Novel viral and host targets to cure hepatitis B

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Abstract word count: 118

Text word count: 3,226

Abstract

Hepatitis B virus (HBV) infection is a global health threat with 240 million chronic carriers at high risk to develop hepatocellular carcinoma. Current antiviral treatment can efficiently control viral replication and reduce liver inflammation, but is still quite far from achieving a cure. Significant progress has been made in understanding the virus life cycle and virus-host interaction in the past few years. With identification of the HBV receptor, cell-culture infection systems have become available that allow drug screening and establishing a pipeline of potential antivirals targeting either viral or host factors. Most of the candidate antivirals summarized in this review are still in preclinical development, but some have already entered or are about to enter early clinical trials.

Introduction

Currently, an estimated 240 million individuals are chronic HBV carriers, and HBV infection and its consequences account for 680,000 deaths / year (WHO 2016). Even though HBV infection can be prevented by a vaccine, there is still no curative treatment for individuals who are already chronically infected. “Cure” is defined as clearance of circulating hepatitis B surface antigen (HBsAg) and seroconversion to anti-HBs+, a status in which antibodies against HBsAg are detectable in the blood. Anti-HBs seroconversion occurs spontaneously in about 0.2-1% of chronically infected patients per year [1]. Interferon (IFN)-based therapies achieve this goal in <10% of treated patients [2]. However, even after anti-HBs seroconversion, low level persistence of HBV is observed for decades [3] indicating on the one hand a functional rather than a complete cure and on the other hand that the immune system is able to keep HBV in check.

Therapy with nucleoside or nucleotide analogs (NAs) controls virus replication and mitigates inflammatory liver disease but does not result in a “cure” of HBV infection because it does not directly affect nuclear covalently closed circular HBV DNA (cccDNA), the HBV persistence form. <1% of HBeAg-negative and 0-10% of HBeAg-positive patients achieve Anti-HBs seroconversion after 3-5 years of continuous therapy [2], a rate that doesn’t significantly exceed that of spontaneous functional cure [1]. In addition, the risk of developing hepatocellular carcinoma (HCC) remains significant despite NA treatment (approx. 7% in 5-7 years) [4,5] and even after anti-HBs seroconversion [1] . A recent study predicts that only a combination of a better vaccination coverage, innovations in prevention of mother-to-child transmission, and ambitious population-wide testing and treatment will be able to eliminate HBV as a major public health threat in the coming decades [6]. Barriers to implementing this and costs are obviously high, but could be largely reduced if a curative treatment would exist [6]. Thus, there is a high medical need for development of new antiviral strategies to achieve at least a functional cure of HBV and reduce HBV-related disease burden.

The hepatitis B virus life cycle

HBV is a small, enveloped DNA virus [7]. It attaches to heparan sulfate proteoglycans (HSPGs) [8], and is taken up upon binding to the hepatocyte-specific bile acid transporter NTCP (sodium taurocholate co-transporting polypeptide) serving as HBV receptor [9,10]. After fusion of the HBV envelope with endosomal membranes, the HBV capsid releases the

partially double-stranded relaxed circular DNA genome (rcDNA) into the nucleus, where rcDNA is converted to cccDNA by cellular enzymes (for details see: [11]). cccDNA serves as transcription template and is the viral persistence form. Viral RNAs transcribed from cccDNA in the nucleus are exported to the cytoplasm and translated into viral proteins. One of the viral transcripts, termed pregenomic RNA (pgRNA) is packaged into a newly formed viral capsid and therein reverse transcribed into rcDNA. The rcDNA-filled mature capsid are either enveloped with cellular membranes containing small, medium and large (S, M, L) HBV envelope proteins and secreted from the infected cell or transported back to the nucleus to maintain a cccDNA pool (Figure 1).

Several unique features in HBV infection make the virus difficult to be successfully targeted by antiviral approaches. Firstly and most importantly, one to five copies of cccDNA hide as episomal minichromosomes in the nucleus of infected hepatocytes, and can be replenished via nuclear reimport of rcDNA bypassing the receptor-mediated entry (Figure 1). Secondly, viral DNA synthesis exclusively occurs inside the capsid and thereby the viral genome and replicative intermediates are shielded from cytoplasmic pattern recognition receptors during the entire life cycle. This, together with lack of STING expression in hepatocyte [12], seems to result in impaired innate immune DNA-sensing. Thirdly, HBV massively sheds non-infectious subviral particles mainly consisting of S protein (HBsAg) that have no apparent role in viral replication and are believed to serve as an immunological decoy. Fourthly, HBV exhibits a strict hepatic tropism and the liver provides a hostile environment for pathogens under distinct conditions [13]. Last but not least, HBV is divided into 10 genotypes which differ in clinical course after infection [2] and in their response to IFN treatment [14]. Thus, different HBV genotypes may also react differently in particular to new immune stimulatory therapies. Preclinical studies should therefore include the most prevalent HBV genotypes and clinical studies should not only include HBeAg-positive and –negative patients, but also monitor if all genotypes are treated with comparable efficacy.

Since host-directed therapies are more prone to elicit side effects than antivirals directly targeting the virus life cycle, a detailed understanding of the HBV life cycle and viral-host interactions are needed to develop novel and ideally curative treatment strategies. Here we summarize key viral and host targets for anti-HBV therapy that are currently exploited.

Viral targets

The viral transcription template: HBV cccDNA

Persistence of HBV relies on nuclear cccDNA that is refractory to current NA therapeutics [11]. Once cccDNA is established, it is extraordinarily stable and seems not to be completely lost via hepatocyte turnover since it can be delivered into daughter cells during cell division by recycling of viral capsids although it does not contain a nuclear retention signal. Owing to technical challenges in selective and sensitive cccDNA detection [11] and restriction of the models used, previous studies gave a wide range of values in terms of the half-life of cccDNA. We carefully determined by Southern blot analysis that the half-life of cccDNA following inhibition of viral replication is approximately 30 days in HepG2-NTCP cells, and cccDNA is not completely lost even after several rounds of cell division (C Ko et al., unpublished). Hence, complete loss of cccDNA without direct targeting strategies appears unlikely as it in average would take more than a lifetime [15].

Of note, a recent finding showed that the stable cccDNA pool can be directly targeted for degradation. We demonstrated that IFN-α, lympotoxin-β receptor agonists, and T cell-derived cytokines (i.e., IFN-γ and TNF-α) can specifically purge cccDNA from the nucleus of infected cells in noncytolytic manner [16-18]. Upon treatment, nuclear cytidine deaminases (APOBEC3A or APOBEC3B) are recruited to the cccDNA, deaminate cytidines and render the cccDNA molecule sensitive to degradation. Completion of this finding by identifying downstream cellular glycosylase and nuclease (D. Stadler et al., unpublished), which are responsible for abasic site formation followed by cccDNA fragmentation, may finally allow translation of this basic research finding into the clinic.

Molecular scissors such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the more recently discovered CRISPR/Cas9-based gene editing approach appears to be promising tools to directly target and reduce the viral cccDNA reservoir [19,20]. To minimize undesired off-target effects and increase efficacy of cleavage of the HBV genome, approaches such as a double-nicking strategy by Cas9-nickase and multiple targeting strategy using two or three guide RNAs in combination have been performed [21,22]. However, improved strategies are needed for *in vivo* delivery and to steer the fate of cccDNA upon cleavage (degraded vs. repaired) and target specificity (episomal cccDNA vs. integrated HBV genome).

HBV transcripts

Another approach is not to directly target the nuclear cccDNA, but to target HBV RNA transcripts. This may be achieved by cytokine-induced pathways with type I IFN destabilizing

viral RNA or IL-6 inhibiting HBV transcription [23]. Alternatively, one can block translation using an antisense RNA (asRNA) approach [24] or to induce degradation of the RNAs by employing RNA interference in a sequence-specific fashion. The latter can be achieved using either short interfering RNA (siRNA) that is brought into infected hepatocytes or short hairpin RNA (shRNA) that is expressed from a transgene which was delivered into the infected cell by a viral gene therapy vector [25]. Targeting HBV RNAs is particularly attractive as all HBV open reading frames use the same polyadenylation site downstream of the X ORF and therefore all HBV transcripts share a common 3´ terminus. This allows simultaneous suppression of all viral transcripts (including that of the HBV pregenome) and antigens with one single RNA-targeting molecule. Immune stimulatory and siRNA function may even be combined in a single molecule [26]. As data accumulate revealing immunomodulatory as well as carcinogenic properties of the secreted HBV antigens (HBsAg and HBeAg) as well as HBx, this approach is very appealing. Anti-HBV siRNAs are already in early clinical trials, developed by Arbutus Biopharma (phase II) and Alnylam Pharmaceuticals (phase I). A clinical trial by Arrowhead Pharmaceuticals was halted (company decision; potential toxicity of the delivery agent used in this trial in primates at very high doses). Although siRNA therapeutics are well tolerated in general, a drawback of the approach is that these strategies targeting HBV transcripts do not affect cccDNA persistence or turn-over and thus need to be applied repeatedly. However, one may hope that suppressing viral proteins does not only suppress viral replication, but allows to restore antiviral immunity and enhance spontaneous clearance rates - or at least improve efficacy of co-administered immune stimulatory drugs.

HBV polymerase: an enzyme with multiple activities

Since HBV polymerase (HBV Pol) is the only viral protein with enzymatic activities [DNA polymerase and ribonuclease H (RNase H) activity], it has been a primary target for drug development [27]. Consequently, five FDA-approved NAs that all target the reverse transcriptase activity of HBV Pol are in the mainstay of current therapies [28]. In addition, next-generation prodrugs of NAs (e.g., Tenofovir alafenamide, CMX157, AGX-1009, Besifovir, and Lagociclovir valactate) with the goal of enhancing antiviral efficacy, oral bioavailability, and long-term tolerability are currently undergoing evaluation in clinical trials.

Enzymatic RNase H activity of HBV Pol is also essential for viral DNA synthesis since it removes the pgRNA template during HBV minus-strand DNA synthesis. Recently, it was reported that chemical compounds being active against HIV RNase H are able to inhibit HBV

replication by blocking HBV RNase H activity [29,30]. Nonetheless, specific RNase H inhibitors have not received much attention for several decades probably due to early introduction of potent NAs into hepatitis B therapy and the interdependence between DNA polymerase and RNase H activity.

Targeting HBV Pol may also allow to target encapsidation of the HBV pregenome. Viral DNA synthesis begins with specific recognition of a stem-loop structure at the 5' end of pgRNA by HBV Pol, followed by incorporation of both into an assembling capsid. Subsequently, HBV Pol, which is covalently attached to a short DNA oligonucleotide (5'-GAA-3'), acts as a protein primer initiating reverse transcription. Attempts to develop specific inhibitors blocking those two largely unexplored steps are undergoing [31,32].

Capsid-forming core protein

Core protein (Cp) that assembles into the viral capsid is involved in many aspects of the HBV life cycle (Figure 1), suggesting that Cp represents a valid target for antivirals. Targeting Cp may lead to simultaneous inhibition of multiple steps. Interference with the capsid-forming activity of core protein has been thoroughly studied due to the importance for subsequent viral DNA synthesis. As a result, two groups of Cp allosteric modulators (CpAM) which misdirect core assembly and thereby induce either empty capsid (e.g., AT130) or aberrant capsid formation (e.g., HAPs) were discovered [33]. Much effort to increase antiviral efficacy of known CpAM by chemical optimization as well as the search for new classes of CpAM are ongoing and the first compounds have entered early clinical trials (e.g., NVR 3-778 and GLS-4).

Although CpAMs and clinically approved NAs suppress HBV DNA accumulation, it is tempting to speculate that CpAMs may have additional antiviral activities e.g. via targeting the incoming capsid that establishes cccDNA in a newly infected cell or activation of innate immunity by abnormal Cp aggregates. HBV rcDNA contained in incoming capsid must be delivered to and released into the nucleus; otherwise, no cccDNA formation is expected. In fact, we observed that a novel CpAM significantly reduced cccDNA levels when it was administered prior to cccDNA establishment in a *de novo* HBV infection (C Ko et al., unpublished). Given that the HBV capsid has a certain degree of flexibility and needs to dissociate at the nuclear pore to release the rcDNA genome [33,34], it is conceivable that CpAMs have an effect on establishing HBV infection through interference with capsid dissociation. In addition, Cp has been described to be associated with cccDNA [35],

although it is not clear if Cp regulates viral transcription or exerts particular function by altering the structure of the cccDNA minichromosome which may also be influenced by CpAMs.

Regulatory HBx protein

HBx is a small regulatory protein that is essential for viral gene expression and has been implicated in the pathogenesis of HCC [36-38]. It also seems to play a role in a number of cellular processes influencing the HBV life cycle including epigenetic control of the cccDNA minichromosome. Similar to cellular chromatin, cccDNA is organized as a viral minichromosome wrapped around histone and non-histone proteins in the nucleus of HBV-infected hepatocytes [39]. The cccDNA minichromosome can be in a transcriptionally open conformation carrying high levels of active chromatin markers [40]. HBx, as a viral protein attached to the cccDNA minichromosome, may play a role in the establishment of an accessible chromatin by regulating the recruitment of chromatin modifying enzymes [41-44].

HBx, however, seems to exert its main function by interacting with distinct cellular proteins and the Cul4-DDB1 E3 ubiquitin ligase machinery [45]. Thus, HBx targets the Smc5/6 complex, which assembles around the cccDNA and prevents transcription of HBV genes [46,47]. This finding was sufficient to bring all the interest in HBx back to the stage and to consider it an antiviral target. It may be worth tracking if additional restriction factors exist that are targeted by HBx. One drawback is that HBx seems to be an intrinsically unfolded protein [48] with a short half-life. Another drawback is that targeting HBx will have to be continuous as experiments have shown that cccDNA persists in the absence of HBx and is reactivated as soon as HBx becomes available again [36]. The fact that cccDNA persists even in patients with resolved chronic hepatitis B (CHB) and can be reactivated under immunosuppression, strongly supports the notion that influencing epigenetic control of HBV transcription does maximally lead to functional control of HBV and needs to be persistent.

HBV envelope proteins

The HBV envelope proteins are primarily responsible for virus entry. Thus, targeting S or L protein (e.g., by neutralizing antibodies) allows to prevent HBV infection. This is exploited using polyclonal antibodies from human serum as a passive vaccination in neonates or after accidental exposure to HBV. Alternatively, a number of monoclonal antibodies have been

developed that neutralize also escape-variant HBV and can serve this purpose [49].

Since HBsAg that is released in large amounts from infected cells serves as an antibody decoy and probably also skews T cell responses, blocking its release is also an interesting strategy which may be achieved by administration of nucleic acid polymers (NAPs). A recent report revealed that NAP monotherapy in CHB patients led to reduction of serum HBV DNA and concurrent appearance of anti-HBs antibodies even after treatment cessation, suggesting that NAPs may have a clinical benefit by restoring host immune response to some extent [50].

In addition, three independent studies recently showed that tetherin/BST-2, an IFN-inducible antiviral protein targeting various enveloped viruses, inhibits HBV virion egress [51-53]. In turn, HBsAg or HBx were proposed to counteract tetherin-mediated antiviral activity [52,53], suggesting that targeting these viral antagonists might be one therapeutic option to trap the infectious particle and thereby prevent virus spread.

Clinical studies showed that serum HBV DNA in chronic patients declines 3-4 log10 scales after loss of HBeAg, whereas HBsAg reduction was significantly lower by only 1 log10 scale [54]. This intriguing finding suggests that HBV DNA that becomes integrated into the host genome already early during HBV infection [55] is a significant source of HBsAg. This renders HBsAg an interesting “marker” of cells either infected with HBV or carrying an integrated HBV genome and thus bearing a risk for HCC development. Targeting HBsAg on the surface of these cells using single chain antibodies allows the development of T-cell retargeting strategies to eliminate the cells. This can either be achieved using chimeric antigen receptors for T cell grafting [56,57] or by applying bispecific antibodies to redirect T cells (U. Protzer et al., unpublished). This immunotherapeutic approach to induce killing of hepatocyte expressing HBsAg through specific recognition by redirect T cells is a potentially promising, alternative antiviral strategy.

Host targets

Achieving HBV cure means targeting cccDNA persistence. Besides directly targeting the cccDNA molecule as discussed above, the only alternatives are to either prevent establishment or at least replenishment of a cccDNA pool. At the level of the host, this may be achieved by preventing HBV entry into cells or by preventing the formation of cccDNA.

Viral entry

Viral entry into host cells is mediated by a number of host factors in a highly orchestrated manner. Viral protein motifs mediating entry are highly conserved. Targeting the host factors involved in viral entry thus is a reasonable approach to prevent both initial viral infection and virus propagation. HBV entry initiates by adsorption of viral particles to HSPGs [8], followed by a specific engagement of viral particles with NTCP via motif aa 9-NPLGF(F/L)P-15 that is located in the N-terminal preS1 domain of the L protein [9,10] and highly conserved among different human and primate HBV genotypes (Figure 1, summarized in: [58]). Specific receptor recognition drives the internalization of virus particles, most likely via receptor-mediated endocytosis, but the underlying endocytic pathway as well as the detailed fusion events remain elusive [59,60]. The cellular factors involved in endocytosis and endosomal fusion may serve as therapeutic targets.

A synthetic peptide spanning this motif and neighboring regions (aa 2-48) in the preS1 domain of L has been identified that inhibits HBV entry by competitively occupying its liver-specific receptor NTCP. This peptide inhibitor, called Myrcludex B, has proven its efficacy at low picomolar concentrations in preclinical models, seems safe in first-in-men trails and is currently in phase II clinical trials [58,61]. Alternatively, compounds targeting NTCP function (e.g., cyclosporine A, ezetimibe, and irbesartan) have been shown to inhibit HBV infection [62-65]. Taken together, antivirals targeting HBV entry may serve as an alternative to neutralizing antibodies to prevent virus transmission (e.g., after liver transplantation or from mother to child) if safety proves equivalent, but also in combination with other antivirals or immune therapies to prevent virus spread or re-establishment of infection.

Conversion of rcDNA to cccDNA

The molecular mechanism by which rcDNA is converted to cccDNA is still enigmatic. Recent studies proposed tyrosyl-DNA-phophodiesterase (TDP2) as the enzyme responsible for removal of covalently attached HBV Pol from rcDNA *in vitro* [66,67], and the cellular DNA polymerases κ being crucial to fill the plus-stand DNA gaps [68]. Further studies are needed to identify additional cellular factors, for instance a cellular exonuclease responsible for removing a short pgRNA remnant which serves as primer for the plus-stand DNA synthesis, or a cellular DNA ligase responsible for ligating the end of each stand. In this regard, a novel screening strategy aiming at identifying drug candidates that prevent the conversion of rcDNA to cccDNA would draw much attention [69,70]. However, targeting host molecules

involved in this process risks side effects.

Conclusion

Currently available NAs are well-tolerated and can control HBV replication. Although a variety of viral and host targets are currently exploited for the development of new antivirals, the holy grail of HBV cure, viral persistence via nuclear cccDNA, is only targeted with very few of the approaches summarized above. To achieve the final goal, HBV cure, approaches not directly targeting cccDNA would either require the host immune system to spontaneously "recover" and control the virus or would have to be combined with a potent trigger increasing HBV-specific immunity.

Conflict of interest

UP serves as an ad hoc advisor for Arbutus, VirBio, Gilead, Jansen and Roche. The laboratory currently receives research funding from Alnylam and Roche.

Financial support

The authors' work is supported by the German Center for Infection Research (DZIF), the German Research Foundation (DFG) via TRR 36 and TRR179, the EU via the HepCar consortium within Horizon 2020 and the Helmholtz Association via the iMed Initiative and the Helmholtz Validation Fund. TM currently receives a clinical leave stipend from DZIF.

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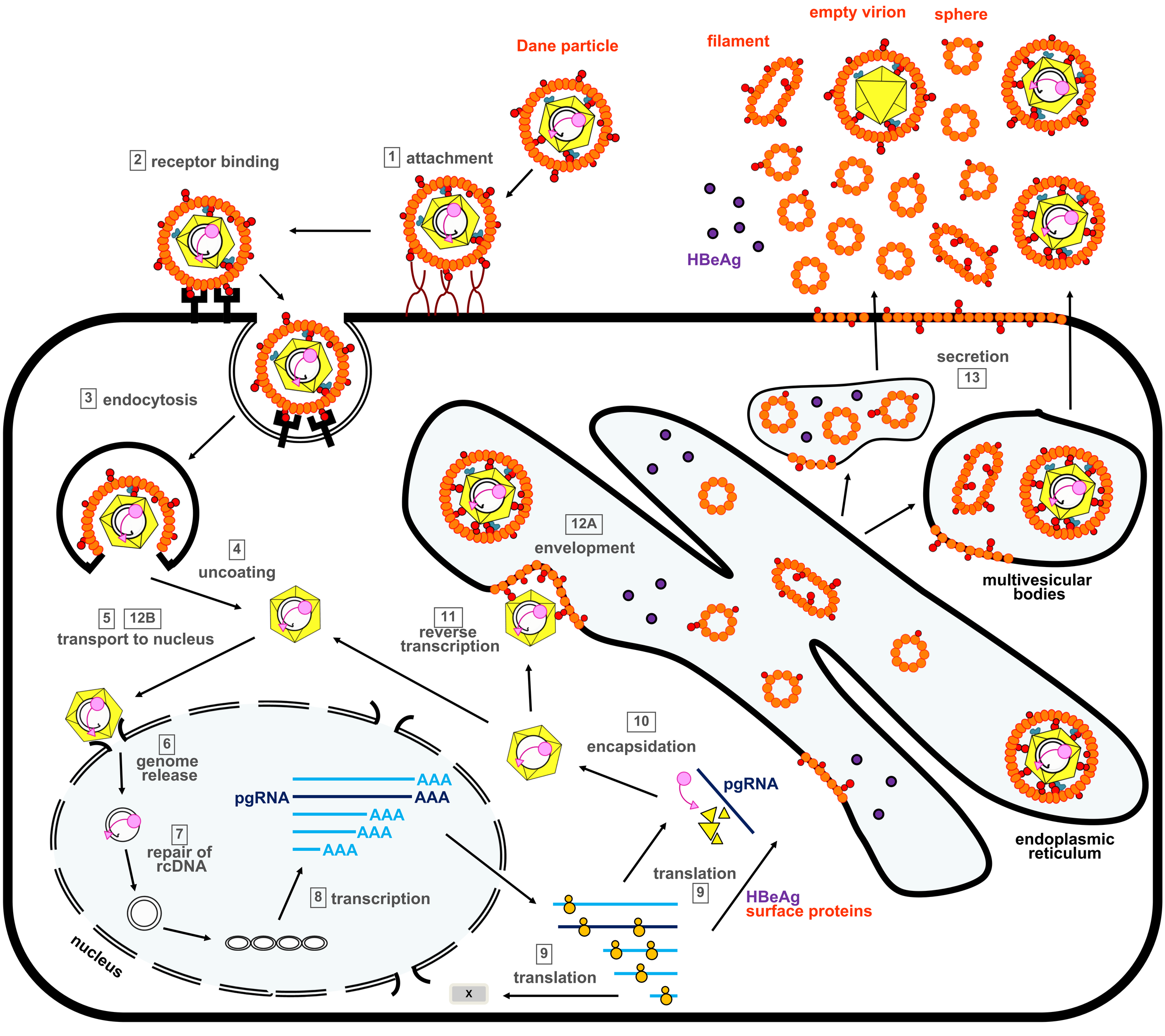
Figure 1. Schematic representation of the HBV life cycle: (1) unspecific and reversible attachment of HBV to heparin sulfate proteoglycans, (2) specific binding to NTCP, (3) endocytosis, (4) uncoating and (5) intracellular trafficking of the viral capsid, (6) release of the rcDNA genome into the nucleus, (7) conversion of rcDNA to the cccDNA persistence form, (8) transcription, (9) translation, (10) encapsidation and (11) reverse transcription of a pregenomic RNA, (12A) envelopment and (12B) re-import of mature capsid into the nucleus, (13) secretion of progeny virions.

Figure 1



myristoylated preS1-domain of HBV L-protein

preS2-domain of HBV L- and M-protein

S protein / S-domain of HBV L- and M-protein

HBV core protein

HBV polymerase

HBeAg

X

HBx

heat shock cognate protein 70 (Hsc70)

ribosome

heparan sulfate proteoglycans

sodium taurocholate cotransporting polypeptide (NTCP)

Highlights

· Current antiviral treatment of HBV can control viral replication, but still is far from achieving a cure.

· A detailed understanding of the HBV life cycle and viral-host interaction might enable the development of curative therapies.

· A variety of viral and host factors are currently exploited for the development of new antivirals.

· Key viral and host targets for anti-HBV therapy and potential targeting approaches are discussed