

Gene therapeutic approaches to inhibit hepatitis B virus replication

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Abstract

Acute and chronic hepatitis B virus (HBV) infections remain to present a major global health problem. The infection can be associated with acute symptomatic or asymptomatic hepatitis which can cause chronic inflammation of the liver and over years this can lead to cirrhosis and the development of hepatocellular

carcinomas. Currently available therapeutics for chronically infected individuals aim at reducing viral replication and to slow down or stop the progression of the disease. Therefore, novel treatment options are needed to efficiently combat and eradicate this disease. Here we provide a state of the art overview of gene therapeutic approaches to inhibit HBV replication. We discuss non-viral and viral approaches which were explored to deliver therapeutic nucleic acids aiming at reducing HBV replication. Types of delivered therapeutic nucleic acids which were studied since many years include antisense oligodeoxynucleotides and antisense RNA, ribozymes and DNazymes, RNA interference, and external guide sequences. More recently designer nucleases gained increased attention and were exploited to destroy the HBV genome. In addition we mention other strategies to reduce HBV replication based on delivery of DNA encoding dominant negative mutants and DNA vaccination. In combination with available cell culture and animal models for HBV infection, *in vitro* and *in vivo* studies can be performed to test efficacy of gene therapeutic approaches. Recent progress but also challenges will be specified and future perspectives will be discussed. This is an exciting time to explore such approaches because recent successes of gene therapeutic strategies in the clinic to treat genetic diseases raise hope to find alternative treatment options for patients chronically infected with HBV.

Key words: Gene therapy; Hepatitis B virus; Antisense nucleic acid; RNA interference; Designer nuclease; Ribozyme; DNzyme; Dominant negative mutant; External guide sequence; DNA vaccination

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Core tip: With various successful clinical trials ongoing, gene therapeutic approaches gained increasing

attention in the community over the recent years. Here we introduce gene therapy as a versatile platform for treatment of hepatitis B (HBV) virus infection. Newest delivery methods based on non-viral and viral techniques combined with most advanced technologies for inhibition of HBV replication based on DNA, RNA and designer nucleases are discussed. An overview of various gene therapeutic systems which were explored *in vitro* and *in vivo* is provided. Advantages but also limitations of the different strategies to inhibit HBV replication are mentioned.

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INTRODUCTION

Hepatitis B virus

The hepatitis B virus (HBV) is an enveloped, partially double-stranded DNA virus which replicates through an RNA intermediate. Upon cell entry the DNA containing core particle is transported to the nucleus where the DNA is released. Next the partial double-stranded DNA is repaired by host enzymes to form the covalently closed circular DNA (cccDNA). The cccDNA serves as a template for transcription of viral proteins and reverse transcription of new viral genomes by the viral polymerase. The large 3.5 kb HBV transcript represents the pregenomic RNA serving as a template for virus replication. Furthermore, there are three additional mRNAs with a length of 2.4, 2.1, and 0.9 kb. The HBV genome is 3.2 kb in size and contains four overlapping major open reading frames tightly arranged that encode polymerase, surface (HBsAg), core (HBcAg) and X proteins (HBx)^[1,2]. In addition, especially early during infection the HBV early antigen (HBeAg) can be detected which is a proteolytic product of the pre-core protein.

HBV infection counts as a major global health problem since more than two billion people show evidence of a past or present infection with the virus. This hepatotropic virus can cause acute and chronic infection of the liver. Fortunately, for most people the infection proceeds nearly without symptoms when taking an acute course of disease and complete recovery is likely. However, 240 million people suffer from chronic HBV infection and more than 780000 people die every year because of hepatitis B related secondary diseases. Mostly newborns and infants are prone to develop the chronic type of the infection^[3] and so far no treatment is available that reliably cures those patients.

Current therapeutics for chronic HBV infection are

intended to reduce viral replication and slow down or stop the progression of the disease. To date there are seven Food and Drug Administration (FDA) approved compounds for the treatment of chronic hepatitis B. These include interferon alpha and pegylated interferon alpha, nucleoside analogues (lamivudine, entecavir and telbivudine) and nucleotide analogues (adefovir, dipivoxil and tenofovir)^[4].

Interferon alpha has an antiviral effect by inhibiting the synthesis of viral DNA and activating antiviral enzymes and additionally, acts in an immunomodulatory way by enhancing the cellular immune response against infected cells^[5]. It has to be administered daily or three times a week as unmodified version and once in a week in the pegylated form. The main disadvantages of interferon alpha are the parenteral administration causing discomfort to the patients and potential adverse effects such as flu-like symptoms in the beginning of treatment and later on for instance fatigue and low blood counts. It is only given to selected patients because under certain conditions administration of interferon alpha is contraindicated^[4]. In a long-term follow-up study of HBeAg-positive patients, 11% lost HBsAg after treatment with interferon alpha^[6] which is considered as a cure of the disease.

Nucleos(t)ide analogues interfere with the HBV replication primarily by targeting the HBV polymerase functions such as reverse transcriptase and DNA polymerase activity^[5]. These drugs are administered orally as a daily dose. The major limitation associated with nucleos(t)ide analogues is the emergence of antiviral drug resistance and that life-long treatment can be indicated in the presence of chronic infection. In this context failure of medication adherence is another problem, because viral relapse is common when ending the treatment. Another prognostic marker of HBV infection is the presence of HBeAg which correlates with high viral replication rates. However, HBeAg seroconversion can be achieved with nucleos(t)ide treatment. In addition, HBV DNA levels can be decreased to an undetectable level but at the same time HBsAg is not lost^[4]. These features demonstrate another peculiarity of the hepatitis B virus. After entry into a cell the cccDNA is maintained as an episomally maintained template in the nucleus. It is not attacked by nucleos(t)ide analogues nor by interferon in general, so that it is able to serve as a reservoir from which previously cleared or treated infections can recur^[4]. The clinical management of chronic hepatitis B infection is reviewed in detail by Santantonio and Fasano^[7].

The viral reservoir and potential reactivation of the virus represents a major problem when developing novel HBV treatments options and this is a challenge that could be faced by gene therapy. Researchers seek to inhibit viral replication in a long-lasting manner without the need for continuous drug administration through gene therapeutic approaches.

The more ambitious goal is to completely eradicate the viral cccDNA depot and hence find a true cure for chronic hepatitis B virus infection. Here we discuss gene therapeutic approaches as a versatile platform to combat HBV infection.

Gene therapy

Gene therapy is a strategy to transfer therapeutic nucleic acids into the desired target cell for treatment of a variety of different diseases. To efficiently deliver the genetic payload, multiple gene transfection techniques were explored which can be subdivided into two major groups: virus-based and non-viral vector systems for delivery of respective therapeutic nucleic acid. Both delivery techniques were also utilized in gene therapeutic approaches to treat chronic infectious diseases such as HBV infection. Since HBV infection resides in liver, the majority of gene transfer approaches were focused on targeting hepatocytes.

Non-viral vectors are based on delivery of naked RNA or DNA which in combination with chemical and physical means can result in efficient delivery of the nucleic acid into the respective target cell^[8,9]. Chemical methods in the context of non-viral vector delivery rely on various chemical formulations such as cationic lipids^[10] and polymers including polyamidoamine dendrimers and polyethylenimine (PEI)^[11]. All chemical reagents were explored in different approaches and there are several commercially available transfection reagents which are commonly used for transfection of DNA and RNA resulting in sufficient transfer efficiencies in many cell lines *in vitro*. Major constraints of these methods are transfection reagent-associated toxicity and the difficulty to cross the nuclear membrane. In addition to chemical transfer methods, physical transfer techniques were explored involving needle injection^[12], gene gun^[13], electroporation^[14], sonoporation^[15], magnetofection^[16], and hydrodynamic gene transfer^[17]. These methods directly deliver therapeutic nucleic acid into the cytosol of the target cell and compared to chemical methods these techniques harbor a reduced risk of transfection-mediated side effects due to dispersion of the transfection reagent. However, limitations of these methods are exposed by the difficulty to cross the nuclear membrane, potential cellular damage caused by the transfection method, and the requirement of costly instruments.

Virus-based transfection techniques were utilized in numerous pre-clinical and clinical gene therapeutic applications. Predominantly used viral vectors can be attributed to three viruses which were converted into viral vectors by deletion of essential viral genes: adenovirus, adeno-associated virus (AAV) and retrovirus. All viral vector systems display advantages and disadvantages which were discussed in more

detail in previous reviews^[18]. Adenoviruses combine a large transgene capacity of up to 36 kilo bases (kb), an episomal nature of the adenoviral genome reducing the risk of genotoxicity, the possibility to produce high viral titers and the ability to transduce dividing and non-dividing cells at high efficiencies *in vitro* and *in vivo*^[19]. However, one major obstacle for *in vivo* applications are the innate and the adaptive immune responses induced by the incoming adenoviral particle. AAV vectors were explored in clinical trials, are non-pathogenic, lead to a reduced immune response and predominantly exist as extrachromosomal vector genomes in the transduced cell^[20]. One major disadvantage, however, is the small transgene capacity which is below 5 kb. Lentiviral vectors^[21] were broadly explored in clinical trials to treat rare genetic diseases in *ex vivo* gene therapeutic approaches. Various generations of lentiviral vectors are available which carry a transgene capacity of up to 8 kb. Although commonly used lentiviral vectors integrate their genetic cargo into the host genome, newest versions of these vectors can circumvent side effects associated with somatic integration by changing their integration profile.

Various non-viral and viral transfer techniques were exploited to combat HBV infection *in vitro* and *in vivo* which will be discussed in the following paragraphs.

GENE THERAPEUTIC APPROACHES AGAINST HBV

Various gene therapeutic approaches to treat HBV infection were studied in cell culture models and in animal models for HBV infection. Within the viral life cycle in an infected cell there are various points of attack which can serve as targets in gene therapeutic approaches to inhibit HBV replication. Figure 1 schematically shows the life cycle of HBV infection and indicates points of attack when considering a gene therapeutic treatment.

As shown in Figure 1 the mechanisms of viral inhibition in gene therapeutic approaches can be on the level of RNA (HBV derived transcripts), DNA (cccDNA) and proteins. On the RNA level antisense oligodeoxynucleotides and antisense RNA, catalytic nucleic acids such as ribozymes and DNazymes, RNA interference and external guide sequences (EGS) can be considered. On the level of DNA (cccDNA) as a potential target designer nuclease such as zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN) and the *clustered regularly interspaced short palindromic repeats* (CRISPR)/Cas9 technology can be used. On the level of proteins, dominant negative HBV mutants and a strategy based on capsid-targeted viral inactivation (CTVI) were studied. Another technology

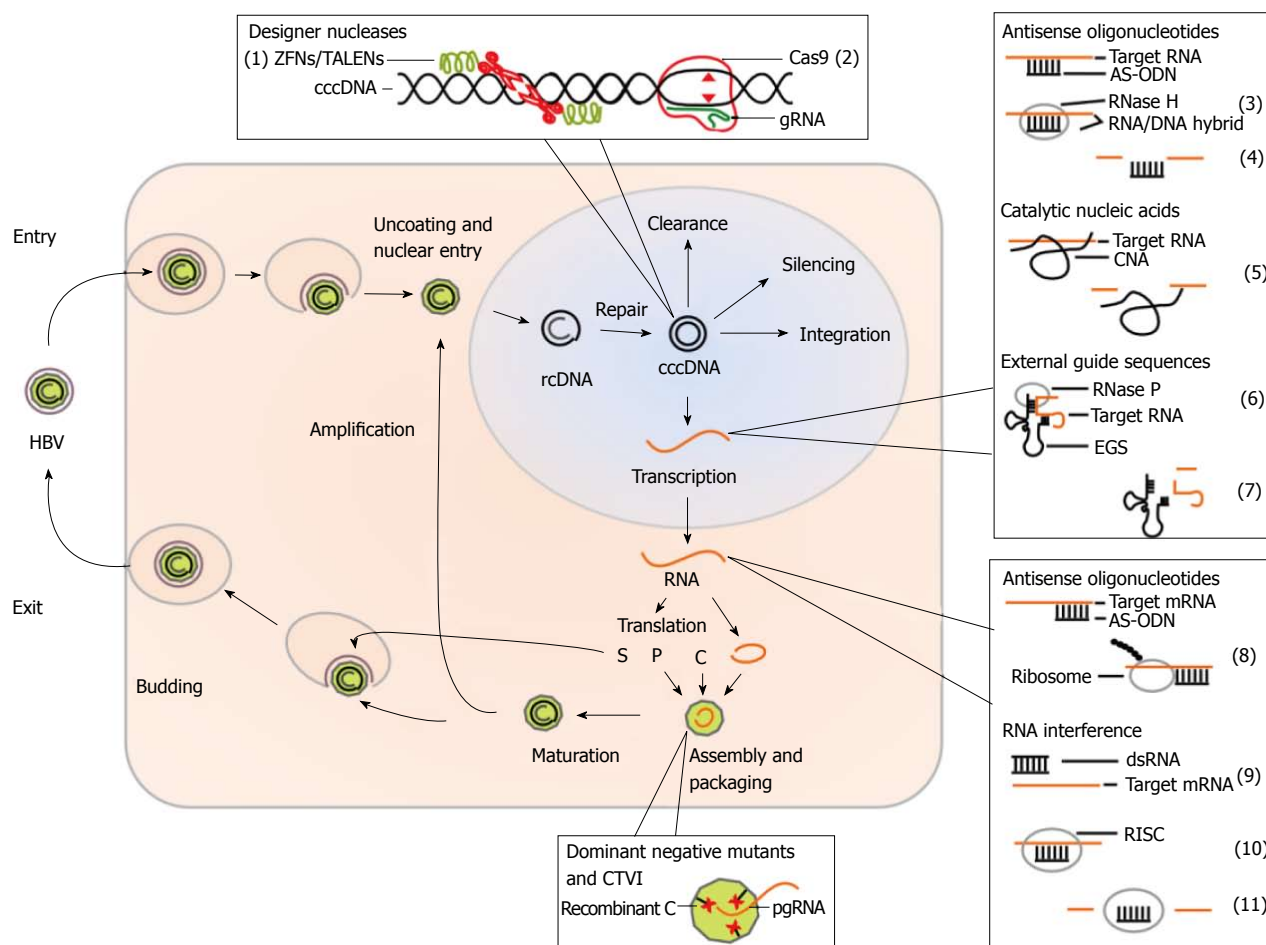


Figure 1 Hepatitis B virus replication cycle and gene therapeutic strategies. Enveloped virions of hepatitis B virus (HBV) infect liver cells *via* attachment to the cell membrane and endocytosis. The capsid with the relaxed circular (rc) DNA is released into the cytoplasm and the DNA is uncoated upon nuclear entry. In the nucleus the rcDNA is repaired to form the covalently closed circular (ccc) DNA. The cccDNA can be eventually cleared out, silenced or integrated into the host genome. Predominantly, it persists as an episome in the nucleus and is transcribed and translated by the host cell machinery. One of the transcripts forms the pregenomic (pg) RNA which is encapsulated together with the translated viral polymerase (P) by the translated viral capsid proteins (C). In the newly assembled nucleocapsid the pgRNA serves as a template for the viral polymerase which synthesizes the rcDNA. The nucleocapsid either migrates back to the nucleus to increase the pool of cccDNA or is internalized by the endoplasmic reticulum (ER). In the latter process it is enveloped with ER-membrane that harbors translated viral surface proteins (S) and finally released from the cell. Gene therapeutic strategies act on several steps of the viral replication cycle. Designer nucleases are intended to promote disruption of the cccDNA. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (1) use protein-based DNA-binding modules, while the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 nuclease system (2) is directed by a guide RNA (gRNA) to the target site. Antisense oligonucleotides (AS-ODNs) act inhibitory on the viral replication in two ways. In the nucleus they recruit RNase H (3) after the formation of RNA/DNA hybrids, which cleaves the viral RNA (4). In the cytosol they bind to the viral RNA which leads to a steric blockade of subsequent processes (8). Catalytic nucleotides (CNA) as ribozymes and DNAzymes are able to cleave targeted RNA by themselves (5). External guide sequences (EGS) are designed in a way that they resemble precursor tRNAs (6) when they bind to their target RNA and trigger cleavage by RNase P (7). RNA interference can be induced by different dsRNA species (9). Longer dsRNAs can be delivered exogenously or expressed in the cells as shRNAs. The RNAs are processed to approximately 21 nucleotide long dsRNAs termed siRNAs which are used to form the large RNA-induced silencing complex (RISC) (10) which degrades target messenger RNAs (mRNAs) (11). Dominant negative mutants of capsid proteins form a hindrance for proper packaging of viral progenitor RNA. In contrast, capsid-targeted viral inactivation (CTVI) is an approach where the capsid proteins are additionally fused to a destructive compound.

for prevention of infection but also for a potential treatment option of chronically infected patients, DNA vaccination can be considered as an attractive alternative. Although the majority of the described strategies for inhibition of HBV replication were not translated into the clinic so far, we believe that gene therapy may represent a valuable alternative in the future. Studies describing milestones of the various gene therapeutic approaches are listed in Table 1 and are described in more detail in the following sections.

Antisense nucleic acids

The beginning of gene therapy against HBV can be linked to the first tests of antisense oligodeoxynucleotides (AS-ODNs) directed against the HBV genome^[22-24]. With respect to antisense nucleic acids post-transcriptional inhibition is achieved by blockade of ribosomal access, inhibition of ribosomal assembly and induction of RNase H cleavage^[25].

First *in vivo* studies showed applicability of this approach in duck-HBV-infected Peking ducklings. Infected animals were treated daily by intravenous

Table 1 Advances in hepatitis B virus infection gene therapy in chronological order

Year	Strategy	Milestone	Ref.
1990	AS-ODN	First <i>in vitro</i> application	[22]
1992	AS-ODN	First non-viral transfection (targeted polycation peptide complex)	[28]
	Ribozyme	First <i>in vitro</i> application	[44]
1993	AS-ODN	First <i>in vivo</i> application	[26]
	DNA vaccination		[75]
1994	Dominant negative mutants	First <i>in vitro</i> application	[65]
1997	AS-ODN	First viral transduction (retroviral)	[39]
	Ribozyme		[46]
1998	DNAzyme	First <i>in vitro</i> application	[60]
	EGS		[87]
2001	CTVI		[69]
2003	RNAi	First <i>in vitro</i> application	[93]
		First <i>in vivo</i> application	[94]
2004	Ribozyme	First <i>in vivo</i> application	[56]
	DNA vaccination	First clinical trial in chronic HBV carriers	[81]
2008	CTVI	First <i>in vivo</i> application	[72]
2010	ZFN	First <i>in vitro</i> application	[138]
2011	RNAi	First clinical trial in chronic HBV carriers	[117]
2013	EGS	First <i>in vivo</i> application	[89]
	TALEN	First <i>in vitro/vivo</i> application	[146]
2014	CRISPR/Cas9	First <i>in vitro/vivo</i> application	[148]

AS-ODN: Antisense oligonucleotides; HBV: Hepatitis B virus; EGS: External guide sequence; CTVI: Capsid-targeted viral inactivation; RNAi: RNA interference; ZFN: Zinc finger nuclease; TALEN: Transcription activator-like effector nuclease; CRISPR: Clustered regularly interspaced short palindromic repeats; Cas9: Cas9 nuclease.

injection of AS-ODNs for ten days. The treatment resulted in nearly complete inhibition of viral replication which was assessed by liver DNA analysis for DNA replicative intermediates and blockade of viral gene expression as demonstrated by disappearance of surface antigen in serum and core antigen in liver^[26]. AS-ODNs proved to be most effective when directed against the initiation site of the HBsAg-gene^[22] or at the encapsidation signal^[27]. Except for a study published by Wu *et al.*^[28] in which already targeted DNA complexes were used, delivery of AS-ODNs was initially restricted to simple cellular uptake and binding of the unmodified antisense DNA to its target sites. Soon the system was improved by enhancing stability of the respective nucleic acids and by increasing uptake of AS-ODNs by the chosen target cell^[29-32]. In other studies AS-ODNs were conjugated to ribonuclease H or manganese porphyrin which after binding to the target site can lead to cleavage of the desired target sequences^[33,34]. Furthermore, DNA carrier systems were used and also enhanced by making them targetable to hepatocytes^[28,35,36].

Other oligonucleotide based approaches include antisense RNA delivered by episomally replicating expression vectors^[37,38] or retroviral vectors^[39,40]. The advantage of these approaches is the fact that antisense RNAs can be expressed in the cells, whereas AS-ODNs have to be exogenously delivered. This allows for experimental settings with long-term effects. For instance efficacies lasting longer than ten months were observed after stable transfection of antisense RNA expression vectors^[37]. Antisense RNA-

mediated inhibition functions preferentially through destabilization of the sense RNA by targeting the antisense/sense-RNA duplex to dsRNase^[41].

A completely different idea unrelated to complementary antisense RNA was introduced by Hafkemeyer *et al.*^[42] and is based on so-called "antisense-toxin-RNA". In this approach the authors took advantage of the HBV reverse transcriptase in infected cells to selectively kill those cells through *Pseudomonas* exotoxin expression from reverse transcribed antisense-toxin-RNA.

Site-specifically cleaving nucleic acids

There are two types of catalytically active nucleic acids, ribozymes and DNAzymes. Ribozymes are naturally occurring RNA molecules that can execute enzymatic activity on itself in the absence of proteins (in *cis*) or on extrinsic targets (in *trans*). DNAzymes were generated by *in vitro* evolution. They resemble ribozymes and do not exist in nature. The substrate for both species is RNA. There are various types of ribozymes known, hammerhead and hairpin ribozymes being the most popular ones. All catalytic nucleic acids have in common that they consist of an antisense sequence recognizing the target site and a catalytic domain mediating cleavage^[43]. Scientists were able to manipulate the recognition of target sites, rendering such nucleic acids very attractive for enhanced transient knockdown of gene expression.

The first experiments with ribozymes targeting the HBV genome were performed in 1992 using a triple ribozyme construct. In this study three hammerhead ribozymes were encoded on a single

DNA template and it was shown that they were simultaneously active *in vitro*. However, cleavage kinetics were similar to single ribozyme constructs. Nonetheless, this approach may still be favorable because it enables facing high target variability and emergence of viral resistance^[44]. However, first studies performed in a cellular context disclosed a first drawback of the hammerhead ribozymes because they were active after *in vitro* transcription and in Mg²⁺-supplemented cell extracts, but not in intact cells. The authors suggested some non-viral block, or inappropriate target site selection being responsible for the lack of intracellular activity^[45].

However, to overcome these problems novel strategies were pursued using other types of ribozymes. Welch and colleagues^[46] designed several hairpin ribozymes against different conserved regions of the HBV genome and tested them in a human hepatoma cell line (Huh7) transfected with full-length HBV genomes. The ribozymes were transduced into target cells using a retroviral vector system. The HBV production could be inhibited to up to 83% assayed through an endogenous polymerase assay. In the following years further *in vitro* studies were performed using different types of modified ribozymes^[47-51]. These were predominantly delivered *via* transfection of an expression plasmid into various cell lines which were additionally transfected with a HBV genome-containing plasmid resulting in varying effects on inhibition of HBV replication^[52-55].

In 2004 the first *in vivo* experiment in a transgenic mouse model was conducted by Pan *et al.*^[56]. For this study a self-processing triple-ribozyme cassette was used, with ribozymes acting in *cis* and in *trans*. The constructs were packaged in liposomes that were targeted to hepatocytes in the presence of asialofetuin. Quantitative PCR analysis for quantification of HBV genome copy numbers in murine liver showed a more than 80% decrease of HBV genome copy numbers and immunohistochemistry revealed a robust reduction in the number of hepatocytes staining positive for HBV core antigen. This was the first proof of concept demonstrating *in vivo* feasibility of viral RNA degradation mediated by ribozymes.

Next, recombinant hepatitis D virus (HDV)^[57] as well as lentiviral vectors were utilized as delivery vehicles for respective ribozymes which achieved effective reduction of HBV mRNA levels over four months^[58]. Furthermore, HDV-derived ribozymes were also utilized which were delivered by a pseudotyped retroviral vector (Moloney murine leukemia virus)^[59]. According to the authors the main advantages of these ribozymes are the natural activity of HDV ribozymes in human cells even at physiological Mg²⁺-ion concentrations and the comparably highest cleavage rates among all known ribozymes. Their results revealed significant reduction in the intracellular HBV DNA concentration

in HepG2.2.15 cells, which secrete infectious HBV virions. Furthermore, decreased extracellular HBsAg and HBeAg levels were observed after treatment with HDV ribozymes in comparison to the negative control. The conclusion was that using this strategy, HBV can be effectively inhibited at post-transcription and replication levels.

DNAzymes cleave RNA substrates based on a similar mechanism also used by ribozymes. However, they may be superior to ribozymes because their production is comparably straight forward and DNAzymes are less sensitive to chemical and enzymatic degradation. The first DNAzyme directed against HBV mRNA was created by Asahina *et al.*^[60]. They targeted the direct repeat 1 (DR1) and polyadenylation signal regions of HBV. In this study the authors used stabilized forms of DNAzymes that on the one hand lost some degree of activity compared to unmodified versions but on the other hand the degradation level was less pronounced. The DNAzyme was tested in Huh7 cells on an HBV-luciferase fusion reporter system where it exhibited 48% suppression compared to untreated control groups. Further *in vitro* studies^[61-64], however, revealed a major disadvantage of this system because intracellular expression of these DNA species is not feasible. DNAzymes have to be transfected directly because they act on post-transcriptional level which is clearly disadvantageous if long-term administration for instance in clinical applications is required.

Dominant negative mutants and CTVI

Dominant negative mutants of viral proteins are able to inhibit viral replication by interfering with the function of the wild type protein. The first study on the molecular effects of dominant negative mutants on the HBV replication was conducted in 1994 by Scaglioni *et al.*^[65] They mutated the core protein and observed an inhibition of viral replication by 90%-95%^[65] and it was concluded that this was the result of the disruption of the viral nucleocapsid assembly process. In a follow-up study the authors provided a delivery system using retroviral and adenoviral expression vectors^[66]. von Weizsäcker *et al.*^[67] showed that carboxy-terminal, but not amino-terminal core mutants inhibit viral replication. Furthermore, it was discussed that rather the packaging of the viral pre-genome and the reverse transcription reaction within the particles than the nucleocapsid formation itself is restrained by dominant negative core mutants^[68]. However, it was also shown that some mixed particles retain replication competency suggesting a possible mechanism of viral escape.

Using the principle of CTVI may represent an interesting variant of capsid modification, as the goal is to introduce a destructive element into the virus. The first time this technology was pursued

was by fusing a Ca²⁺-dependent nuclease from *Staphylococcus aureus* to the HBV core protein. This led to inhibition of proper synthesis of viral DNA inside the capsid and rapid viral DNA degradation^[69]. Later human eosinophil-derived neurotoxin was fused to the HBV core protein by another group and thoroughly studied also *in vivo*^[70-72].

Therapeutic DNA vaccination

Initial considerations to use vaccination in a therapeutic manner against HBV infection were made in 1993 by a group around Christian Bréchet^[73,74]. This approach was based on classical vaccination compounds. Also in 1993 the first genetic immunization with expression vectors for HBsAg was introduced in mice^[75]. In a further step Mancini *et al.*^[76] proved that a combination of both strategies is feasible and they showed efficacy in a HBsAg transgenic mouse as a model for chronic HBV infection. A single intramuscular injection of HBV envelope encoding plasmid DNA resulted in the elimination of serum HBV antigen levels as early as 4 wk after administration of the therapeutic DNA in some mice. Notably no liver cell injury was detected. Soon genetic immunization employing retroviral delivery was tested first in rhesus monkeys^[77] and then in chimpanzees chronically infected with HBV^[78]. The first time a DNA vaccine encoding HBsAg delivered with a gene gun was tested in healthy humans was in 1999 and it turned out to be ineffective at the applied dosage (0.25 µg)^[79]. However, the vaccine was well tolerated. A subsequent study used higher dosages (1, 2, or 4 µg) and this time antigen-specific CD8⁺ T cells, T helper cells, and protective levels of antibodies could be induced^[80]. Therapeutic DNA vaccination for chronic HBV infection was first tested in 2004 and in other subsequent studies^[81-83]. It was shown that HBV DNA vaccination was safe and immunologically effective because T-cell responses were activated in some chronic HBV carriers who did not respond to current antiviral therapies. These results indicated that in combination with conventional therapy, DNA vaccination may have a synergic effect leading to complete recovery from HBV infection.

EGS and RNA interference

The recruitment of ribonuclease P (RNase P) by EGS represents another interesting gene interference strategy. RNase P is an enzyme that removes the leader sequence of tRNA precursors by recognizing a common structure shared amongst all tRNAs. Using a custom-designed EGS that hybridizes with an mRNA to form a tRNA-like structure, RNase P can be recruited to cleave the target mRNA^[84,85]. With respect to HBV infection, this strategy was first proposed by Werner *et al.*^[86] in 1997. They took advantage of EGS designed to target HBV sequences

as proof-of-concept for the use of EGS as anti-viral or anti-cancer therapeutics^[87,88]. The EGS technology gained more attraction in 2013, when EGS was considered as a sufficient method to antagonize HBV replication. One study was conducted in cell culture and in mice using *Salmonella*-mediated delivery. Oral inoculation of attenuated *Salmonella* carrying the EGS construct led to up to approximately 95% inhibition of HBV gene expression levels and a approximately 200000-fold reduction of viral DNA levels in the livers and sera of the treated mice transfected with a HBV plasmid^[89]. In a follow-up study efficacy could be improved about 50-fold by modifying the EGS derived from natural tRNA through *in vitro* selection^[90].

RNA interference (RNAi) is a natural intracellular antiviral immune response mechanism triggered by double-stranded RNA (dsRNA). After conversion of dsRNA into guide molecules (siRNA), dsRNA induces the degradation of RNA *via* the RNA-induced silencing complex (RISC). Synthetic siRNA can be delivered exogenously or produced endogenously in the form of precursor small hairpin RNAs (shRNA) from plasmid DNA or viral vectors^[91]. When RNAi was first discovered^[92] it released a wave of multiple studies and applications. Also in HBV therapy it gained attention. In 2003 several studies were published^[93-97] and marked the beginning of the most intense exploitation of a gene therapy method as a valuable treatment option for HBV infection.

Shlomai *et al.*^[93] were the first to publish the use of RNAi in an *in vitro* cell culture model of HBV replication. They found that HBV gene suppression was achieved with different efficacies for different gene targets. Analysis of HBV transcripts revealed a reduction of about 68% on the level of all viral transcripts for RNAi targeting HBx protein. However, RNAi against two different targets on the same HBcAg open reading frame, which is exclusively encoded by the large 3.5 kb transcript, resulted in about 13% and 50% reduction on the level of the 3.5 kb transcript, respectively. This led to the conclusion, that RNAi target sequences have to be evaluated carefully.

In the next study McCaffrey *et al.*^[94] tested the RNAi-based approach *in vivo* in immunocompetent and immunodeficient mice. DNAs containing a HBV expression plasmid and a shRNA expression plasmid were co-transfected into mouse liver by hydrodynamic plasmid delivery. The authors could show that RNAi can inhibit all the steps of HBV replication that occur in cell culture and in mice. This was indicated by reduced secreted HBsAg levels in the supernatant of transfected cells and in mouse serum, reduced HBV RNAs levels in mouse liver, reduction of HBV genomic DNA to undetectable levels in mouse liver, and decreased numbers of cells stained positive for HBcAg.

The first viral vectors used to transduce RNAi sequence expression cassettes were either based on a prototype foamy virus (PFV) or an adeno-associated virus (AAV)^[98]. The vectors expressing the respective RNAi molecule were assessed in 293T.HBs cells, a cell line stably expressing HBsAg and HepG2.2.15 cells. In 293T.HBs cells HBsAg was knocked down by approximately 90% if directly compared to controls cells. HBsAg expression was also inhibited in HepG2.2.15 cells even in the presence of HBV replication. Uprichard *et al.*^[99] introduced recombinant adenovirus vectors for the delivery of shRNAs. Additionally, they used for the first time HBV-transgenic mice to show that ongoing HBV replication *in vivo* can be cleared by RNAi-targeted suppression of viral RNA for at least 26 d. Other viral vectors used included retroviral vectors^[100,101], AAV-7, -8 and -9 pseudotyped vectors^[102-104], recombinant human foamy virus^[105], gene-deleted adenoviral vectors^[106,107], lentiviral vectors^[108,109], and recombinant baculovirus^[110].

Further studies of the RNAi system included lipid-encapsulation of chemically modified siRNAs for non-viral delivery^[111] and the assessment of efficacy and pharmacodynamic properties of different RNAi target sequences and constructs, including methyl-modified siRNAs and plasmid based DNA vectors^[112]. Moreover, high-throughput generation and screening of siRNAs was established^[113] and expression systems introducing multiple siRNAs were developed^[105,114-117]. Ely *et al.*^[116] found that a Pol II promoter may be advantageous compared to a Pol III promoter, which was traditionally used for shRNA expression. The Pol III promoter can result in shRNA overexpression and saturation of the endogenous microRNA pathway leading to serious toxic effect *in vivo*^[118]. This could be restricted with the Pol II promoter, which provides the possibility to control the production of RNAi activators^[119].

Another non-viral vector system which was used in shRNA approaches is the episomal replicating plasmid vector pEPI-1. Herein, the transcription unit is linked to a scaffold/matrix attachment region (S/MAR) which ensures that the vector is mitotically stable in transfected cells. It was shown that it provides long-term expression of shRNAs which resulted in suppression of HBV gene expression, intracellular HBV DNA replication and release of progeny HBV over 8 mo^[120].

Besides therapeutic DNA vaccination, siRNA is the only gene therapeutic approach that was translated into clinical trials. In 2006 a Phase Ib, first-in-human safety and tolerability study of an RNAi-based therapy (NUC B1000) in patients with mild to moderate chronic HBV infection was conducted^[117]. NUC B1000 is composed out of four expressed shRNAs on one plasmid carried on a nanoparticle (cholesteryl spermine complex) and administered through intravenous infusion. The results revealed elevated cytokines and no HBV DNA or HBsAg

decrease in the patients. However, the safety profile of RNAi therapy conducted among patients with HBV was considered as reasonable. A second compound, ARC-520, a liver-tropic cholesterol-conjugated siRNA (chol-siRNA), transported by the proprietary Dynamic Polyconjugate delivery system is just reaching a Phase II clinical study^[121,122].

In summary the RNAi system was thoroughly exploited for treatment of chronic HBV infection in the past and research will be ongoing on this topic to overcome major hurdles like evocation of immune responses and maintenance of long-term suppression. Long-term suppression is required because it was shown that the HBV cccDNA depot in the host cells is not affected by this approach^[110]. Interestingly, another limitation might be the manipulation of the host RNAi defense by the HBx protein that potentially functions as a RNA-silencing suppressor (RSS)^[123].

Designer nucleases

For sequence-specific DNA targeting designer nucleases such as zinc finger nucleases (ZFNs)^[124-126], transcription activator-like effector nucleases (TALENs)^[127,128] and the clustered, regularly interspaced, short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) system^[129-131] can be applied. They combine customizable DNA binding molecules for sequence-specific DNA-binding and a nuclease for introduction of double-strand DNA (dsDNA) breaks. The induced dsDNA breaks activate different cellular DNA repair pathways. The two most exploited pathways in gene therapy are homologous recombination (HR) and nonhomologous end-joining (NHEJ). In the presence of a respective homologous donor DNA, cells are able to repair the dsDNA break *via* HR by exchanging the respective sequence^[132]. Without any homologous donor DNA cells repair dsDNA breaks *via* NHEJ. This error-prone repair mechanism can lead to insertions or deletions of one or several base pairs and may cause specific knockout of a gene^[132]. Therefore, designer nucleases are valuable tools to specifically introduce knock out mutations at a desired DNA locus.

The DNA binding domains (DBD) of ZFNs commonly contains 3-4 zinc fingers. Each zinc finger consists of 30 amino acids and forms two β -sheets and one α -helix. Upon DNA-binding the α -helix is placed in the major groove of the dsDNA and directs contact with a certain base pair triplet. Depending on the amino acids within the α -helix and the number of zinc fingers, ZFNs can be designed specifically to target defined stretches of DNA triplets with high affinity^[133-135]. The DBD is connected to a sequence independent cleavage domain of the type II S restriction enzyme *FokI* which causes double strand breaks after dimerization^[136]. Therefore two ZFN monomers are necessary to create the desired dsDNA break in the spacer between the binding sites of

the ZFNs. The double strand breaks will be repaired *via* NHEJ, causing insertion and deletion mutations (indels) of several base pairs within the sequence. These indels can lead to frame shifts within the open reading frame or translation abortion by newly formed stop codons. Both options result in dysfunctional proteins.

There are some approaches using zinc fingers to target HBV cccDNA. Zimmerman *et al.*^[137] were the first to use zinc fingers in conjunction with HBV although they did not yet use ZFNs. Instead, they created several zinc finger proteins (ZFPs) targeting the enhancer region of duck HBV (DHBV), which probably form a steric hindrance for the RNA polymerase. After screening the candidates for binding efficiency, the two most efficient ZFPs were expressed in a special DHBV tissue culture system *via* transfection and both the transcription of viral genomic RNA and viral protein production was assessed *via* quantitative PCR and western blot analysis. They showed that both ZFPs significantly reduce transcription from cccDNA compared to controls. The authors concluded that ZFPs designed to target HBV DNA are able to substantially reduce viral transcription and interfere with viral replication of cccDNA.

In another study, Cradick *et al.*^[138] showed that ZFNs can mediate inhibition viral replication *in vitro*. They generated ZFN pairs targeting several conserved regions of HBV genomic DNA and chose the most robust pairs for further studies. Huh7 cells were transfected with plasmids encoding ZFNs under the control of a cytomegalovirus (CMV) immediate early promoter and the target plasmid pTHBV2. This target plasmid contains a 1.3-fold HBV genome which is capable of full transcription of the viral RNAs, translation of the proteins and production of infectious virus. Three days post transfection, Southern blotting analyses revealed about 26% linearized DNA and about 10% cleaved target plasmids being rejoined in a tail to tail orientation. Both DNA species indicate that ZFN-specific cleavage at the intended ZFN target site occurred. Linear genomes are the result of direct cleavage without any subsequent repair while concatamer formation is caused by NHEJ after ZFN cleavage. To investigate if NHEJ led to indels in recircularized genomes or head-to-tail concatamers, a *Xba*I site was inserted within the spacer region of the ZFN dimer. After NHEJ occurred this site should be destroyed. Target sites of treated samples were amplified by PCR, digested with *Xba*I and resistant amplicons were sequenced. 13 of 16 samples showed a frameshift which would lead to dysfunctional proteins. Furthermore this study indicated a 29% reduction of pregenomic RNA in northern blot analysis compared to controls. Besides these results it could also be demonstrated that the ZFN pairs caused only moderate toxicity. In conclusion, Cradick *et al.*^[138] revealed that

specifically designed ZFNs targeting regions which are conserved among many different HBV serotypes can significantly reduce viral replication which is associated with negligible toxicity.

A third study by Weber *et al.*^[139] deals with the delivery of functional ZFNs into target cells *via* a viral vector system. Here, in contrast to the latter study, obligate heterodimeric ZFNs were designed, which are not able to form homodimers. This measure minimizes off-target effects because ZFNs that are able to form homodimers might cleave at unintended genomic loci by tolerating some mismatches. The three designed ZFN pairs targeted the open reading frames of HBx, HBcAg and polymerase. For delivery a self-complementary AAV-vector (scAAV) was used, which shows higher transduction efficiencies compared to single stranded AAV vectors^[140]. Since scAAV vectors have a reduced transgene capacity each ZFN of a pair was delivered individually in co-transduction experiments. Transduction of each ZFN pair into HepAD38 cells, a model cell line for controllable HBV replication, revealed mutation rates ranging from 9.8% to 34%. Transduction of all three ZFN pairs simultaneously resulted in mutation rates of 8% to 20%. Off-target mutagenesis for seven potential off-target sites was investigated using single molecule real time (SMRT) sequencing which detected indels (> 1 nt) in four sequencing reads out of 9290 filtered reads. It is of note that indels of only one nucleotide were not taken into account because they could not be distinguished from sequencing artefacts. In order to test if ZFN treatment has an effect on HBV replication, ZFNs were transduced into HepAD38 cells in which HBV replication was shut down. After turning HBV replication on, the controls showed a 30-fold and 323-fold increase in HBV marker levels in cells and in the supernatant, respectively. In ZFNs treated cells no significant increase of cellular or supernatant HBV marker levels could be detected, leading to the conclusion that ZFN-mediated cleavage resulted in replication-deficient HBV. In summary, it was demonstrated that HBV specific ZFN can be delivered successfully *via* AAV vectors into target cells *in vitro*, resulting in an efficient inhibition of HBV replication.

TALENs are a promising new class of designer nucleases that can be specifically designed to bind DNA sequences of interest and to introduce dsDNA breaks. Comparable to ZFNs, TALENs are chimeric proteins consisting of a N-terminal nuclear localization signal, a central DNA binding domain and a C-terminal *Fok*I nuclease domain. The DBD originates from transcription activator like effectors (TALEs) of bacterial plant pathogens of the genus *Xanthomonas*, which secrete these proteins to regulate gene expression within their host cells^[141]. The DBD is comprised of a repeat region consisting of a number of incomplete tandem repeats containing repeat-variable diresidues (RVD)^[127]. The

RVD of each repeat is specific for binding a corresponding nucleotide in their contiguous target DNA sequence^[127,141]. Similar to ZFNs, the TALEN-DNA binding domain is combined with a non-specific endonuclease activity which is dependent on dimerization of two *FokI*-cleavage domains. Binding of the DBDs upstream and downstream of a DNA target brings the *FokI* nuclease domains of a TALEN pair in close proximity and dimerization of the *FokI* monomers introduces dsDNA breaks. Note that the spacer between the binding sites of a pair needs to be considered in the TALEN design to ensure optimal cleavage^[127,128]. Up to now several techniques are available to specifically design^[127,142] and to assemble TALENs for the desired application^[127,143-145]. Assembly can be performed without highly complex screening procedures or the need for special equipment, making it cheap, simple and fast and thereby with that respect superior to the ZFN technology.

Bloom *et al.*^[146] designed TALENs with target sequences in the HBsAg or HBcAg expressing region of the HBV genome. TALEN efficacy was determined after co-transfection of Huh7 cells with TALEN expression plasmids together with the pCH-9/3091 HBV replication-competent plasmid. They observed that the HBsAg production was diminished in cells expressing TALENs. In a more stringent experimental model of HBV replication, the HepG2.2.15 cell line was transfected with TALEN expression plasmids. After three subsequent transfections and culturing cells under hypothermic conditions, HBsAg-specific TALEN expression resulted in disruption of cccDNA molecules with efficiencies of approximately 31%. This was confirmed in a T7E1 mutation detection assay and correlated with a decrease of HBsAg secretion of these cells. Expression of the HBcAg-specific TALEN pair only mutated 12% of cccDNA molecules which was not sufficient to inhibit HBsAg secretion. After hydrodynamic tail vein injection of replication-competent HBV DNA together with HBsAg-specific TALEN expression plasmids, serum HBsAg levels were decreased by more than 90% and circulating viral particle equivalents (VPEs) were decreased by approximately 70%. The T7E1 assay demonstrated mutation rates of 58%-87% in HBV-DNA extracted from livers of TALEN treated mice.

Chen *et al.*^[147] used three TALEN pairs targeting regions of HBV genomic DNA conserved among HBV genotypes A-D. Huh7 cells were transfected with the monomeric linear full-length HBV DNA of subgroups A, B, C, or D, respectively and plasmids expressing one of the respective TALEN pairs. Cells containing HBV DNA simulated the complete HBV replication cycle, including the nuclear generation of cccDNA. Suppression of HBeAg and HBsAg production by the TALEN expression was observed for all four HBV genotypes. Additionally HBcAg-RNA as well as pregenomic HBV RNA levels were decreased in

TALEN expressing cells. Furthermore T7E1 mutation detection assay confirmed that mutations were successfully induced at the respective TALEN target site within the HBV genome leading to a 10%-50% decrease of cccDNA levels. In combination with interferon alpha treatment TALENs expression led to synergistic effects further increasing the inhibition of HBV transcription in Huh7 cells. After delivery of monomeric linear full-length HBV DNA and TALEN expression plasmids into C3H/HeN mice by hydrodynamic tail vein injection, serum levels of HBeAg, HBsAg and cccDNA as well as liver pregenomic HBV RNA significantly decreased compared to control animals. In summary, HBV-targeting TALENs were shown to be active in cell culture models as well as in *in vivo* models and are capable of introducing mutations at their target sites reducing HBV gene expression levels and cccDNA genome numbers.

The most recent approach exploited the CRISPR/Cas9 system for genome engineering^[131]. Because sequence specificity is achieved by a guide RNA (gRNA) which can be produced from an adaptable expression cassette, this system is easier to manipulate in comparison to ZFNs and TALENs where the DBD is based on a protein sequence. Lin *et al.*^[148] were the first to apply this system for achieving HBV genome degradation^[148]. They co-expressed the Cas9 nuclease together with eight HBV specific guide RNAs individually and in addition combined two of them on one expression vector. They tested all constructs in Huh7 cells which were transfected with a HBV-expression vector. Inhibition varied among the different guide sequences. A maximum inhibition of 70% of intracellular HBsAg expression was reached after using one guide RNA and Cas9 nuclease expressed from individual vectors and up to 96% inhibition was reached when they were combined on one expression vector. Next, they multiplexed the two most effective gRNAs, an approach which proved to be even more effective. Finally, the expression vectors that contained expression cassettes for one gRNA and the Cas9 nuclease were tested in the HBV-hydrodynamic mouse model. Serum HBsAg levels were significantly reduced two days post-injection but increased again on day seven. However, Southern blot analyses revealed a reduction of intrahepatic HBV-expression levels of 20% to 60%. In conclusion the RNA-guided Cas9 nuclease system may be a useful technique for achieving inhibition of HBV replication.

CONCLUSION

Numerous and highly diverse gene therapeutic approaches were pursued to combat chronic HBV infection. Although strategies that solely rely on nucleic acids like antisense oligonucleotides and catalytic nucleic acids have a great advantage in

their simplicity, these technologies are also limited due to their instability and imprecision. RNAi was most adopted and thoroughly investigated not only in the field of HBV therapy and this generated a deep knowledge regarding this technique. However, especially in the case of HBV, RNAi may be disadvantageous, because it does not affect the HBV cccDNA. It can rather be considered as an alternative to nucleos(t)ide analogues based therapy potentially associated with an improved ability to also respond to escape mutants. In addition, if long-term expression is required, RNAi-based approaches are superior compared to conventional therapy based on daily administration required for nucleos(t)ide analogues.

We believe that the most recent technology to combat HBV infection based on designer nucleases may be one of the most promising approaches to be explored in the future. This strategy bears the potential to actually eradicate cccDNA species in infected cells. Especially in combination with compounds that inhibit the HBV replication cycle this could be an attractive therapeutic option. However, major obstacles are the production and delivery of the designer nucleases and for translation of this approach into the clinic, this methodology needs to be further improved. The most promising results to date were obtained with genetic vaccines which were also pursued in the clinic. Also this strategy may hold great potential for eradicating chronic hepatitis B infection.

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