Hepatitis delta virus: insights into a peculiar pathogen and novel treatment options

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Abstract | Chronic hepatitis D is the most severe form of viral hepatitis, affecting ~20 million HBV-infected people worldwide. The causative agent, hepatitis delta virus (HDV), is a unique human pathogen: it is the smallest known virus; it depends on HBV to disseminate its viroid-like RNA; it encodes only one protein (HDAg), which has both structural and regulatory functions; and it replicates using predominantly host proteins. The failure of HBV-specific nucleoside analogues to suppress the HBV helper function, and the limitations of experimental systems to study the HDV life cycle, have impeded the development of HDV-specific drugs. Thus, the only clinical regimen for HDV is IFNα, which shows some efficacy but long-term virological responses are rare. Insights into the receptor-mediated entry of HDV, and the observation that HDV assembly requires farnesyltransferase, have enabled novel therapeutic strategies to be developed. Interference with entry, for example through blockade of the HBV–HDV-specific receptor sodium/taurocholate cotransporting polypeptide NTCP by Myrcludex B, and inhibition of assembly by blockade of farnesyltransferase using lonafarnib or nucleic acid polymers such as REP 2139-Ca, have shown promising results in phase II studies. In this Review, we summarize our knowledge of HDV epidemiology, pathogenesis and molecular biology, with a particular emphasis on possible future developments.

In 1977, Mario Rizzetto and colleagues discovered a novel antigen by immunohistochemical staining of liver biopsy samples from HBV-infected patients with severe liver disease[1]. This antigen, detected in 19% of HBsAg carriers in Italy at that time, was named 'delta' and was initially thought to be a novel viral marker for HBV infections[2]. However, transmission of sera from patients with delta antigen to HBV-infected chimpanzees revealed that the delta antigen was associated with a satellite virus that depends on HBV[3]. This satellite virus turned out to be a virusoid and was named hepatitis delta virus (HDV)[3,4]. Almost 40 years later, HBV and HDV co-infections constitute a global health problem responsible for the most severe form of virally induced liver diseases, with unsatisfactory treatment options. In this Review, we provide a short update on HDV epidemiology, pathogenesis and clinical virology. We also elaborate on the peculiar life cycle of this virus, emphasizing key steps of viral replication that could be targeted for therapeutic intervention. Finally, a summary of current treatment options and novel treatment regimens under clinical development is provided.

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Molecular virology

Measuring 35–37 nm in diameter, the HDV virion is smaller than HBV (~42 nm) but larger than spherical subviral HBV particles, which are formed by self-assembly of HBV surface proteins (HBsAg) in HBV-infected cells[4,5] (FIG. 1). In contrast to HBV, no high-resolution structure of HDV is currently available. The virion contains a single-stranded circular RNA genome of negative polarity, composed of 1,672–1,697 nucleotides (genotype dependent), and constitutes the smallest mammalian virus genome identified so far[6–9]. The highly self-complementary (~74% base pairing) rod-shaped circular genome encloses only a single functional open reading frame encoding the hepatitis delta protein, serologically defined as hepatitis delta antigen (HDAg), which is expressed in two forms, the small HDAg (S-HDAg, 195 amino acids, 24 kDa) and the large HDAg (L-HDAg, 214 amino acids, 27 kDa). Each form serves distinct functions in virus replication. Within the HDV virion, 70–200 HDAg proteins are associated with the genomic HDV RNA to form a ribonucleoprotein complex[10,11]. During assembly,

Key points

- Co-infection with HBV and HDV is considered to be the most severe form of viral hepatitis, affecting 15–20 million individuals worldwide
- HDV is an RNA virusoid that depends on HBV envelope proteins for its assembly, and host cellular proteins to facilitate its replication
- Current treatment options for chronic HDV infection are limited to interferon regimes
- Three novel antivirals targeting virus entry or particle assembly and/or secretion have shown promising results in phase II clinical trials

the ribonucleoprotein complex becomes enveloped by an endoplasmic-reticulum-derived lipid bilayer containing the three HBV envelope proteins: small (S-HBsAg), middle (M-HBsAg) and large (L-HBsAg) (FIG. 2).

Sharing the envelope proteins with HBV, HDV exploits identical cellular entry factors, which are responsible for the strong liver tropism and host specificity of both viruses[12]. Initially, the virus encounters heparan sulfate proteoglycans (HSPGs) on the hepatocyte surface. Glypican-5 has been suggested as a major (but not the only) HSPG mediating HDV attachment[13]. This step is mediated by both the antigenic loop of the HBV envelope proteins and the preS1 region of the L-HBsAg protein[14–18]. Subsequently, and probably after induction of a conformational change in the *N*-terminal preS1 domain, HDV binds to its receptor, the human sodium/taurocholate cotransporting polypeptide (NTCP), encoded by *SLC10A1*, via the myristoylated *N*-terminal receptor-binding site of the preS1 domain (amino acids 2–75)[19,20]. NTCP is a bile salt transporter exclusively expressed on the sinusoidal (basolateral) site of parenchymal liver cells (hepatocytes)[21]. Subsequent steps of virus entry are less well characterized. Thus, it is unclear whether membrane fusion of HDV occurs at the plasma membrane or after endocytosis and whether HBV and HDV differ in this respect. Once released into the cytoplasm, the ribonucleoprotein complex is transported to the nucleus, possibly guided by the HDAg, which contains a nuclear localization signal that can be recognized by the cellular nuclear import machinery[22,23] (FIG. 2). HDV RNA replication takes place exclusively in the nucleus. As the virus does not encode an RNA-dependent RNA polymerase, replication and mRNA synthesis involves the activity of host polymerases. Strong evidence indicates that RNA polymerase II catalyses both processes[24,25]; however, some reports also claim a role for RNA polymerase I, which localizes to nucleoli[26,27]. Similar to plant viroids, which are smaller in size owing to the lack of an encoded protein but structurally closely related to HDV[28], replication proceeds via a rolling-circle mechanism of RNA synthesis. In the first step, an exact complement of the genome, called the antigenome, is produced. The subsequent double rolling-circle replication steps generate RNA multimers of genomic or antigenomic polarity[29]. These multimers cleave themselves into monomers by ribozymes embedded in both the genomic and antigenomic strands[30–32] (FIG. 2). Monomers of either polarity become ligated to form circular antigenomes and genomes. Whether HDV recruits (a) cellular ligase(s) or whether the ribozymes themselves possess ligase activity is presently unknown[33,34]. The sequence encoding S-HDAg resides in the antigenome; however, owing to its exclusively nuclear localization and its lack of an internal ribosome entry site element, the antigenome cannot encounter the ribosomal translation machinery directly. Accordingly, a 5′-capped and 3′-polyadenylated ~800 nucleotide mRNA is produced, exported into the cytoplasm and translated[35–37]. The copy numbers of HDV genomic, antigenomic and mRNA are approximately 300,000, 60,000 and 600 respectively, per average infected cell[38].

In chronically infected patients, experimentally infected chimpanzees[39,40] and cell culture systems[41], the L-HDAg protein sequence contains the entire S-HDAg polypeptide plus an additional *C*-terminal extension of 19 or 20 amino acids (genotype dependent). L-HDAg is encoded in the same RNA segment as S-HDAg; however, during replication, the antigenomic RNA becomes edited in such a way that the encoded amber stop codon (UAG) of S-HDAg is mutated to UGG, allowing elongation of the S-HDAg sequence by an additional 19 or 20 amino acids. This protein is called L-HDAg[42]. The RNA editing is catalysed by the host enzyme ADAR1 (REFS 43,44), which exists in a nuclear (ADAR1-S) and a cytoplasmic (ADAR1-L) isoform. Both isoforms are able to mediate editing of the HDV genome. As the antigenomic template is localized exclusively in the nucleus, ADAR1-S is believed to mediate the majority of editing events[44]. However, some reports also speculate about a role for the interferon-induced ADAR1-L in editing[45,46]. Although incoming and *de novo* synthesized S-HDAg is required for viral replication[47,48], L-HDAg, as an ADAR1 editing product, and thus expressed later in infection, represses replication and directs the viral life cycle towards assembly and secretion of progeny virus[41]. Both S-HDAg and L-HDAg share several functional domains, including RNA-binding domains, a coiled-coil motif, a helix–loop–helix motif and a nuclear localization sequence[49]. Interaction screens using recombinant protein revealed ~100 cellular binding partners of S-HDAg including several subunits of RNA polymerase II[50]. This finding indicates that S-HDAg would guide the HDV ribonucleoprotein complex to RNA polymerase II and alter its substrate specificity from double-stranded DNA to self-complementary RNA. Both HDAgs undergo post-translational modifications, including methylation, acetylation, phosphorylation and sumoylation within their S-HDAg region[38]. Within its *C*-terminal extension, the L-HDAg contains a nuclear export signal[51], a motif involved in HBsAg binding[52] and a prenylation site (Cys211), on which a farnesyl moiety becomes covalently linked by cellular farnesyltransferase[53,54]. Farnesylation of L-HDAg is important for negatively regulating viral replication later in the life cycle[55] and simultaneously promoting the envelopment of ribonucleoproteins and release of viral particles[53,56]. Inhibition of farnesyltransferase by lonafarnib inhibits L-HDAg prenylation thereby preventing the interaction of ribonucleoproteins with a Trp-rich domain in the cytosolic loop of HBsAg[57] (FIG. 1c). As replication proceeds, genomic RNA and RNA–protein complexes are

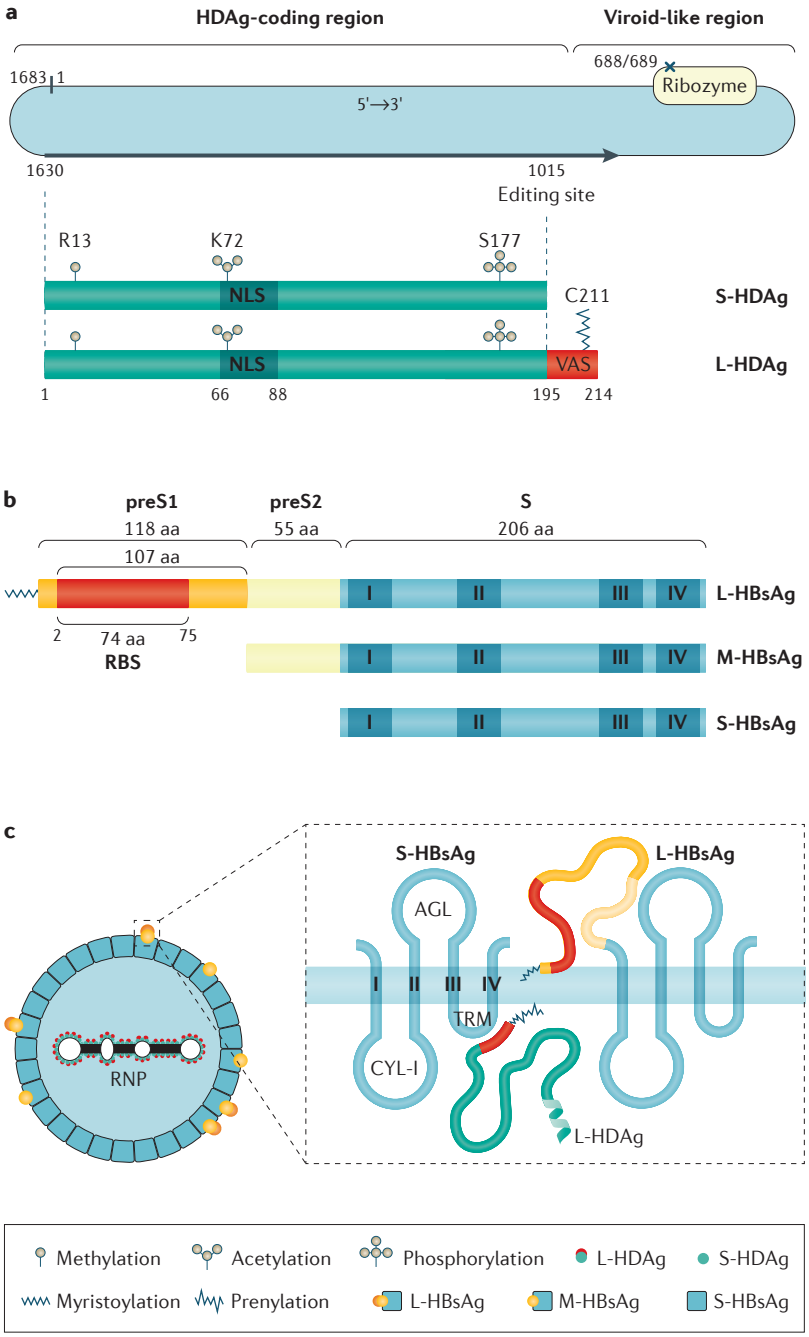

Figure 1 | **Structure of hepatitis D virus (HDV). a** | The HDV genome (1,683 nucleotides for genotype I)[156] comprises a viroid-like ribozyme-harbouring region. During virus replication, RNA editing results in an mRNA encoding L-HDAg (large HDAg). Both S-HDAg (small HDAg) and L-HDAg undergo methylation, acetylation and phosphorylation at R13, K72 and S177, respectively. The cysteine residue (C211) in the virus assembly sequence (VAS) region of L-HDAg becomes prenylated by farnesyltransferase. **b** | The three HBV-encoded envelope proteins of the virion contain the same C-terminal S-protein. M-HBsAg (middle HBsAg) has a preS2 domain, and L-HBsAg (large HBsAg) additionally contains a preS1 domain with 107–118 amino acids (genotype dependent). The receptor-binding site (RBS) is located within the myristoylated N-terminal part of preS1 and contains an essential NTCP-binding site. **c** | The HDV ribonucleoprotein (RNP) is surrounded by an envelope embedded with HBsAg. The cytosolic loop I (CYL-I) is crucial for envelopment and the antigenic loop (AGL) determines the antigenicity and contains an infectivity determinant. During HDV assembly, L-HDAg binds to the tryptophan-rich motif (TRM) of the envelope proteins via the prenylated moiety and the proline-rich VAS domain. NLS, nuclear localization signal; S-HBsAg, small HBsAg.

exported into the cytoplasm, where complete assembly of ribonucleoproteins occurs. For unknown reasons, antigenomic RNA cannot be exported[58].

HDV can only be assembled and released from cells expressing the HBV envelope protein. In a natural infection, these cells replicate HBV and express HBsAg from covalently closed circular DNA (cccDNA). However, hepatocytes containing rearranged HBV integrates that encode functional HBsAg but are replication deficient might contribute to HDV dissemination within the liver (for example in HBeAg-negative patients). The latter scenario has been shown *in vitro*, where hepatoma cell lines with stable HBV integrates are able to assemble HDV particles[59], and cell lines that co-express NTCP and only the HBV surface proteins are able to fully support the HDV replication cycle in the absence of HBV[60]. In contrast to HBV, which requires the interaction of the preS-encoded matrix domain (17 *C*-terminal amino acids of preS1 followed by the *N*-terminal five amino acids of preS2) with the HBV nucleocapsid for envelopment, HDV assembly only requires S-HBsAg[61,62]. Thus, secreted HDV particles might become enveloped by only the S-HBsAg protein and are consequently noninfectious[63]. To what extent ribonucleoprotein-containing HDV particles in the serum of patients co-infected with HBV and HDV are L-HBsAg deficient is presently unknown. The L-HBsAg with its myristoylated preS1 domain is needed for infectivity in order to interact with HSPGs and NTCP[64]. Whether HDV particles are secreted via multivesicular bodies (as shown for HBV[65]) or use the subviral particle secretion pathway via the Golgi remains unclear.

Cell culture and animal models of HDV

For many years, viral entry, including the establishment of authentic replication complexes of HDV (and HBV), could only be analysed in primary human hepatocytes (PHHs)[14] or differentiated HepaRG cells[66]. This restriction is mostly caused by the down-modulation of NTCP in commonly used human hepatoma cells lines (HepG2 or HuH7) or the species-specific receptor activity of NTCP[19,20]. Thus, chimpanzees have been the only immune-competent animal model for the study of HBV and HDV, but the use of Hominidae is ethically questionable[67]. An alternative *in vivo* system is immune-deficient mice partially transplanted with PHHs[68,69]. Such mice have been used to study HBV infection, HBV and HDV co-infections and the activity of antiviral agents[70]. Surprisingly, HDV can infect woodchucks, which replicate woodchuck hepatitis virus (WHV) and are released at very high titres as HDV ribonucleoproteins enwrapped by WHV envelope protein[71]. This model can be used to study the pathology of HDV replication. However, as infection with WHV-enveloped HDV ribonucleoproteins cannot be blocked by the NTCP-specific inhibitor Myrcludex B, entry by another pathway has to be assumed (K. Schöneweis & S. Urban, unpublished work), and this model cannot be applied to study the natural infection and/or co-infection events of HBV and HDV. Interestingly, one publication using this pseudotyped virus showed that normal mice can be

abortively infected at a low level[72]. If this finding can be replicated, it would indicate the presence of a partially functional receptor for WHV in mice. Stable transduction of human NTCP into mouse hepatocytes renders them susceptible to HDV but not HBV[19,73,74]. However, the reported infection efficacy is poor when compared to PHHs, indicating that some early infection event is still limited[19,75]. Consistent with these *in vitro* findings, transgenic mice expressing human NTCP become susceptible to HDV at very low levels when compared to PHH-transplanted immune-deficient animals[76,77]. Knockout of interferon signalling in NTCP transgenic animals only slightly increases infection efficacy, indicating that HDV-induced interferon responses might not

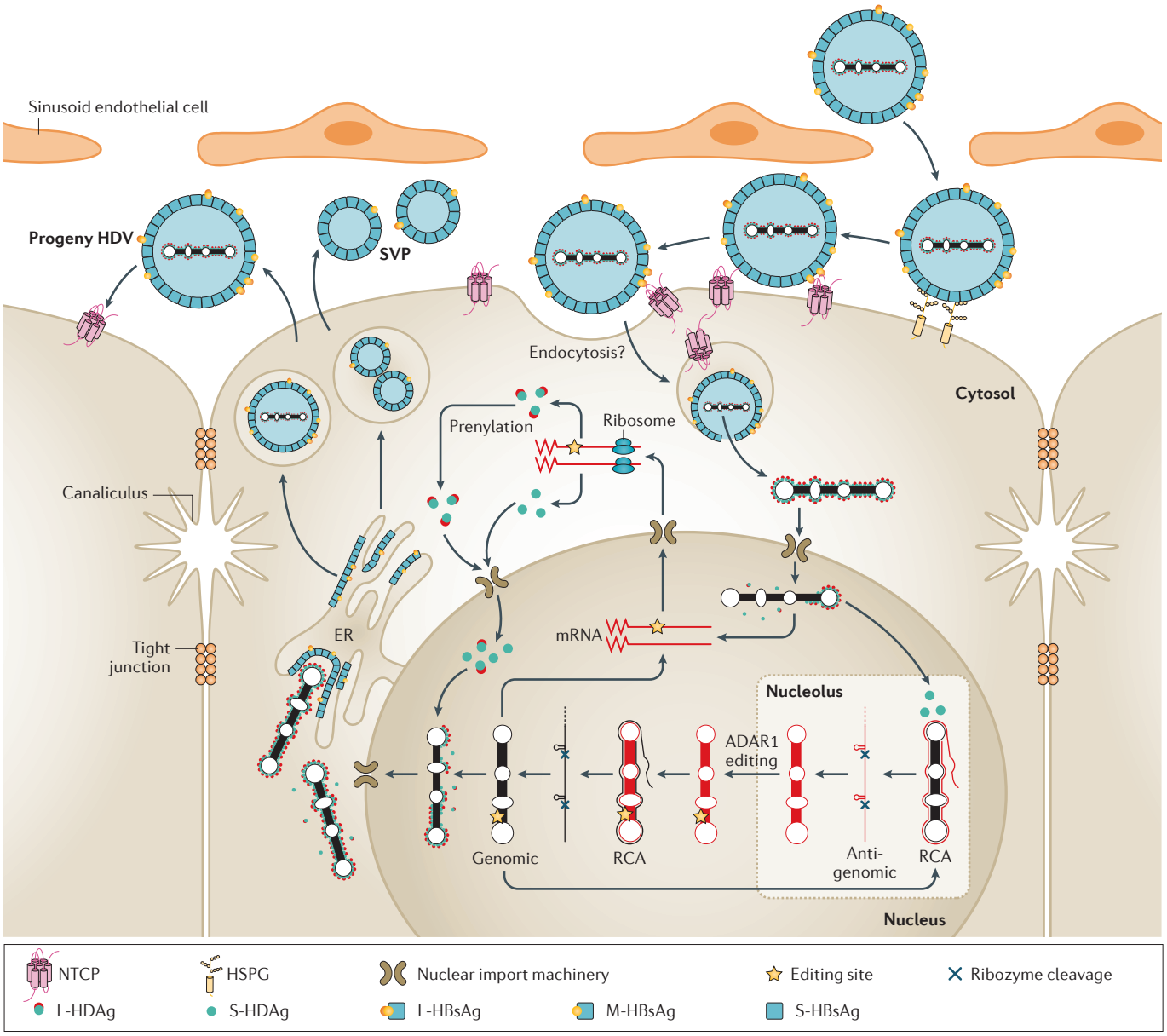

Figure 2 | **The replication cycle of HDV.** Hepatitis D virus (HDV) passes through fenestrated endothelium to enter the space of Dissé, where it interacts with heparan sulfate proteoglycans (HSPGs), binds to NTCP, and fuses either with the plasma membrane or undergoes an endocytosis-mediated fusion process to release the ribonucleoprotein (RNP) complex into the cytosol. The RNP complex is transported into the nucleus to initiate RNA replication. The genomic RNA with negative polarity serves as the template for the first rolling-circle amplification (RCA) step. The resulting antigenomic RNA oligomer is cleaved in *cis* by intrinsic ribozyme activity and ligated into the circular viral antigenome. The antigenomes undergo a second RCA. The resulting genomic RNA oligomer is autocatalytically cleaved and ligated into the circular genomic RNA. These genomic RNAs are used as the template for mRNA transcription in an RNA-polymerase-II-dependent manner. The heterogeneous mRNAs are translated into S-HDAg (small HDAg) and L-HDAg (large HDAg) and transported into the nucleus to regulate virus replication or bind to circular virus genome to form RNPs, which are exported to the cytosol. L-HDAg is responsible for RNP interactions with the HBV envelope proteins. Synthesis of HBV envelope proteins depends on covalently closed circular DNA (cccDNA)-dependent transcription or transcription from integrated HBV genomes (not shown)[157]. HDV is presumably released through the classic secretory pathway and progeny viruses infect neighbouring hepatocytes. L-HBsAg, large HBsAg; M-HBsAg, middle HBsAg; S-HBsAg, small HBsAg; SVP, subviral particle.

have a key role in the restriction. For future studies on the immunology and pathogenesis of HDV (and HBV) it will be crucial to identify additional host factors involved in modulating infection efficacy of both viruses, thereby enabling generation of immune-competent small animal models[78]. Some important findings with clinical relevance made using established models (mostly transplanted uPA-SCID mice) are: HDV RNA persists in the liver in the absence of HBV for at least 6 weeks and HDV propagation can be triggered upon superinfection with HBV[76]; HDV induces pronounced innate immune responses in HBV-infected animals[79]; and cell divisions of HDV-infected hepatocytes (in contrast to HBV-infected cells[80]) allows HDV replicative intermediates to spread into progeny cells without the need to secrete virus particles. Extrapolated into a clinical setting these findings could mean that HBsAg-negative hepatocytes, which replicate HDV RNA, might propagate RNA genomes by cell division in the liver and therefore provide an intracellular reservoir of genomes. Upon superinfection with HBV these cells might reassemble infectious particles for extracellular dissemination. This finding might imply that HDV antiviral treatment (unless it results in a complete eradication of replicative genomes) has to be sustained for a long time until HBsAg clearance can be achieved (discussed later).

Epidemiology

HDV prevalence in patients infected with HBV describes the percentage of anti-HDAg antibody-positive individuals in HBsAg-positive patients. HDV epidemiology has been reviewed in detail elsewhere[81]. Based on estimations from the 1980s and 1990s, ~5% of chronic HBV carriers worldwide are co-infected with HDV, which corresponds to an absolute number of 10–20 million patients infected with HDV. Regional variations in the prevalence of HDV are noticeable, with the highest rates in low-income and developing countries. Some evidence suggests that the prevalence of HDV does not proportionally correlate with the prevalence of HBV[81]. Following HBV vaccination programmes that started in the 1980s, HDV prevalence among patients infected with HBV decreased in most industrialized countries; for example in Italy, HDV prevalence decreased from 25% in 1983 to 8.3% in 1997 (REFS 82,83), and in central Turkey from 29% before 1995 to 12% after 1995 (REF. 84). In the past decade, however, HDV prevalence rates have remained unchanged in many European countries, ranging from 8–11% in Germany, Italy and England[85,86]. This figure is owing to immigration of people from HDV-endemic regions; in Germany, 75% of patients infected with HDV are immigrants, mostly from Turkey and Eastern Europe[86]. In the USA, prevalence data are limited and were only recently reassessed. In some US states, rates were comparable to Europe at 3–8%[87,88]. Remarkably, one report identified up to 50% HDAg-specific antibodies in HBsAg-positive injecting drug users[89]. One important factor for underestimating HDV prevalence is the practice in many hospitals to not routinely test for HDAg-specific antibodies in HBsAg-positive individuals[85,87,90,91]; one study found that testing rates varied substantially, with some as low as 8%[88]. In China, a country with >100 million people infected with HBV (>10% HBV prevalence), data on the prevalence of HDV need to be readdressed in the future. An early analysis of 2,346 liver samples from 17 areas in China indicated that 9.5% of HBsAg-positive samples also stained positive for HDAg[92]. In developing countries, high endemic areas with >20% prevalence are reported for Central Africa, Romania, Mongolia, Pakistan, Iran, in the mountainous region of Venezuela and Colombia as well as in the Amazon Basin in South America[93–96]. HBV vaccination protects against HDV infection; however, as the 240 million individuals with chronic HBV infection cannot be protected by a HDV-specific vaccine they are at risk of becoming superinfected with HDV.

Pathogenesis

Chronic HBV and HDV co-infection is considered to be the most severe form of viral hepatitis, causing liver cirrhosis and hepatocellular carcinoma. Patients co-infected with HBV and HDV have a twofold higher risk of developing cirrhosis, a threefold higher risk of developing hepatocellular carcinoma and a twofold increased mortality rate compared with HBV monoinfected individuals[97,98]. Similar to HBV, HDV is transmitted via the parenteral route, through contact with infectious body fluids. Accordingly, intravenous drug users are at very high risk of infection owing to contaminated syringes. Sexual transmission has been reported[99,100] as well as intrafamilial spread, mostly in regions of high endemicity[101,102]. The fact that HDV prevalence among individuals infected with HBV shows distinct geographical hotspots suggests that transmission within those regions could also be related to as yet unknown routes (such as by biting insects). In contrast to HBV, perinatal transmission of HDV is uncommon[103]. As HDV depends on HBV, two modes of acquiring infection can be distinguished. Co-infection designates a simultaneous infection of naive individuals with both HBV and HDV. In the majority of cases, HBV and HDV co-infection is self-limited and proceeds similar to an acute HBV monoinfection with clearance rates of >95% in immune-competent adults. However, compared with HBV monoinfection, an increased rate of fulminant hepatitis cases has been observed[104]. HDV superinfection describes HDV infection of an already chronically infected HBV carrier. Such superinfections cause severe acute hepatitis that progresses to chronicity in >80% of cases[105].

Our present knowledge on the cellular and molecular pathogenesis of hepatitis D is very limited. As for HBV and HCV, pathogenesis is believed to be mostly immune-mediated, as virus replication per se is not cytolytic and expression of HDAg shows few cytotoxic effects in hepatic cells or HDAg-transgenic mice[106,107]. However, debate exists on whether the cytolytic potential of HDV might differ in resting or regenerating hepatocytes, and some observations indicate that clearance of HDV-infected cells can occur quite fast in resting hepatocytes (see results of the Myrcludex B trial discussed later). In contrast to HBV, HDV infection induces a strong expression of type I interferons and

subsequently interferon-stimulated genes[77,79]. Using HDV-transfected HuH7 cells, HDV replication has been shown to interfere with IFNα-induced translocation of STAT1 to the nucleus[108]. If this finding holds true in a natural infection of PHHs with therapeutic doses of PEG-IFNα, the limited antiviral treatment response during IFNα therapy of patients with chronic HDV infection might be explained by a natural counteraction of the virus. Moreover, an altered phenotype of natural killer cells in patients infected with HDV has been described; although the absolute frequency of natural killer cells in the peripheral blood was increased, cells were less activated and showed impaired cytolytic function[109,110] indicating that similar to HBV, exhaustion of the cellular immune system might have a role in eliminating the virus. A $CD4^+$ T-cell response against HDAg has been shown in a small cohort of patients with chronic HDV infection. Interestingly, only patients negative for HDV RNA could mount a response[111]. In another study, a specific cytokine pattern was identified in patients with chronic HDV infection, which differed in patients who responded to IFNα treatment versus nonresponders[112].

Owing to its error-prone way of replication, the genome of HDV is highly variable. Accordingly, different HDV genotypes have evolved in distinct geographical regions[81]. Today, eight genotypes, with <20% divergence within one genotype and up to 35% divergence between different genotypes, have been described. Genotype 1 has a worldwide distribution but predominates in Europe and North America, genotype 2 is mostly present in Asia, genotype 3 has exclusively been found in South America, genotype 4 is present in Taiwan and Okinawa, and genotypes 5–8 are predominant in West and Central Africa[93]. Knowledge about the clinical outcome of chronic infection with the different genotypes is limited. Genotype 1 infections have a lower remission rate and more adverse outcomes than infections with genotype 2 (REF. 113). Genotype 3 is the most divergent of all genotypes and is often associated with severe liver disease and outbreaks of fulminant hepatitis in South America[114,115]. The high genomic variability also results in the generation of quasispecies populations and co-evolution of such quasispecies within an infected patient[116]. To what extent quasispecies formation contributes to pathogenesis, immune escape and possible resistance formation in upcoming treatment options will be interesting to investigate.

Current treatment

The peculiar life cycle of HDV, with its predominant dependence on host cellular enzymes, limits the development of direct-acting antiviral agents for possible treatment of chronic HDV infection. Obviously, the preferred treatment of HDV infection would be a functional control of HBV replication comprising the complete suppression of HBV envelope protein production (HBsAg negativation). Ideally, this suppression would be associated with a sustained positive anti-HBsAg antibody status. Current treatment options, however, are limited to IFNα, which was introduced against chronic HDV infection in the 1980s[117]. Following the introduction of PEG-IFNα in 2006, which has an increased half-life and improved safety profile, several studies have been performed showing HDV negativity rates of 17–47% but no HBsAg seroconversion[81,118,119]. The largest multicentre HDV clinical trial so far (HIDIT-1) consisted of three treatment arms: PEG-IFNα alone, PEG-IFNα in combination with adefovir dipivoxil, or adefovir dipivoxil alone for 48 weeks. HDV RNA negativation was observed in 31%, 26% and 0% of patients, respectively, 24 weeks after end of treatment[120]. However, 56% of these initially RNA-negative patients relapsed during long-term follow-up, indicating that 24 weeks post-treatment is too early as an end point for definition of a sustained virological response[121]. Moreover, whether a sustained virological response can be achieved at all without HBsAg loss and anti-HBsAg seroconversion remains unclear. In a successive study (HIDIT-2), PEG-IFNα treatment was prolonged to 96 weeks and was administered either alone or in combination with tenofovir disoproxil fumarate[122]. Interim results at week 24 post-treatment showed RNA negativation in 23% of patients treated with PEG-IFNα and 30% of patients treated with PEG-IFNα and tenofovir, indicating that treatment prolongation does not improve the outcome when compared with the HIDIT-1 results[123], especially as prolonged treatment was associated with a high frequency of severe adverse events. In both studies, combination treatment with adefovir or tenofovir did not substantially enhance HDV RNA response. Owing to the low response rate and high frequency of adverse events in IFNα-based therapy, novel treatment options are urgently needed.

Development of novel therapies

Viral targets

Unlike other negative-strand RNA viruses, HDV replication does not depend on a virally encoded RNA-dependent RNA polymerase, but requires host-specific DNA-dependent RNA polymerases (discussed earlier). Targeting these enzymes is possible (for example RNA polymerase II by α-amanitin) and has been shown to affect HDV replication *in vitro*[124]; however, owing to the essential cellular functions these molecules have, such drugs cannot be regarded as potential therapeutics. Other essential steps during RNA replication, such as cleavage of multimeric linear genomic or antigenomic RNAs, or ligation of the autocatalytically processed monomers, are mediated by the viral ribozymes. Inhibition of the ribozyme functions could be highly specific (and might even be achieved by small molecules[125]), but might also involve targeted strategies like RNA interference. In addition, small interfering RNA (siRNA)-directed degradation of HDV mRNA or replicative RNA intermediates could help to control virus replication. Few reports have shown that such approaches might work *in vitro*[126]. Whether they are successful in animal models remains to be demonstrated. Although such approaches seem to be far away from clinical applications, the progress in targeting HBV-specific transcripts[127,128] in patients infected with HBV might accelerate such approaches for HDV.

The only HDV-encoded targets that could be affected by direct-acting antiviral agents are S-HDAg and L-HDAg. As both these viral proteins modulate HDV replication at different stages of replication (S-HDAg by stimulation of nuclear replication and L-HDAg by facilitating egress from an HBV co-infected cell) they are excellent molecules for specific repression of HDV replication. Unfortunately, information on compounds that target HDAgs and might modulate their activities is lacking. This gap in our knowledge is mostly related to the historical lack of appropriate cell culture systems that support the full replication cycle of HDV (including entry) and enable high-throughput screening approaches, which was not possible with the previous systems that were based on transient co-transfection. However, the discovery of the HBV–HDV receptor NTCP[19,20] and the generation of cell lines that support the complete HDV replication cycle[60] provide the basis for the discovery of such novel antiviral compounds.

Cellular targets

HDV replication mostly depends on host cell proteins. Most of these molecules, such as HSPGs, RNA polymerase II or farnesyltransferase[14,24,53], are involved in important cellular functions and are ubiquitously expressed. This expression pattern fits the observation that HDV replicates in many different cell types once HDV cDNA or HDV RNA are artificially introduced in those cells (such as by transfection of plasmids encoding antigenomes)[48]. In contrast to these ubiquitously expressed factors, NTCP is exclusively expressed in differentiated hepatocytes, where it acts as a transporter for conjugated bile salts within the enterohepatic pathway of bile acid regeneration[21]. Accordingly, NTCP is a key factor determining the hepatotropism (and also host specificity) of HDV[19,75,129,130]. Generally, therapeutic interference with HDV replication through inhibition of cellular proteins bears the risk of causing intolerable adverse events when the protein becomes inactivated by the drug. Considering that treatment of chronic HDV infection probably requires long-term treatment to control or in the best case eliminate the virus, this aspect is of particular importance. Thus, druggable targets have to be carefully evaluated in order to allow a tolerable degree of adverse effects that might occur during long-term administration.

Novel treatments for HDV infection

Three novel therapeutic approaches are currently being investigated in phase II clinical trials: lonafarnib, an orally administered inhibitor of farnesyltransferase, prevents L-HDAg prenylation and consequently assembly and release of HDV particles[131–133]; REP 2139-Ca, an intravenously injected compound, inhibits virus attachment to HSPGs and affects HBsAg[134–136]; and Myrcludex B, which is a preS-derived lipopeptide that specifically blocks NTCP -mediated entry of HDV and HBV into hepatocytes[137–139]. Lonafarnib and Myrcludex B received orphan drug status from the EMA and FDA, and follow-up studies are currently ongoing.

Lonafarnib. As described above, HDV assembly requires post-translational farnesylation of the L-HDAg by cellular farnesyltransferase[53]. Lonafarnib, an inhibitor of this enzyme originally tested for its antineoplastic effects[140], potently affects this modification, interfering with the envelopment and release of the viral ribonucleoprotein complex[141]. These effects have been shown *in vitro* and *in vivo* using mouse models[142,143]. Thus, lonafarnib is able to directly interfere with HDV particle release by infected hepatocytes, thereby acting in a similar way to inhibitors of HBV, for example nucleoside analogues, which prevent reverse transcription and block virus production. However, lonafarnib inactivates an essential cellular enzyme and therefore influences important cellular events, including the farnesylation of signalling molecules such as c-Ras. Lonafarnib has been explored in many clinical trials against different forms of cancer with only little clinical benefit[144,145]. Remarkably, clinical data revealed that lonafarnib dose-dependently reduced serum levels of HDV RNA when administered in patients with chronic HDV infection[131]. This finding demonstrates that the drug reduces HDV assembly in HDV-infected human livers; whether this reduction results in elimination of infected hepatocytes (for example by inducing cytolytic mechanisms through accumulation of intracellular replicative intermediates or activation of cytolytic immune responses) remains to be seen. Managing the adverse effects associated with the enzymatic inhibition of the transferase for long-term treatment durations, which are probably required for treating chronic HDV infection, will be a further challenge.

Nucleic acid polymers. The second approach being investigated for chronic HDV infection is based on intravenous administration of highly negatively charged nucleic acid polymers, which have been hypothesized to interfere with the attachment of HBV and HDV to HSPGs. This interaction is required before specific NTCP binding of both HBV and HDV[14,18]. Nucleic acid polymers have been shown to interfere with entry of duck HBV, which uses a distinct pathway for cell entry to that of HBV, but can also be blocked by highly negatively charged molecules[146]. In addition to the inhibitory effect on viral entry, nucleic acid polymers are assumed to interfere with the assembly of HBsAg. However, the detailed molecular mechanism remains to be analysed. In addition to the results obtained in duck HBV, the entry inhibitor activity of a related nucleic acid polymer, REP 2055, has been verified for HDV[147]. Nucleic acid polymers have entered phase II trials in HBV-monoinfected and HBV–HDV co-infected patients. In a single-arm clinical trial including 12 HBeAg-negative patients co-infected with HBV and HDV, 500 mg REP 2139-Ca was administered once a week for 15 weeks, followed by weekly co-administration of 250 mg REP 2139-Ca and 180 µg PEG-IFNα for another 15 weeks. PEG-IFNα was maintained for another 33 weeks and patients were followed up for 24 weeks post-treatment. Interim results of this analysis showed that REP 2139-Ca was well tolerated and resulted in

a strong reduction of serum HDV RNA levels under REP 2139-Ca therapy. In parallel to the decline in HDV RNA levels, detectable HBsAg decreased substantially in the serum of patients. Both a decline in HDV RNA levels and reduced measurable HBsAg is promising, and it will be interesting to see whether a sustained virological response can be achieved in follow-up studies. Current information on the developmental status of this drug can be found elsewhere[148].

Myrcludex B. The third therapeutic approach relies on blocking the hepatocyte-specific NTCP receptor. Synthetic lipopeptides that mimic the receptor-binding site within the preS1-domain of the L-HBsAg (FIG. 1B) have been shown to potently inhibit HBV and HDV infection in cell culture and in a mouse model at subnanomolar concentrations[66,149–151]. A lead candidate of such peptides, Myrcludex B, has been characterized regarding its hepatocyte-binding specificity and its hepatotropism[19,129,130]. Myrcludex B efficiently targets NTCP of different species and accumulates at the sinusoidal membrane of differentiated hepatocytes *in vivo* after intravenous or subcutaneous administrations. It associates *in vivo* with NTCP with half-life times of about 16 h in mice[129,130] and chimpanzees (S. Urban and R. Lanford, unpublished work). Myrcludex B can inhibit the natural bile acid transporter function of NTCP[19,152] and thereby increases conjugated bile acid levels in humans when administered at doses >3–4 mg[153]. Remarkably, the IC_{50} of Myrcludex B for inhibition of HBV and HDV entry (80 pM) differs substantially (>500-fold) from its inhibitory effect on bile salt transport (~47 nM). This difference indicates that blockade of NTCP as a receptor for HBV and/or HDV can be achieved without blocking the transporter function of NTCP. In addition, NTCP-knockout mice are viable and show few NTCP-specific abnormalities[154]. In a case study, a person with highly elevated levels of bile salt was diagnosed with a functional knock down of NTCP without displaying apparent clinical symptoms[155]. Thus, NTCP is presumably eligible for prolonged treatment durations. Myrcludex B has successfully passed phase I safety studies[138] and has entered into phase IIa clinical trials in HBeAg-negative HBV monoinfected and HBV–HDV co-infected patients[137,139]. In the HBV–HDV substudy including 24 patients co-infected with HBV and HDV, Myrcludex B was applied at non-saturating doses (2 mg daily) either alone or in combination with PEG-IFNα for 24 weeks[137,139]. PEG-IFNα alone was used in the control arm. Myrcludex B was well tolerated and only a slight increase in levels of bile salts was observed. At week 24, HDV RNA levels declined by more than 1 $\log_{10}$ (tenfold decrease) in all cohorts and became negative in two patients in the Myrcludex B arm and two patients in the PEG-IFNα control arm. Remarkably, negativation was observed in five of seven evaluable patients in the Myrcludex B and PEG-IFNα combination arm, indicating a synergistic effect of PEG-IFNα and Myrcludex B. HBV DNA levels were also reduced at week 24 in the Myrcludex B and PEG-IFNα cohort[137,139].

Conclusions

Chronic HDV infection is a global health problem with very limited treatment options. Through major advances in understanding the molecular biology of the virus, especially the discovery of NTCP as the receptor for HBV and HDV and the subsequent development of cellular and animal models, fundamental tools for the discovery of novel antivirals have become available. Although the unique life cycle of the virus and the absence of virally encoded enzymes hamper the identification of possible therapeutic targets, it might become feasible in the near future to develop novel drugs for treating this disease. Three antiviral agents targeting different mechanisms of action are currently in clinical development and show pronounced antiviral potency. It will be interesting to follow whether one of these compounds alone or a combination of the novel drugs with one another and/or PEG-IFNα can control HDV replication and slow down the progression of HBV and HDV-related liver disease. Whether elimination of HDV can be achieved without sustained control of HBV replication is questionable. Therefore, the optimal therapeutic regimen for chronic hepatitis D would be sustained control (functional cure) of HBV, including HBsAg negativation and seroconversion to an anti-HBsAg positive state. However, this aim might not be achievable in the near future. Accordingly, HDV-specific therapies that are needed now should be compliant with long-term application in patients, reduce HDV serum levels, control intrahepatic virus turnover and normalize liver functions.

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F.A.L. and S.U. researched data for the article. Y.N. prepared the figures. All authors provided substantial contribution to discussion of content and writing of the article. S.U. reviewed and edited the manuscript before submission.

Competing interests statement

S.U. is co-applicant and co-inventor of patents protecting Myrcludex B. S.U. is also a consultant for Gilead and Humabs BioMed SA. F.A.L. and Y.N. declare no competing interests.