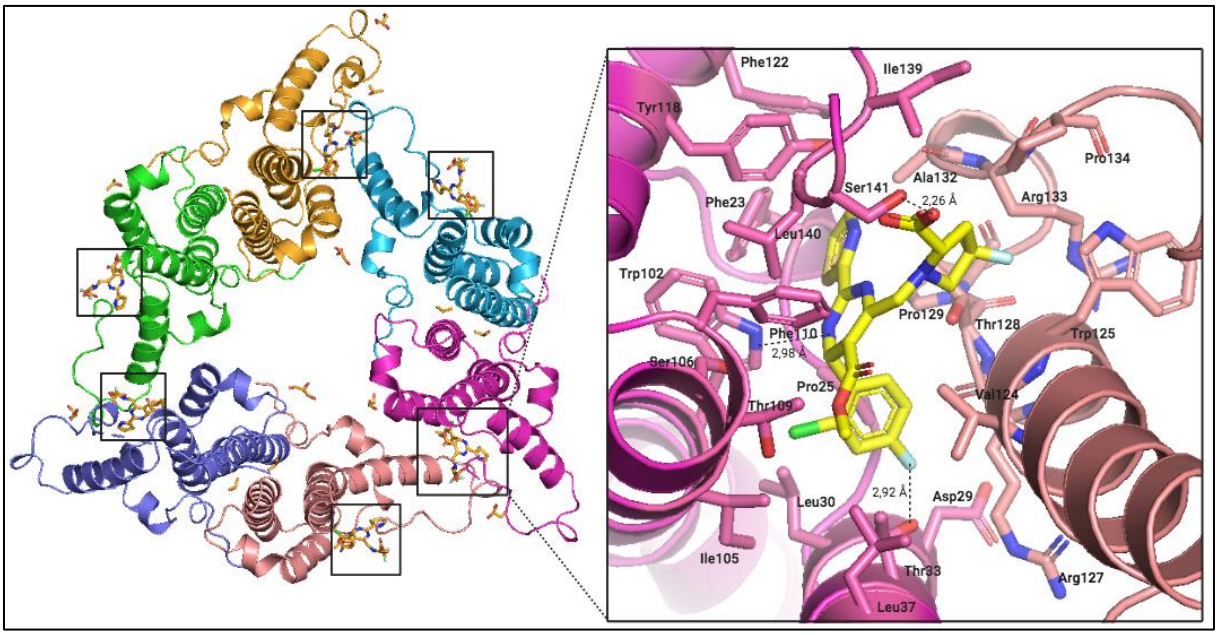

Figure 7. Hexamer of the Y132A core protein with HAP_R01 bound to the hydrophobic binding sites. Binding site from the BC dimer interface is zoomed in, showing the amino acid residues of the chains in pink (chain B) and salmon (chain C) color. HAP_R01 is colored yellow. Made with Pymol (DeLano Scientific). PDB code: 5WRE.

HAP_R01 is another target, which accelerates assembly, by binding of the thiazole group of HAP_R01 to the dimer interface of the hexamer. This hexamer has 6 hydrophobic binding pockets. In figure 7 is shown the hydrophobic binding site for HAP_R01 at the dimer-dimer interface from the core protein Y132A hexamer structure. Further, HAP_R01 structure will form hydrogen bonds with Thr33, Trp102 and Ser14. The residues Phe23, Tyr118, Phe122 and Ala132 are decisive for icosahedral assembly of the capsid. Mutations of these residues, especially Phe23 and Ala 132 residues, will enhance disruption of capsid formation.
Looking at the structures, only helix 5 from the C-chain of HAP_R01 shows structural changes. Deformation of the 5-helix induces steric collision with Ala132 and Ala131 of the B-chain. Y132A hexamer is important for assembly capsid formation. Alterations in this hexamer

pattern may interrupt the icosahedral architecture of the core protein, resulting in depletion of HBV replication. [11]

HAP-TAMRA (PDB: 6BVF)

Deformation and interruption of a capsid is a new technique for inhibition of HBV, using HAPs as mentioned before. A variant of HAP is the HAP-TAMRA, which is a flour atom labeled HAP13 compound. It will induce capsid assembly in such a way that it will deform the capsid, inhibiting virus formation. In addition, conformational change of the capsid occurs and crosslinks of disulfide bonds appear. The core protein contains two binding pockets in chain B and C (figure 8), which contain the amino acids Trp102, Thr109, Phe110, Tyr118 and Leu140. Researchers expect that when HAP-TAMRA binds to the binding site, it gives disturbance of the interaction between the core protein and HAP binding site. Nevertheless, more research needs to be done to get a better insight into this drug candidate. [12]

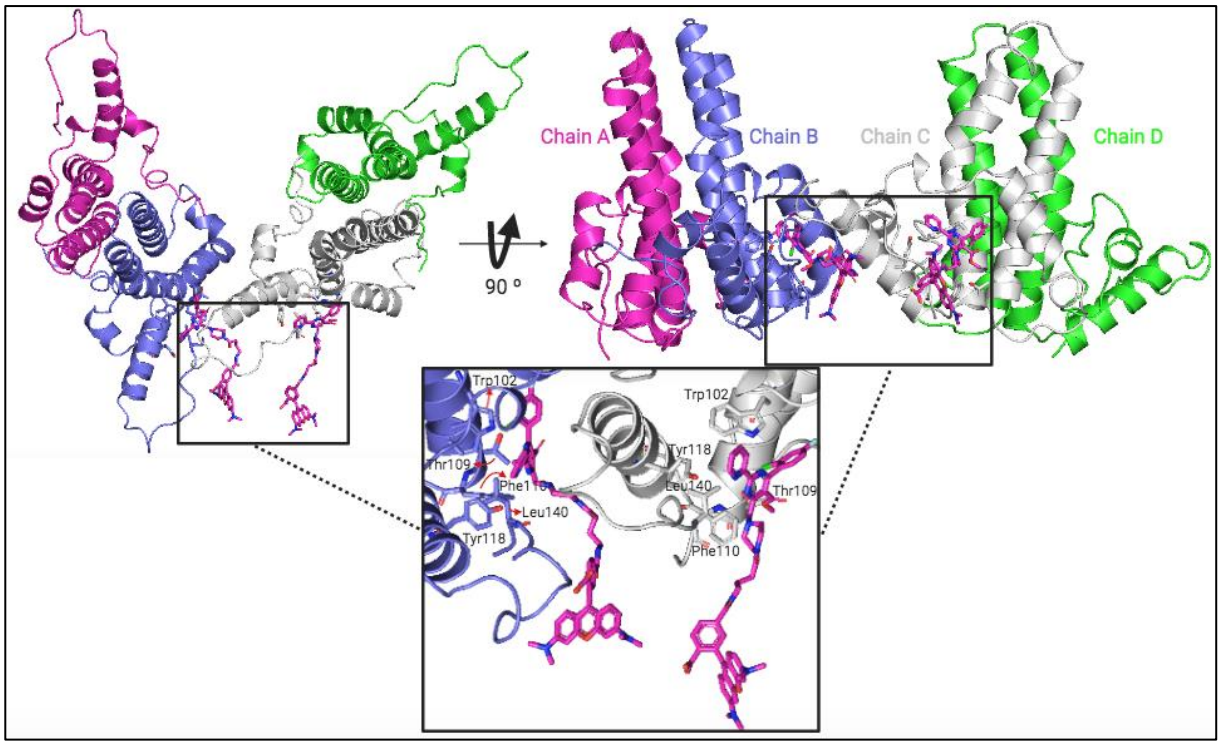

Figure 8. Core protein 150 with two binding sites for HAP-TAMRA, showed in blue (chain B) and grey (chain C). Pink colored structure is HAP-TAMRA. Made with Pymol (DeLano Scientific). PDB code: 6BVF.

NVR-010-001-E2 (PDB: 5E0I)

One of the powerful inhibitors of the HBV replication is Y132 mutant in combination with HAP molecule NVR-010-001-E2 (figure 9), which is able to bind to the HBV core proteins and initiate capsid assembly. Moreover, it will stabilize protein-protein interaction and can cause deformed capsids. This is measured in cell culture of HepG2.2.15 cells, showing reduction of HBV DNA. The side chains of NVR-010-001-E2 contribute to the binding affinity with the amino acids residues of the binding pocket. The NVR-010-001-E2 consists of a central pyrimidine that lies at the bottom of the hydrophobic binding site and is secured through hydrogen bonds to residues Trp102 and Thr33. In addition, the nitrogen of the thiazole group makes a hydrogen bond with the backbone of Leu140. [13]

The bromofluor phenyl side group has a well-defined attachment to Trp102, Pro25, Leu30, Asp29, Thr33, and Ile105 (figure 9). Furthermore, the thiazole side group fits into the residues Trp102, Tyr118, Phe122 and Phe23. Additionally, the ester group of NVR-010-001-E2 has an attachment with Thr109, Phe110, Thr33 and Leu37. These interactions with the core proteins lead to an assembly of the NVR-010-001-E2 with the hydrophobic pocket site leading to induce assembly. [13]

When looking at mutations, a couple of them can be explained. Normally, Thr109 forms a polar binding with the ester group of NVR-010-001-E2. The antiviral activity declines when Thr109 is replaced with methionine, a larger and less polar amino acid. However, when it is replaced with Serine, the antiviral activity is ameliorated 2,5-fold due to the polarity. [13]

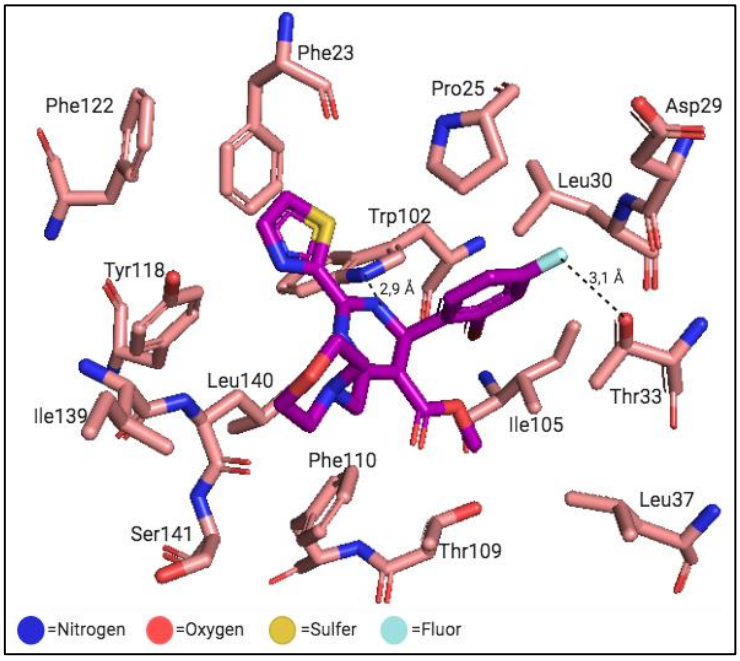

Figure 9. NVR-010-001-E2 (in purple color) with the amino acid residues from the core proteins (salmon color). Two hydrogen bonds are shown in dotted lines. Made with Pymol (DeLano Scientific), PDB code: 5E0I.

Moreover, BAY41-4109 is a HAP compound and is a capsid inhibitor too, whereby Y132 mutant can form a complex, but this compound differs in structure from NVR-010-001-E2. A reason for this is that it has no morpholino group, the thiazole is substituted by a difluoropyridine and BAY41-4109 consist of chlorine instead of bromine. These differences make the compound less potent. However, the absence of the morpholino group causes a reduction in binding interaction with the lid, which reduces the stability of BAY41-4109 with the binding sites. Therefore, reduction of the assembly of BAY41-4109 with the core protein leads to a deficiency of antiviral activity. [13]

DBT1 (PDB: 6WFS)

DBT1, known as dibenzothiazepine, is correlated with capsid formation accelerator. This compound stabilizes the interaction between proteins and provoke divergent formation of capsids and capsid assembly. In addition, it stabilizes also mistakes in protein- protein interactions, which leads to defective capsids. However, disassembly of preformed capsids happens and dissociate, by DBT1. This has several consequences. First consequence that can occur is disassembly of mature viruses, because the viruses become metastable, by forming of asymmetric capsid, which will lead to structural defects and inhibition of new infections. Another result is that capsid formation can occur with a defective or empty capsid, which will lead to the suppression of new virus formation. [14]

Preformed capsids are used to study the structure when DBT1 is present (figure 10A). The DBT1 structure will be compared with the HAP molecule NVR-010-001-E2, using the binding site of chain A. Besides, they have different binding affinities. Both structures, DBT1 and NVR-010-001-E2, interacts with, among others, Val124, Phe24, Thr109 from the binding site (figure 10B). Nevertheless, NVR-010-001-E2 has a collides with Val124 on helix-5 of the neighboring capping chain. Although, DTB1 fits well into the binding site and has no collisions with the binding pocket residues. Due to the fact that it has a flexible structure, DTB1 will twist around the capping chain and avoid Val124. [14]

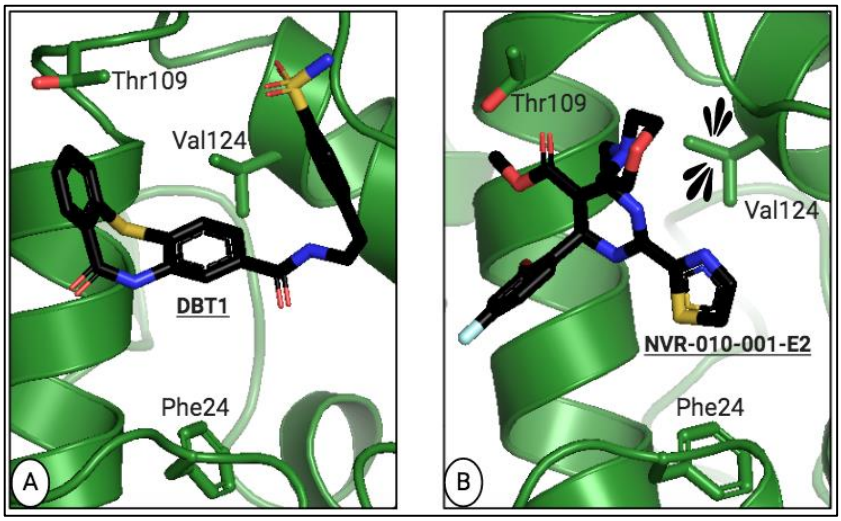

Figure 10. Binding sites from chain A with DBT1 (A) and NVR-010-001-E2 (B). Showing the residues Phe24, Thr109 and Val124 of chain A. Made with Pymol (DeLano Scientific), PDB code: 6WFS (DBT1), 5E0I (NVR-010-001-E2).

When the different drug target families of the capsid formation accelerators are compared with each other, there can be seen that the various structures of core protein allosteric modulators (cpAMs) can bind at the same binding site, while the molecular structures are different from each other and have different assembly functions (figure 11). [14]

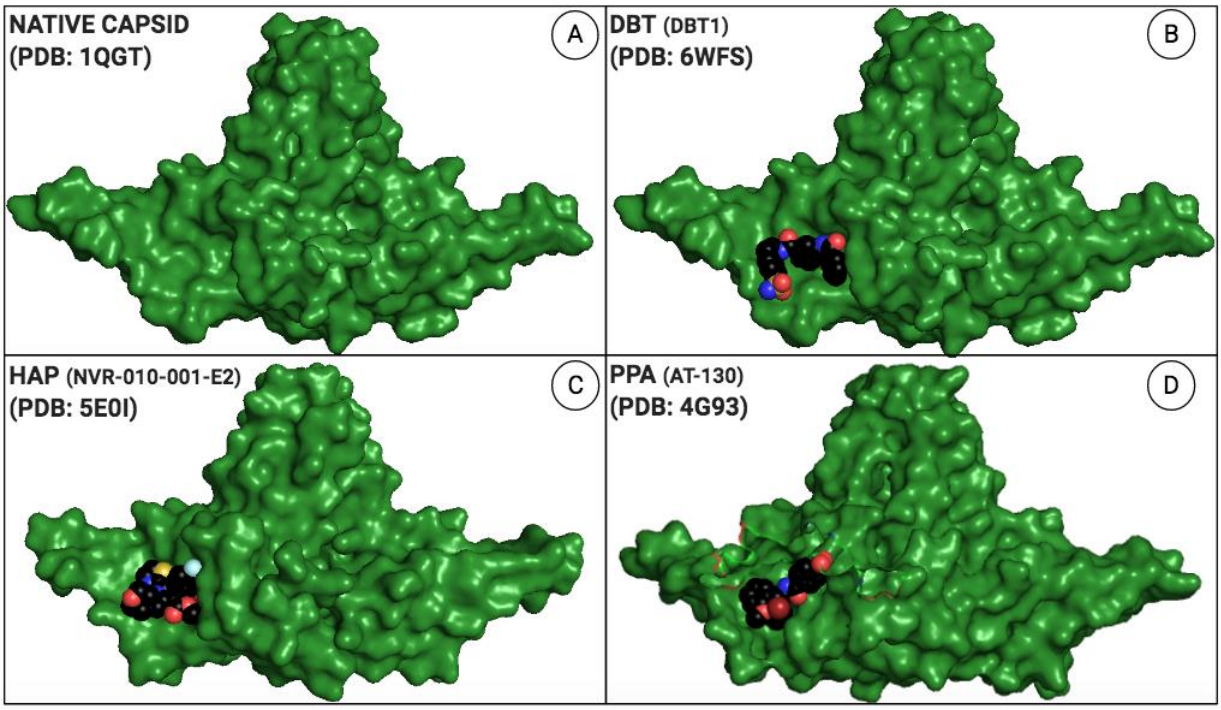

Figure 11. Native capsid (A) with the same binding site for different cpAMs drug targets (B, C, D). Native capsid shown in green surface. The cpAMs are shown in spheres; black= carbon, red=oxygen, light blue= fluor, blue= nitrogen, gold=sulfur atoms. Made with Pymol (DeLano Scientific), PDB code: 1QGT (Native capsid), 6WFS (DBT1), 5E0I (NVR-010-001-E2) and 4G93 (AT-130).

Antibodies

2H5-A14 antibody (PDB: 5YAX)

One of the antibody clinical candidate for the HBV treatment and prevention is the 2H5-A14 antibody (figure 12, 13), which blocks the binding of Sodium Taurocholate co-transporting polypeptide (NTCP) to preS1 domain by capturing the preS1 peptide.

Besides, it induces immunological effector functions that are antibody-Fc dependent, leading to a repression of viral infection in mice, which are infected with HBV. This leads to degradation of small envelope antigen levels and a reduction in viral DNA. [15]

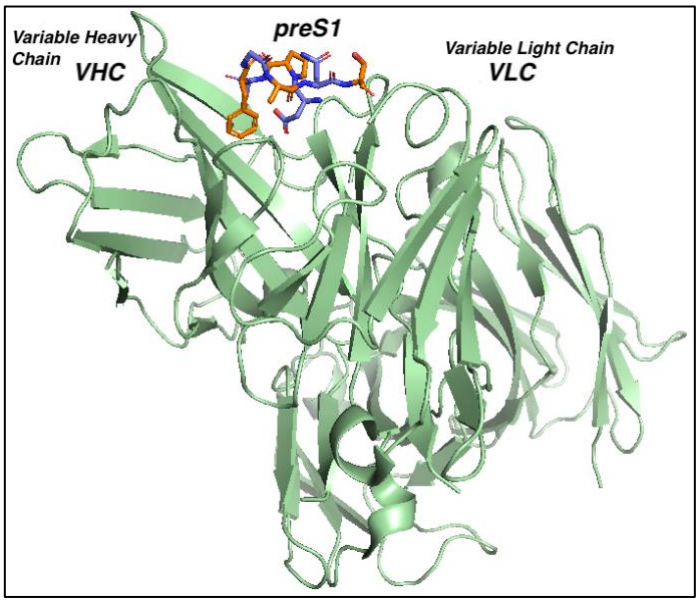

Figure 12. 2H5-A14 antibody (green) with the U-shaped residues 20-27 of preS1 peptide (blue and orange). On the linker side is shown the variable heavy chain (VHC) and in the right variable light chain (VLC), made with Pymol (DeLano Scientific), PDB code: 5YAX.

Moreover, the U-shaped residues (20 tm 27) of preS1 peptide are captured by residues of the complementarity-determining-region loops of the variable light (LCDR) and heavy (HCDR) chain of the 2H5-A14 antibody (figure 13). [15]

The residues (colored in purple) of the different loops (HCDR1,2,3 and LCDR1 & 3) bind direct to the residues of preS1 peptide. Phe23 in preS1 clamps deep into the hydrophobic pocket, due to the fact that it has a large benzyl group.

In addition, Asp20 (in preS1) forms electrostatic interaction with Arg55 of the HCDR2-loop. The residue Ala22 (in preS1) forms hydrogen bonds with Arg59 of the HCDR2-loop and Asp20

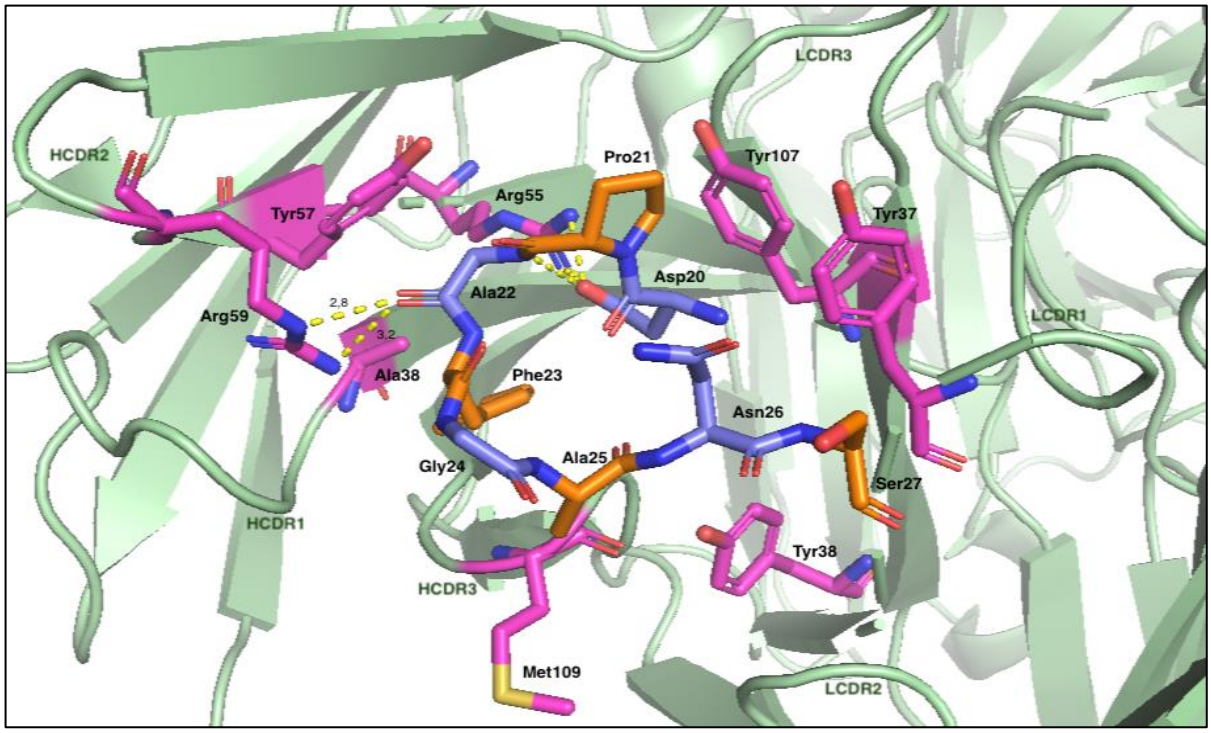

Figure 13. 2H5-A14 antibody (purple and green) with the U-shaped residues 20-27 of preS1 peptide. Made with Pymol (DeLano Scientific), PDB code: 5YAX.

with Arg55. Ala22 is involved in the binding of 2H5-A14 with preS1, because if Ala22 mutated to Leu19, the binding activity becomes reduced.
There are also several hydrophobic- and van der Waals interactions with the residues of the 2H5-A14 and the preS1. Further, Ala22 and Ala25 have hydrophobic interactions with Tyr57 and Met109. Pro21 goes an interaction with Tyr37 and Tyr107. Besides, Asn26 and Ser27 have van der Waals interactions with Tyr37 and Tyr38 residues. Looking at the sequence of 2H5-A15 and its function, the 2H5-A14 antibody can provide a wide protection against the superiority of HBV genotypes. [15]

H015 antibody (PDB: 6VJT)

An additional possibility for HBV treatment are combinations of complementary broadly neutralizing antibodies (bNAbs) against hepatitis B surface antigen (HBsAg). The H015 antibody is a bNAb, which binds to HBsAg on the position Lys141-Pro142-Ser143-Asp144-Gly145 of the HBsAg epitope residue (figure 14). This epitope contains mutated amino acids, like alanine mutations, which plays a role during infections. Mutations in Lys141 and Pro142 decrease viral infection as well as it stabilizes the structure of the epitope. In addition, the epitope has a hairpin shape, as a result of salt bridges between the Lys141 and Asp144 and the hydrogen bond among Lys141 and Gly145. During infection, each residue of the epitope has an important role in recognition of the immune system. The residues of the epitope interact with the antibody residues Arg31, Trp52, Phe53, Glu99, Pro101, Leu103 and Leu104 of the heavy chain. From the light chain, they only interact with Pro95 (LCDR3). A combination of complementary bNAbs with non-overlapping epitopes will dramatically reduce HBsAg levels and they are consequently involved in HVB treatment. [16]

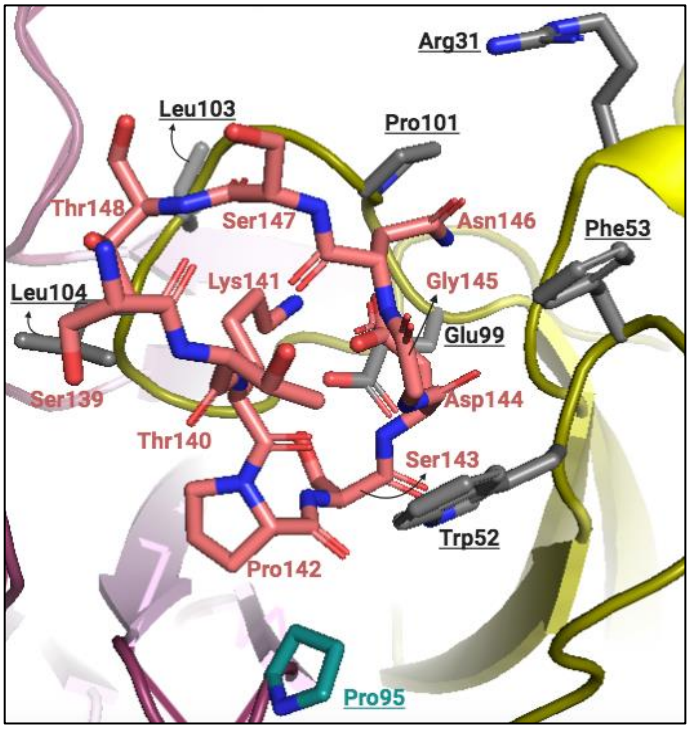

Figure 14. H015 antibody residues colored grey (heavy chain) and cyan (light chain) in complex with the hepatitis B surface antigen epitope, which has a hairpin shape and colored rose. Made with Pymol (DeLano Scientific), PDB code: 6VJT.

ScFv e13 (PDB: 6CVK) and Fab e21 (PDB: 6CWT)

The capsid of HBV contains hepatitis B core antigen (HBcAg) and hepatitis B e-antigen (HBeAg) and are involved in HBV life cycle. HBcAg plays an important role in transport of the viral genome, capsid formation and finishing of genome duplication. However, HBeAg is an immune modulator, which is involved in the improvement of chronic HBV. These antigens could be a considerable target for drug design in the treatment of HBV. Two auspicious drug candidates are single chain variable fragment (scFv) e13 and fragment antigen binding(Fab) e21, which are chimeric immunoglobulin fragments consisting of rabbit and human fragments.

The variable domains of the antibodies are derived from rabbits and constant domains are derived from the human. These fragments scFv e13 and Fab e21 will bind to HBcAg as well as HBeAg. They effectively inhibit the HBcAg dimers (HBcAgd) leading to blocking of capsid assembly. This causes prevention of Dane particle forming, which plays a role in HBV genome transport as well as infecting new hepatocytes. [17]
When the fragments bind to HBeAg dimer, it will prevent immune modulation and inhibit "core like 'decoy' particles" assembly. Researches guess that these "decoy particles" deplete the immune system. Looking at the crystal structure of recombinant HBeAg dimer (rHBeAgd) with scFv e13, the HBeAg was around 50% unfolded by conformational modification. [17]

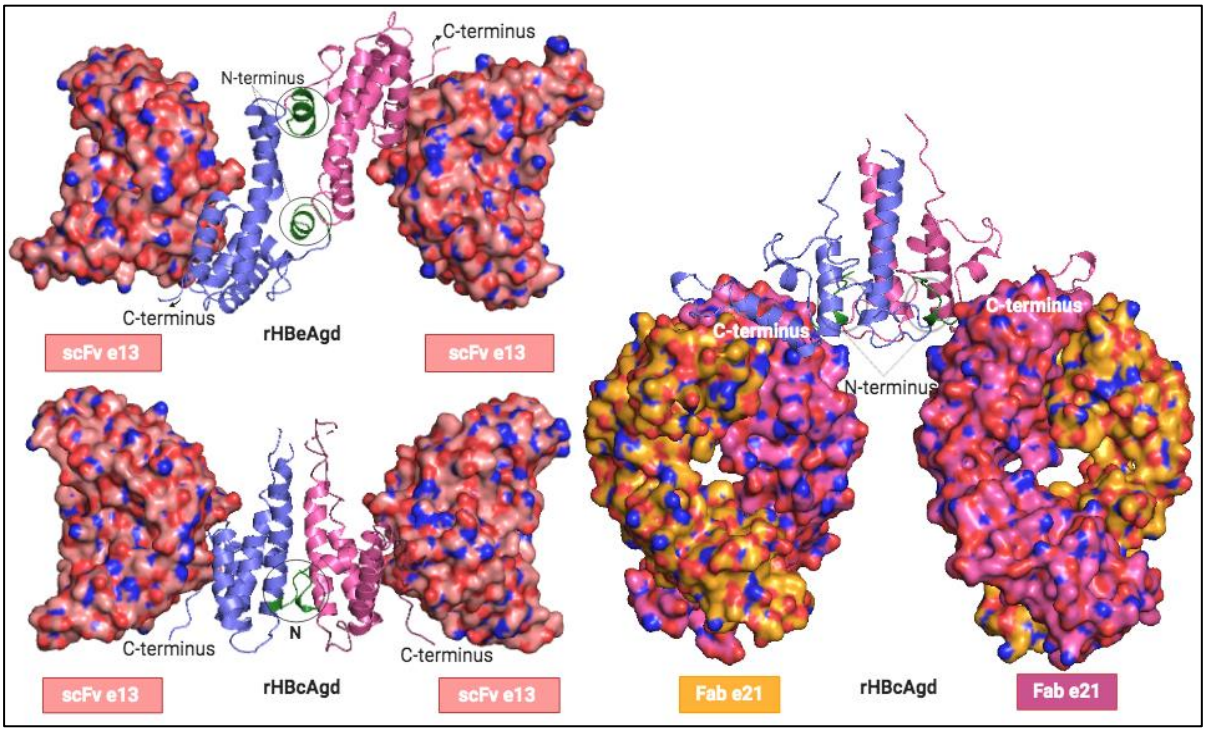

Figure 15. Structures of rHBeAgd in complex with scFv e13 (PDB code: 6CVK) and rHBcAgd in complex with scFv e13 (PDB code: 6CWD) and Fab e21 (PDB code 6WT). Shown the monomers in the color slate and purple. N-terminal is depicted in the color green. The antibody fragments scFv e13 and Fab e21 are shown with surface. Made with Pymol (DeLano Scientific).

In figure 15 is shown the structures rHBcAgd and rHBeAgd in complex with scFv e13 and Fab e21. Fragments scFv e13 and Fab e21 are shown as surface and the dimers as ribbon cartoons. The 10 amino acids of N-terminus of the dimers are shown in green. From the results can be confirmed that scFv e13 binds bivalent to rHBcAgd and rHBeAgd. Therefore, the binding of scFv e13 fragment isn't affected by structural modifications. From this outcome, rHBcAgd is picked up to look at the interactions with Fab e21 fragment and compare it with rHBcAgd in complex with scFv e13 and it will be compared with the native capsid structure (figure 16). The structures of the monomers are obtained and rotated 90 degrees for the view. A distortion of the monomers compared with each other and with the native capsid could be observed. The variation between the two structures is due to the fact that they have high flexibility and have different affinity to the antibody fragments. Besides, an essential motion was observed from the C-terminus of the slate color monomer. This ensures inhibition of capsid assembly without inducing other modifications, leading to reduction of HBV replication. [17]

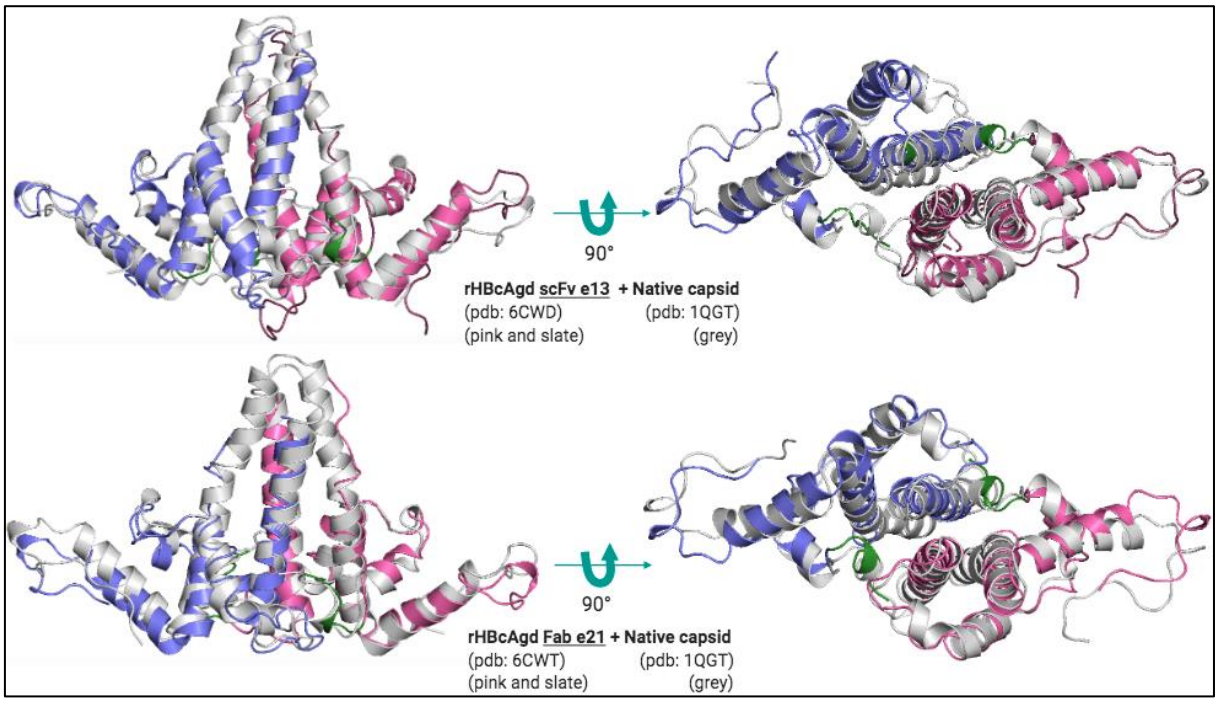

Figure 16. rHBcAgd in complex with scFv e13 and Fab e21 compared with the native capsid structure (PDB code: 1QGT). Showing the monomers in the color slate and pink and the N-terminal in green. Made with Pymol (DeLano Scientific), PDB code: 6CWD (rHBcAgd scFv e13) and 6CWT (rHBcAgd Fab e21).

Humanized E6F6 antibody B11(6L8T)

Another promising treatment for HBV is the use of stable humanized E6F6-1 antibody (huE6F6-1) in complex with B11 HBsAg model. E6F6-1 stands for murine derived monoclonal antibody, which identifies the SA-epitope (residue codes: GPCK(R)TCT) of HBsAg. This antibody induces longtime repression of HBsAg, which provides diminishing of HBV caused hepatic-carcinogenesis. The HBsAg recognition site is formed by three LCDRs and three HCDRs (figure 17). [18]

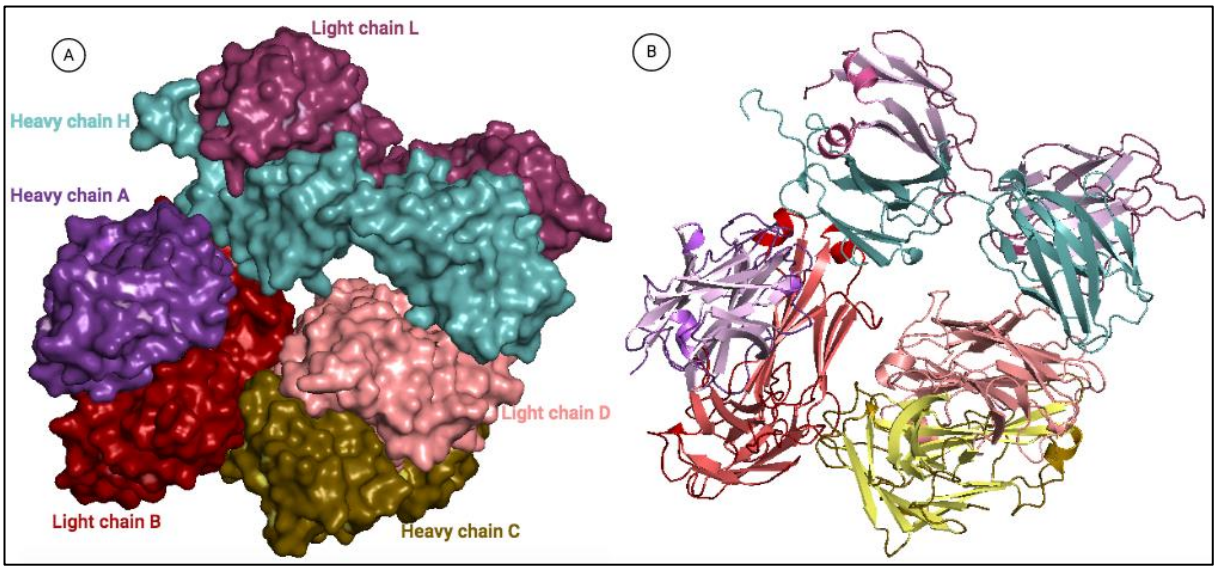

Figure 17. Structure of HBsAg with 6 chains each colored in different colors. There are three heavy chains A, C, H respectively. Chain B, D, L are light chains. There is shown the surface of the HBsAg (14A) and on the right (14B) is shown the structure in alpha-, beta sheets and loops are present. Made with Pymol (DeLano Scientific), PDB code: 6L8T.

When point mutation of huE6F6-1 occurs, hydrophobicity of the binding site increases and it shows a neutralization activity of the huE6F6-1: HBsAg complex. In addition, the binding affinity increased, due to the fact that the binding site becomes more hydrophobic. [18]

Table 1. Interactions between huE6F6-1 antibody and the antigen binding site of B11 HBsAg.

Region	Type of interaction	huE6F6-1 antibody	Binding site	Amount of interaction
HCDR 1	*Hydrogen bond*	Gly27 Ser28 His38	Thr3 Thr4 Thr6, Pro8	2 1 2
	PI interactions	-	-	-
HCDR 3	*Hydrogen bond*	Ser106 Gly107	Thr6 Thr6	1 1
	PI interactions	Phe115	Pro8	1
LCDR 1	*Hydrogen bond*	His31 Ser32 Tyr38	Thr11, Lys10, Cys12 Thr13, Cys12 Thr11	2 2
	PI interactions	His31	Thr11, Lys10, Cys12	2
LCDR 3	*Hydrogen bond*	Thr108	Lys10	1
	PI interactions	Tyr116	Pro8	1

Furthermore, there are different hydrogen bonds and PI interactions between E6F6 antibody and binding site (table 1). Research on this antibody indicates that the structure of huE6F6-1 has more compact and stabilized hydrogen bonds, leading to a higher binding affinity of the antibody to the antigen. From the results of this study, it can be assumed that antibody huE6F6-1 can be taken as a superior therapeutic drug candidate for HBV treatment and purposes to increase the loss of HBsAg. [18]

Polymerase inhibitors

RIV-1 RT 3MB (PDB: 6KDJ)

Polymerase is an important step in the life cycle of HBV and is therefore considerable for the drug development for HBV. Several nucleotides reverse transcriptase(RT) inhibitors (NRTIs), such as lamivudine (3TC) and entecavir (ETV), can inhibit HBV polymerase as well as human immunodeficiency virus 1 (HIV-1) reverse transcriptase. Nonetheless, resistance may develop against these NRTIs. Here there will be observed changes in drugs, which will help to design new targets for ETV and 3TC to conquer drug resistance. HIV-1 reverse transcriptase (RT) mutants F115Y/Y116F/Q151M (3MB) DNA will be used, which mimicking the N-terminal of HBV RT (figure 18) using it in combination with 3TC-triphosphate (3TC-TP) and ETV-triphosphate (ETV-TP). [19]

Residues shown in the color slate are amino acids from the HIV-1 RT and DNA aptamer has salmon color. Hydrogen bonds are shown in dotted lines. There is a hydrogen bond between Asp185 and the DNA residue dG33. 3TC-TP forms hydrogen bonds with dG0 and dG33 with dC1. In addition, magnesium ion is a metal, which has interactions with chelate compounds.

The experiment showed that HIV-1 RT 3MB resistance to ETV and 3TC was caused by Met184. Tight binding conformation in the ETV-TP and 3TC-TP is the consequence of alteration of Met184. The side chain of Met184 is shoved backward, through the methylene group of ETV-

TP and oxathiolane group of 3TC-TP, shown with a green arrow. To solve this resistance, new drug targets has to be obtained, which will prevent conformational change of Met184. [19]

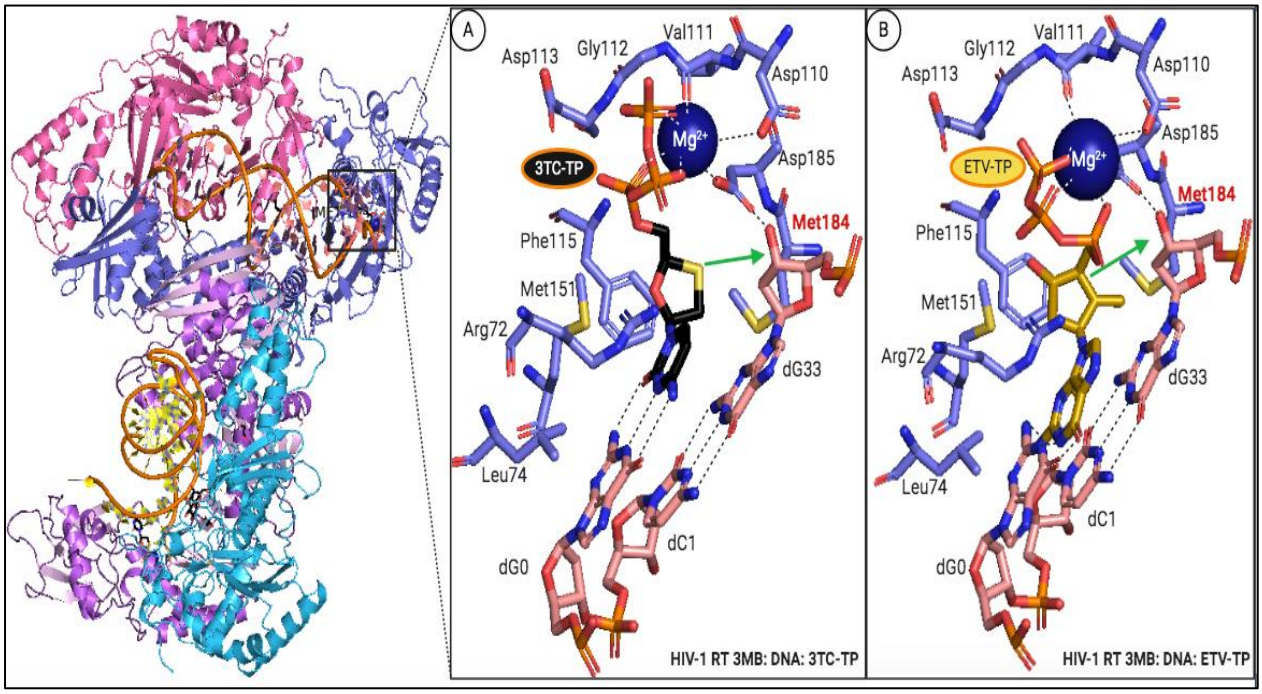

Figure 18. HIV-RT with mutant F115Y/Y116F/Q151M (3MB) and DNA composing 3TC-TP (A)(pdb:6KDJ) or ETV-TP(B)(pdb:6KDM) from the N-site structure. Amino acids from HIV-1 RT are shown in the color slate. The residues of the DNA aptamer are depicted in salmon color. Magnesium (Mg^{2+}) is shown in a dark blue spherical structure. A: 3TC-TP is colored black (3TC) and orange (TP). B: ETV-TP is colored gold (ETV) and orange (TP). Nitrogen atoms are colored blue and oxygen atoms red. Sulfur is colored yellow.

Moreover, when there is a replacement of Met 184 with Val/Ile184, steric clash has taken place among Val/Ile184 and methylene group of ETV-TP and the oxathiolane group of 3TC-TP. A solution for this resistance, could be extending the interactions between RTs and NRTIs to recompense for the Val/Ile184 steric clash. This experiment could help with new drug design for HBV resistance against the two NRTIs, ETV and 3TC. [19]

Q151M mutation (4ZHR)

As mentioned above, NRTIs are important in preventing HBV polymerase and are considerable drug targets for example entecavir and tenofovir. In this crystallographic study, they look at the resistance against NRTIs, which is caused by mutation of Gln151, Q151M, of chain A in HIV-1 RT. This residue is located in 'deoxynucleotide triphosphate' (dNTP) binding pocket and contains resistance to different NRTIs. The residue Gln151 of HIV1-RT is similar to Met151 in HBV. That's why HIV1-RT can be used for imitation of the HBV polymerase. In figure 19 is shown the structure of HIV1-RT with Q151M from chain A. The open and closed structure of dNTP-binding site is induced by conformation in the β2–β3 strands (residue: 60 tm 75). The Q151M mutation (figure 19A) could be a reason for this. [20]

An example to show the resistance is HIV1-RT DNA dNTP-binding site, which is occupied with azidothymidine triphosphate (AZT) target (pdb:3V4I) (figure 19B). A second example is HIV-1 RT with DNA in complex with tenofovir diphosphate (TNV) (pdb:1T05) (figure 19C). The hydrogen bonds were depicted as dotted lines. Looking at the residues of dNTP-binding sites, Lys65 and Arg72 are required for binding to the target (AZT and TNV). [20]

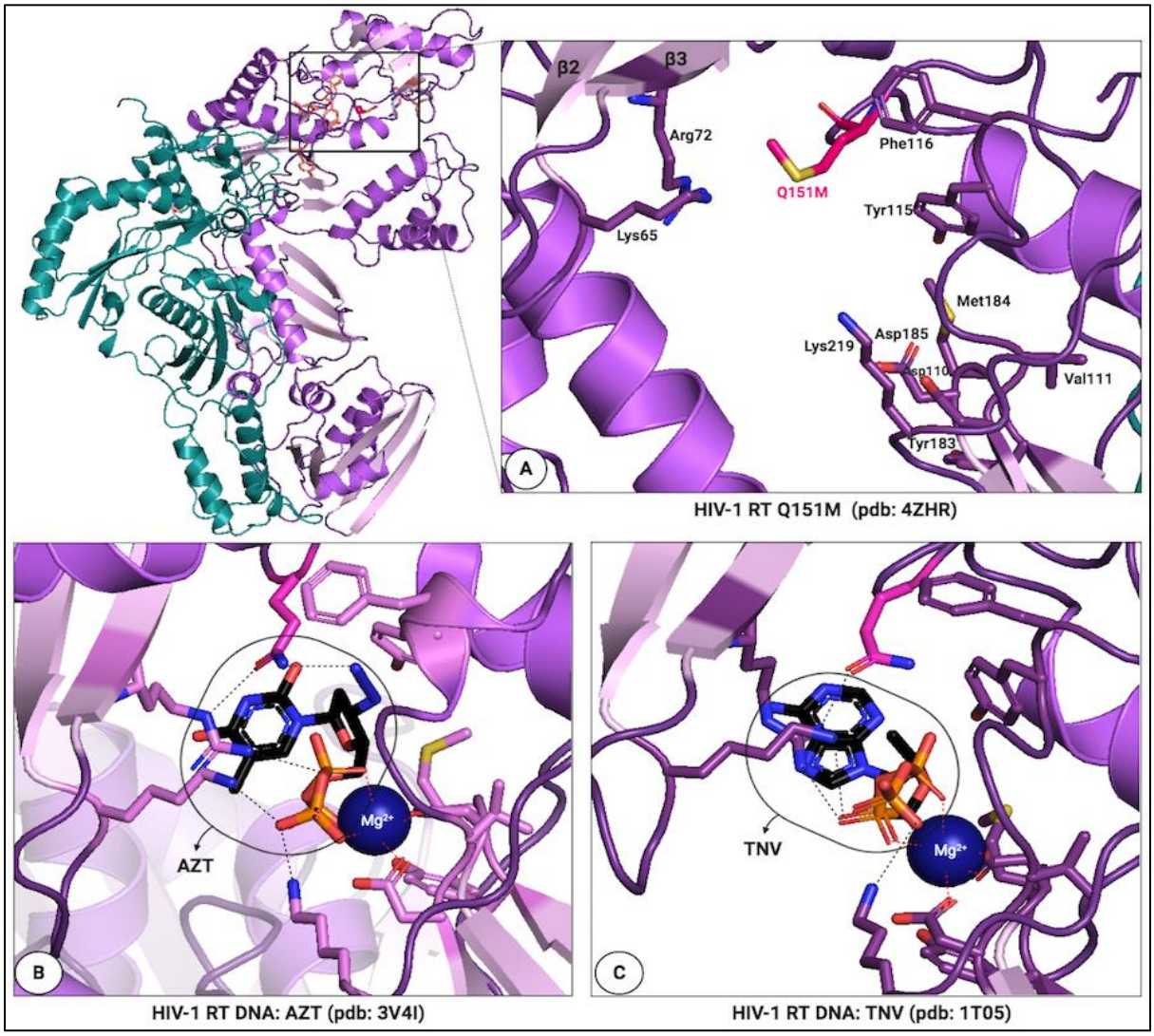

Figure 19. Structure of HIV-1 RT shown with dNTP-binding site. (A): HIV-1 RT in complex with Q151M mutant (PDB: 4ZHR), (B): HIV-1 RT in complex with AZT (PDB: 3V4I), (C): HIV-1 RT in complex with TNV (PDB: 1T05). Made with Pymol (DeLano Scientific).

Residues Asp 110 and Asp185 bind to Mg^{2+} and are necessary for catalysis. Tyr115 identifies the deoxyribose ring of target and distinguishes it among NTP and dNTP. Besides, Gln151 has an interaction with the 3'-OH group of the target and forms a hydrogen bond with Arg72. The side chain of Q151M turned away from the pocket upwards, compared with the Gln151. Furthermore, Q151M causes conformational change of Lys65 and Arg72. The Q151M mutant induces loss of ability to form hydrogen bonds, which causes a decrease in affinity for binding to NRTIs. Consistent with these results, it's potential that hydrogen bonds can't develop anymore in the dNTP-binding pocket with Q151M mutant (figure 19A). This could be a reason for NRTIs resistance due to Q151M mutant. Therefore, more research is needed for this structure to find possible drug targets to prevent or inhibit mutational effects. [20]

Summary

In this review, insight into new alternative drug targets for HBV treatment are given. To improve the drugs of hepatitis B, research has been done on the life cycle of HBV.
One of the effective ways to reduce HBV activity is inhibiting capsid formation by binding of the drug target into the hydrophobic pocket.
Ciclopirox could be a good alternative for a new drug in the treatment of HBV, because it inhibits capsid formation of HBV, by binding to the hydrophobic pockets, where normally assembly modulators bind for capsid formation. The binding pocket is located in the core protein, which consist of a hexamer. When ciclopirox binds to the hydrophobic pocket, the phenyl ring of Phe23 rotates 142 degrees upwards and makes the pocket free. Afterwards ciclopirox is allowed to bind to the pocket and clamps deep into the HBV core protein. This deep binding generates hydrophobic interactions and prevents ciclopirox from releasing, leading to inhibition of capsid formation.
(2S,4S)-4,4-difluoroproline substituted analogue 34a could be an oral drug candidate in the HBV treatment as an inhibitor of capsid formation. This analogue 34a is obtained from 4-methyl HAP. This compound forms van der Waals interactions and hydrogen bonds with the residues from the binding pocket from chain B and C of the hexamer. In addition, the 4-methyl group will provoke steric collision with Thr128.

A second method to increase the anti-HBV activity is by using capsid formation accelerators. These drug targets increase the rate of capsid assembly, which cause a distortion of the capsid or a capsid without the RNA genome, leading to reduction of HBV replication.
HAP1 is a potent capsid misdirector and assembly activator of HBV. HAP1 binds to its binding pocket, leading to changing the structure of the protein and misdirection of the capsid assembly. Normally, the residues of the binding site will bind to each other and will stabilize the structure. However, when HAP1 is present, it localizes in this pocket and blocks a part of a groove. Furthermore, this groove is normally used to interact with a nearby dimer. This happens so quickly that it misdirects the assembly. So, HAP1 destabilizes the capsid assembly by misdirection of assembly, leading to disruption of the capsid, which makes it a promising target for antiviral new generation therapeutics
AT-130 structure doesn't disrupt the capsid structure, but it changes the tertiary and quaternary structure of the AB and CD dimers, by binding on the capsid surface. Several studies suggested that AT-130 blocks RNA packaging, which leads to a capsid without the viral genome.
HAP18 speeds up capsid assembly and stabilization of the protein-protein interaction by more than 100-fold and cause misdirection of assembly, leading to incompetent capsid formation. Moreover, this contributes by alterations in the tertiary structure HAP18 interacts with the amino acid residues of the chains, using hydrogen bonds as well as hydrophobic interactions.
HAP_R01 target accelerates assembly. The thiazole group of HAP_R01 binds to the dimer-dimer interface of core protein Y132A hexamer. Deformation of the 5-helix induces steric collision with Ala132 and Ala 131 of the B-chain. Besides, mutations of Phe23 and Ala 132 residues, will enhance disruption of capsid formation.
HAP-TAMRA is flour atom labeled HAP13 compound, which will induce capsid assembly in such a way that it will deform the capsid. Researchers expect that when HAP-TAMRA binds to the binding site, it gives disturbance of the interaction between the core protein and HAP binding site, inhibiting DNA synthesis.

NVR-010-001-E2 in complex with the Y132 mutant fits well into his binding pocket, due to the fact that the side chains of NVR-010-001-E2 target contribute to the binding affinity with the hydrophobic pocket. Moreover, it shares hydrogen bonds with Trp102 and Thr33, which results in stabilization of protein- protein interaction and cause deformed capsids.
DBT1 compound stabilizes the interaction between proteins and provoke divergent formation of capsids and capsid assembly. In addition, it stabilizes also mistakes in protein- protein interactions, which leads to defective capsids. However, disassembly of preformed capsids happens and will dissociate, by DBT1. If DBT1 compared with NVR-010-001-E2, there is shown the same interactions with the residues, expect the Val124 residue. NVR-010-001-E2 has a collides with Val124. Although, DTB1 fits well into the binding site and has no collisions with the binding pocket residues.

Another essential method is using antibodies against HBsAg, HBcAg and HBeAg.
2H5-A14 antibody capture preS1 domain, leading to blocking of NTCP binding to the preS1 domain. The preS1 domain is captured by different residues from the antibody, originating of LCDR and HCDR. To stabilize the binding, several electrostatic interactions, hydrogen bonds and van der Waals interactions are formed between the antibody and preD1 domain.
H015 is a bNAb against HBsAg. Mutations in Lys141 and Pro142 decrease viral infection as well as it stabilizes the structure of the epitope. A combination of complementary bNAbs with non-overlapping epitopes will dramatically reduce HBsAg levels and they are consequently involved in HVB treatment.
scFv e13 and Fab e21 antibodies will bind to HBcAg as well as HBeAg. They effectively inhibit the dimers of HBcAg (HBcAgd), leading to blocking of capsid assembly. This causes prevention of Dane particle forming, which plays a role in HBV genome transport as well as infecting new hepatocytes. Binding of scFv e13 to rHBcAgd and rHBeAgd shows no structural modifications. However, there is shown a structural change between rHBcAgd: scFv e13 and rHBcAgd: Fab e21. Moreover, an essential motion was observed from the C-terminus of the monomer. This ensures inhibition of capsid assembly without inducing other modifications.
huE6F6-1 in complex with B11 HBsAg model induce longtime repression of HBsAg, providing diminishing of HBV caused hepatic-carcinogenesis. There are several hydrogen bonds and PI interactions between the antibody and antigen, leading to higher binding affinity of the huE6F6-1: HBsAg complex.

The last subject reviewed is about polymerase inhibitors, which inhibit DNA polymerase, using NRTIs. This part examines the cause of resistance to NRTIs and the possible solution to the resistance.
RIV-1 RT 3MB in complex with 3TC-TP and ETV-TP shows resistance to 3TC and ETV. The experiment showed that HIV-1 RT 3MB resistance to ETV and 3TC was caused by Met184. Tight binding conformation in the ETV-TP and 3TC-TP is the consequence of alteration of Met184, leading that the side chain of Met184 is shoved backward.
Q151M mutant is one of the causes of resistance against NRTIs. It's a mutation of Gln151, Q151M, which is located in dNTP-binding site. The side chain of Q151M turned away from the pocket upwards, compared with Gln151. Q151M causes conformational change of Lys65 and Arg72. Consistent with these results, it's potential that hydrogen bonds can't develop anymore in the dNTP-binding pocket with Q151M mutant.
To solve these resistances against NRTIs, new drug targets have to be obtained, which must prevent conformational changes of Met184, Lys65 and Arg72.

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