

Molecular Biology and Inhibitors of Hepatitis A Virus

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Abstract: Hepatitis A virus (HAV) is a faeco-orally transmitted picornavirus and is one of the main causes of acute hepatitis worldwide. An overview of the molecular biology of HAV is presented with an emphasis on recent findings. Immune evasion strategies and a possible correlation between HAV and atopy are discussed as well. Despite the availability of efficient vaccines, antiviral drugs targeting HAV are required to treat severe cases of fulminant hepatitis, contain outbreaks, and halt the potential spread of vaccine-escape variants. Additionally, such drugs could be used to shorten the period of illness and decrease associated economical costs. Several known inhibitors of HAV with various mechanisms of action will be discussed. Since none of these molecules is readily useable in the clinic and since the availability of an anti-HAV drug would be of clinical importance, increased efforts should be targeted toward discovery and development of such antivirals. © 2013 Wiley Periodicals, Inc. *Med. Res. Rev.*, 34, No. 5, 895–917, 2014

Key words: hepatitis A virus; virus replication; immune evasion; atopy; antiviral drugs

1. INTRODUCTION

Hepatitis A virus (HAV) is a major cause of enterically transmitted hepatitis worldwide, posing a global burden estimated in 2005 at 119 million infections of which 31 million resulted in symptomatic illness and 34,000 in death.¹ The virus is transmitted faeco-orally, mainly through close contact with infected individuals or by consumption of contaminated food and drinking water. The extreme environmental stability of the HAV particle contributes significantly to its transmission.^{2,3} HAV epidemiology correlates with poor hygiene and living conditions. Consequently, virtually every adult in developing countries is seropositive due to childhood infection. On the contrary, in regions with improved hygiene standards, infection is often postponed to later age. In addition, disease severity is generally age-dependent: infections are usually mild or asymptomatic in young children, whereas at older age, hepatitis A frequently presents with classic symptoms of acute hepatitis (e.g., jaundice, fatigue, general malaise, etc.) and a higher incidence of fulminant hepatitis, which may require liver transplantation.⁴ Fulminant hepatitis occurs especially in those aged over 50 for which mortality rates up to 5.4% have been

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reported.⁵ Additionally, HAV superinfections in chronic liver disease patients (e.g., hepatitis B or C) are believed to increase morbidity and mortality,^{6–8} although these findings are still subject to debate.⁹ Since highly efficient vaccines that provide long-lasting immunity have become available, HAV mortality and morbidity has decreased dramatically.^{8,10} However, occasional outbreaks of hepatitis A, sometimes resulting in fatal outcomes, still occur in industrialized countries. For instance, in the last decade, outbreaks have been linked to the consumption of contaminated green onions, semidried tomatoes, seafood, such as raw oysters and sushi, and other foodstuffs.^{11–14}

As posited earlier,¹⁵ research interest in the molecular biology and pathogenesis of HAV has decreased substantially since the availability of a safe and efficient vaccine. Nonetheless, HAV remains an intriguing and poorly understood virus and hepatitis A is still a public health problem in many countries. Here, we will review key aspects of the biology of HAV with an emphasis on recent findings and the unique characteristics of the virus. The potential clinical use of antivirals against HAV will be discussed as well.

2. HAV GENOME ORGANIZATION AND REPLICATION CYCLE

HAV is a nonenveloped, single-stranded RNA virus with a positive-sense genome. Despite the fact that HAV is classified within the family of the *Picornaviridae*, it exhibits quite some differences compared to other members of this family and is consequently the sole member of the genus *Hepatovirus*.¹⁶ Based on phylogenetic analysis of full length VP1 sequences, the single HAV serotype is divided into six genotypes (I–VI).¹⁷ Genotypes I to III are human and can be subdivided into subgenotypes A and B (and potentially subgenotype IC¹⁸), whereas genotypes IV to VI are of simian origin.¹⁷ The HAV genome (as shown in Fig. 1) is approximately 7.5 kb in length and consists of a 5' untranslated region (UTR), a single open reading frame (ORF) and a 3'-UTR with a polyadenosine tract. Like other picornaviruses, the HAV genome

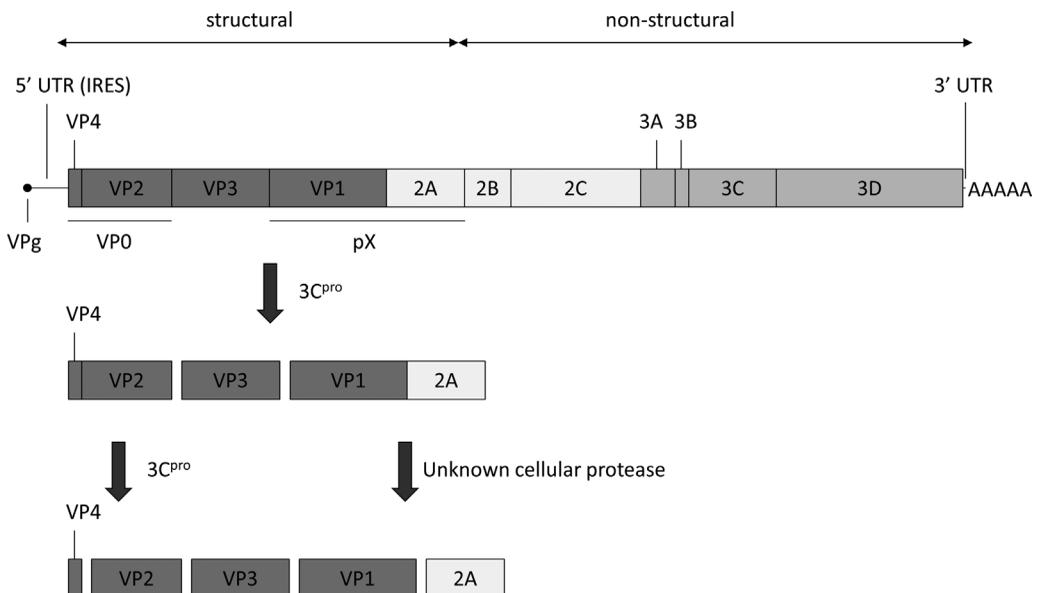


Figure 1. HAV genome organization and proteolytic processing of the structural proteins (IRES, internal ribosome entry site; UTR, untranslated region).

lacks a cap structure; instead a VPg protein (3B) is attached to the 5' end. In the 5'-UTR, six secondary structure domains can be found: domain I contains a hairpin structure, while the second domain comprises two stem loop structures followed by a polypyrimidine tract (pY1).¹⁹ The remaining domains form the type III internal ribosome entry site (IRES) allowing cap-independent translation of the viral genome. The single ORF comprises the structural genes (VP1 to VP4 (= P1) and 2A) and the nonstructural genes (2B–2C and 3A–3D). The short 3'-UTR contains two stem loops and/or a pseudoknot structure which are, together with domains I and II and the polypyrimidine tract of the 5'-UTR, crucial for viral RNA synthesis.²⁰

A. Receptor Binding and Cell Entry

In 1996, the HAV cellular receptor (HAVcr-1) was identified on African green monkey kidney cells (AGMK) as an attachment and probably functional receptor.²¹ Consequently, the human homolog (HuHAVcr-1) was identified and characterized as a human HAV receptor.²² HAVcr-1 is also known to be a marker for acute ischemic kidney injury (in this context referred to as kidney injury molecule 1 or KIM-1)²³ and a regulator of T-cell based immunity (in this context referred to as T-cell immunoglobulin and mucin domain-containing protein 1 or TIM-1).²⁴ Nevertheless, since HAVcr-1 is also expressed on other organs,²² it is likely that additional receptors are required for HAV attachment and entry. For instance, it has been suggested that TIM-3 promotes HAV entry without being a functional receptor.²⁵ In addition, HAV-specific IgA are reported to mediate infection of hepatocytes via the asialoglycoprotein receptor (ASGPR).²⁶ This and the fact that IgA was also identified as a natural ligand for HAVcr-1²⁷ may explain why (and how) IgA-coated HAV can enter the hepatocytes through both HAVcr-1 and ASGPR, thereby promoting enterohepatic circulation and continuous (endogenous) reinfections of the liver.²⁸ This phenomenon is thought to play an essential role in prolonged and relapsing cases of hepatitis A. Only the emergence of avid IgG antibodies can break the cycle and eventually clear the infection.²⁸

B. Uptake and Uncoating

Following receptor-mediated binding to the cell surface, the HAV particle is internalized. This has been suggested to occur through receptor-mediated endocytosis since HAV infection can be inhibited by blockers of endosomal acidification such as monensin, ammonium chloride, and chloroquine.^{29–32} However, the precise mechanism remains unclear to date. Upon binding to its receptor the particle is destabilized, thus initiating the uncoating process and releasing the single-stranded RNA into the host cell cytoplasm.³³ Uncoating has been reported to be a slow and asynchronous process with a reported duration of 4–10 hr, in contrast to under an hour for poliovirus.³⁴ It has been proposed that this asynchronicity may be due to the fact that the HAV inoculum contains a heterogeneous mixture of mature virions and provirions. These provirions still require a maturation cleavage (of VP0 into VP2 and VP4, as discussed below) following entry, prior to uncoating, and might therefore uncoat more slowly than mature virions.^{34,35} During the uncoating process, dense, noninfectious HAV uncoating intermediates are formed. Intriguingly, these particles do not appear to have an altered sedimentation profile, thereby differing from the typical picornaviral A particle.³⁶ Finally, both low pH and Ca²⁺ ions are reported to play an important role in HAV receptor binding, uncoating and during the maturation cleavage of provirions following virus entry (as described above).^{31,32,35,37} Upon completion of uncoating, the single-stranded RNA is released into the host cell by an unknown mechanism.

C. Translation

The viral RNA, being released into the cytoplasm, is then translated into a single polyprotein. Similar to other picornaviruses, HAV employs an IRES, located in the 5' UTR, to direct cap-independent translation of the viral genome using the host ribosomal machinery. Most picornaviruses affect the host cell protein synthesis to favor a more efficient translation of the viral mRNA. This process is mediated by a proteolytic cleavage of eukaryotic initiation factor 4G (eIF4G), thereby inducing a complete shutdown of capped mRNA translation.³⁸ Intriguingly, unlike for other picornaviruses, it is thought that HAV IRES depends on eIF4G, as part of an intact eIF4F complex.³⁹ The fact that HAV has to compete with an intact host cell machinery may also explain its poor replication in cell culture (see Section 5). However, a recent publication by Redondo et al. suggested that HAV IRES-driven translation can occur without intact eIF4G and that another factor may be crucial for this translation.³⁸

Next, the translated polyprotein is processed co- and posttranslationally in a series of proteolytic cleavages into several functional precursor and mature proteins. The primary post-translational cleavage occurs at the junction between P1-2A and 2B (instead of the junction between P1 and 2A as for other picornaviruses) (Fig. 1).⁴⁰ All other processing steps (except the VP1-2A junction, as discussed below) are mediated by 3C^{pro} and its functional precursor 3ABC^{pro}.⁴¹ This is different from other picornaviruses that may use L^{pro}, 2A, 3C^{pro}, and the 3CD^{pro} precursor.⁴² Intriguingly, HAV protein 2A, unlike for other picornaviruses, has no proteolytic activity nor does it contain a ribosome-skipping sequence,⁴² but seems to be implicated in morphogenesis (see Section 2,F).

D. Regulating the Balance between Translation and Replication

Since translation of viral proteins and RNA replication are competing processes, they must be balanced properly to allow efficient viral replication.⁴³ In order to tip the balance from translation to replication, the poly(A)-binding protein (PABP), as part of the eIF4F complex, is cleaved by HAV 3C^{pro}. The N-terminal cleavage product of PABP was shown to have an improved RNA-binding capacity compared to uncleaved PABP and may act as a dominant negative for IRES-mediated translation, thus favoring viral RNA synthesis.⁴⁴ Additionally, proteolytic cleavage of the poly(rC)-binding protein PCBP2, which interacts with the pyrimidine-rich tract pY1 in the HAV 5'-UTR, may also be implicated in regulating the balance between translation and viral RNA synthesis.¹⁹ Other enzymes that bind to the HAV IRES are polypyrimidine tract binding protein (PTB), glyceraldehyde 3-phosphate dehydrogenase, and La autoantigen, which respectively enhance, suppress, and suppress translation.⁴⁵⁻⁴⁷

E. Replication

As for most RNA viruses, replication of the viral genome takes place in replication complexes that consist of rearranged cellular membranes containing both viral and host proteins. Studies revealed important roles for 2BC precursor and 2C proteins in the membrane rearrangements forming the replication complexes. These complexes are described as a tubular vesicular network and were thought to be of endoplasmatic reticulum (ER) origin.^{48,49} However, a recent publication described the mitochondrial localization of the 3ABC precursor protein suggesting that HAV replication complexes may be derived from the outer mitochondrial membrane.⁵⁰ In line with this hypothesis, exchanging the mitochondria-targeting 3A transmembrane domain for the ER-targeting poliovirus 3A transmembrane domain resulted in loss of replication competence.⁵¹

Within the replication complexes, the viral genome is transcribed into antisense RNA, which subsequently serves as a template for the production of new viral genomes. During this process, the 3D protein functions as an RNA-dependent RNA polymerase. The 3B (VPg) protein serves as a starting point for primer-independent transcription and is covalently linked to the 5' genome end. A conserved replication element near the 5' end of the 3D^{pol}-coding sequence likely directs uridylylation of VPg by 3D^{pol}, in such way priming VPg for initiation of RNA replication.⁵² During replication, the 3A/3B junction remains uncleaved and the transmembrane protein 3A serves as an anchor tethering the growing HAV RNA strand and associated proteins to the membranes of the replication complex,^{42,53} as is the case for other picornaviruses.⁵⁴ It has also been reported that the 2C protein of HAV binds to the 3' end of the antisense RNA. In this way, protein 2C may be implicated in anchoring the negative sense RNA template to the membranes of the replication complex.⁵⁵

F. Capsid Assembly

Despite the fact that the multitiered capsid assembly process is only poorly understood, HAV differs at various steps from other picornaviruses. Following the initial cleavage at the 2A/2B junction by 3C^{pro} (as discussed above), the N-terminal part of protein 2A coordinates proper folding of the P1–2A precursor protein. This precursor protein is then processed by 3C^{pro} and its stable precursor 3ABC^{pro} into VP0 (consisting of VP4 and VP2), pX precursor (consisting of VP1 and 2A), and mature VP3 protein.^{42,56} These building blocks assemble into pentamers and subsequently associate with viral RNA to form preprovirions. In a final processing step, VP0 will be cleaved into VP4 and VP2 and precursor pX into VP1 and 2A, yielding mature virus particles with only VP1, VP2, and VP3 proteins. The processing of pX is mediated by a yet unknown cellular protease.⁵⁷ Although the cleavage can be performed by extracellular enzymes like factor Xa and trypsin,^{58,59} maturation can also be executed by the lysosomal proteinase cathepsin L. This may indicate a role for lysosomal proteinases in maturation cleavage and potential targeting of HAV provirions to the early lysosomes for maturation cleavage.⁵⁹ On the other hand, VP0 processing is thought to be a self-catalytic process that is dependent on the presence of encapsidated RNA.⁶⁰ The resulting VP4 is rather small compared to other picornaviruses and lacks an N-terminal myristoylation signal.⁶¹ Note that the mature VP4 protein has never been identified in purified virus stocks and its role in capsid formation remains unclear.^{15,60}

G. Release

HAV is an enterically transmitted virus that replicates mostly in hepatocytes before it is excreted via the bile into the faeces. Despite the fact that the exact details of HAV release remain elusive, the mechanism seems to differ depending on the cell type infected and is thought to involve either a vesicle-mediated cellular protein transport pathway or specialized hepatocellular transport proteins involved in bile secretion.⁶² Blank and colleagues demonstrated that following infection of polarized human intestinal epithelial Caco-2 cells, release of progeny virus was largely restricted to the apical membrane.⁶² In this way, virus is secreted mainly into the intestinal lumen resulting in an amplification of the HAV inoculum in the intestines and thus an increased viral shedding and spreading of the virus. However, it is still unclear by which mechanism HAV reaches the blood stream. A role for transcytosis by M cells present in Peyer's patches in the ileum has been suggested, a mechanism that was found for poliovirus and reovirus as well.⁶² Another study described that infection of polarized human hepatocytes occurred most efficiently via the basolateral plasma membrane, after which more than 95% of progeny virus was exported through the basolateral membrane (into the bloodstream), rather than through

the apical membrane (into the bile channels).⁶³ This contrasts with the *in vivo* observation that bile and faecal titers are considerably higher than serum titers.^{15,64} Reuptake and transcytosis of progeny virus by the hepatocytes into the bile channels is suggested as the mechanism for enteric secretion.

H. Particle Structure

HAV is a nonenveloped icosahedral particle of approximately 27 nm in diameter.⁶⁵ The mature capsid is composed of 12 pentamers each consisting of five copies of VP1, VP2, and VP3. Unlike for other picornaviruses, VP4 appears not to be present in mature HAV particles (see Section 2,F). Attempts to produce high-resolution images of HAV particles remained unsuccessful so far. A 2006 review¹⁵ showed a medium-resolution image obtained by cryo-electron microscopy suggesting the absence of the well-defined canyon surrounding the fivefold axis, in this way differing from other picornaviruses. However, these results have not been confirmed or published separately to date. HAV has a limited number of neutralization antigenic sites. The immunodominant antigenic site is composed of VP1 and VP3 residues and is conformation-dependent.⁶⁶ A second antigenic site is the glycoporphin A binding site by which HAV can bind to erythrocytes, causing hemagglutination. This process is optimal at acidic pH, but is impaired at physiological pH. This suggests that HAV has evolved to escape erythrocyte binding and consequent clearance.^{67,68} Indeed, a mutant of this binding site displayed increased clearance from the blood and lower overall fitness, suggesting evolutionary constraints and explaining the low level of antigenic variability of the glycoporphin A binding site.⁶⁸

3. IMMUNE EVASION MECHANISMS

Hepatitis A is clinically characterized by a prolonged asymptomatic phase before clinical illness becomes apparent and a very limited type I interferon response (only in week 1–2 after HAV challenge).⁶⁹ HAV replication remains largely undetected by the immune system for several weeks after infection and intrahepatic RNA was shown to persist for over 48 weeks in experimentally infected chimpanzees.⁶⁹ To this end, HAV employs several strategies to evade the host immune response that are rather different from those used by other picornaviruses.

A. Targeting MAVS and TRIF

One of these evasion tactics is to ablate the innate immunity's alarming system that induces IFN expression. HAV was shown to inhibit double-stranded RNA (ds RNA)-induced IFN β gene expression⁷⁰ by interfering with the retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) signaling pathways (Fig. 2).⁷¹ RIG-I recognizes single-stranded 5'-triphosphate RNA, while MDA-5 detects single- and double-stranded picornaviral RNA covalently linked to VPg.⁷² Both RIG-I and MDA-5 use an adaptor protein called mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, or Cardif) that is localized on the outer membrane of the mitochondria.⁵⁰ Upon activation by RIG-I or MDA-5, MAVS recruits and activates TANK-binding kinase 1 (TBK1) and inhibitor of NF- κ B kinase ϵ (IKK ϵ). TBK1 and IKK ϵ are both responsible for the phosphorylation of IFN regulatory factor 3 (IRF-3), eventually leading to IRF-3 dimerization, nuclear translocation, and induction of IFN β transcription. HAV proteins 3ABC^{pro50} and 2B⁷² have been described to interfere with MAVS, thereby disrupting the innate cellular antiviral defense mechanism. Precursor protein 3ABC is a stable polyprotein processing intermediate that

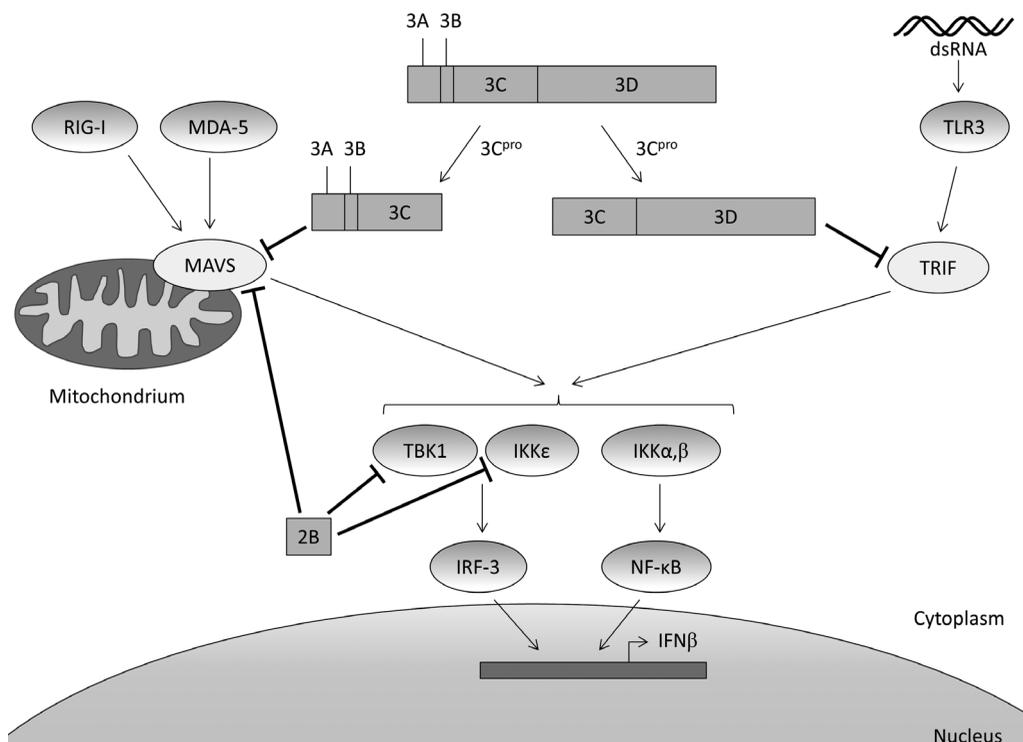


Figure 2. Inhibition of $\text{IFN}\beta$ transcription by HAV through proteolytic cleavage of MAVS (by 3ABC) and TRIF (by 3CD) and through direct inhibition of MAVS, $\text{IKK}\epsilon$, and TBK1 (by 2B). (dsRNA, double-stranded RNA; IKK , inhibitor of $\text{NF-}\kappa\text{B}$ kinase; IRF-3, interferon regulatory factor 3; MAVS, mitochondrial antiviral signaling protein; MDA-5, melanoma differentiation-associated gene 5; RIG-I, retinoic acid-inducible gene I; TBK1, TANK-binding kinase 1; TLR3, Toll-like receptor 3; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing $\text{IFN}\beta$).

requires both the 3A and the 3C^{pro} domain for the cleavage and inactivation of MAVS. The transmembrane domain of protein 3A ascertains mitochondrial localization while the 3C^{pro} catalytic site performs the actual proteolytic cleavage of MAVS. Mere 3C^{pro} , lacking the 3A domain, is incapable to perform this proteolysis.⁵⁰ In addition, it has been demonstrated that protein 2B suppressed both MAVS functioning and the kinase activities of TBK1 and $\text{IKK}\epsilon$, thus synergistically suppressing RIG-I/MDA-5 signaling, although the exact mechanism still remains to be elucidated.⁷²

Toll-like receptor 3 (TLR3) provides an additional recognition mechanism for dsRNA. Activation also results in IRF-3 phosphorylation and induction of $\text{IFN}\beta$ transcription, but unlike RIG-I and MDA-5, the downstream action of TLR3 is mediated through TRIF (Toll/IL-1 receptor domain-containing adaptor inducing $\text{IFN}\beta$), which activates the TBK1 and $\text{IKK}\epsilon$ kinases. A recent study reported the proteolytic cleavage of TRIF by the 3CD precursor.⁷³ In analogy with the specificity of 3ABC^{pro} -induced cleavage of MAVS, 3C^{pro} without the 3D domain is not capable of performing this cleavage. This suggests that the 3D^{pol} sequence is required to modify the substrate specificity without requiring the catalytic polymerase activity. In addition, proteolytic cleavage of TRIF cannot be performed by the 3ABC intermediate.⁷³

Cleavage of TRIF and MAVS has been reported for other picornaviruses as well, for example, coxsackievirus B3 (MAVS and TRIF)⁷⁴ and enterovirus 71 (TRIF).⁷⁵ Unlike for HAV, 3C^{pro} of both viruses appeared to be sufficient for proteolysis. Interestingly, both MAVS and TRIF are also cleaved by the HCV NS3/4A protease. Despite the fact that the HAV and HCV proteases differ in cleavage specificity and are phylogenetically unrelated, they target the

same pair of adaptor proteins, which may be considered a remarkable example of convergent evolution.⁷³

B. Coding Biases

A second immune evasion strategy employed by HAV can be found in its codon bias or nonuniform usage of codons during translation.⁷⁶ Due to the lack of mechanisms to induce cellular shut off, HAV has to compete with host mRNA for translation (as described above). Within this context, HAV has strategically adopted a codon bias toward rare codons, resulting in a highly deoptimized coding usage.^{77,78} This phenomenon contributes considerably to the low replication efficiency of HAV in several ways and seems to serve multiple purposes: first, the deoptimized codon use may reflect a strategy by which rare tRNAs are preferred over abundant tRNAs thereby avoiding competition with cellular tRNAs in the absence of an adequate host cell translation shut off.⁷⁷ Second, due to a decreased translation and replication rate, the virus is capable of keeping the cellular amount of dsRNA to a minimum, thus escaping the host cell antiviral response.⁷⁸ In addition, CpG-containing codons are particularly suppressed in the HAV coding sequence.^{79,80} This particular codon deoptimization is proposed to be associated with the recognition of unmethylated CpG by the innate immunity as a pathogen marker, contrary to the methylated CpG pairs of the host. Also in this way, HAV can evade the attention of the immune system. Third, the use of rare codons, usually grouped in clusters, is important for translation kinetics as such cluster may lead to a transient translation stop, a phenomenon known as ribosome stalling.⁸¹ It has been suggested that this transient stop may be an important prerequisite for proper folding of HAV structural proteins thereby contributing to the low antigenic variability and extreme environmental stability of HAV.^{77,78,82} Consequently, when HAV is adapted to replicate *in vitro* under circumstances of chemically induced cellular shut off, the resulting strains (with a “re-deoptimized” coding sequence) display reduced environmental stability.⁸³ Given the preference of HAV for reduced RNA replication and translation levels, it is now comprehensible why HAV would employ such inefficient type III IRES^{84,85}: a very efficient IRES would not be compatible with escaping innate immunity and with the many ribosome stalls during translation.⁸³

In conclusion, the coding biases found in the HAV genome may result from evolutionary pressures toward (i) environmental stability (and thus controlled translation kinetics to obtain a resilient viral particle) and (ii) evading the host immune system.⁷⁹ Additional insights into the mechanisms at action would prove very valuable with regards to our understanding of virus evolution and virus–host interplay.

C. Modulation of Regulatory T-Cell Activity

In addition to manipulation of the innate immunity, HAV also modifies the adaptive immunity through its receptor binding. HAVcr-1 is a phosphatidylserine (PtdSer) receptor and functions as a potent T-cell costimulatory molecule that regulates activation of and tolerance induction (thus suppressing autoimmune responses) in T cells.²⁴ A recent study reported that HAVcr-1 is constitutively expressed on regulatory T (Treg) cells.⁸⁶ The function of these specialized T cells is to limit the magnitude of the immune response to diverse pathogens in such way avoiding a hyperactive immune response and subsequent collateral (tissue) damage.⁸⁷ Several microorganisms activate Treg in order to limit inflammation and tissue injury, as is the case for chronic HBV and HCV infections.⁸⁷ Conversely, binding of HAV to its receptor inhibits Treg cell functioning.⁸⁶ In this way, the host is overwhelmed by anti-self-responses since Treg cells normally suppress autoimmune reactions, allowing HAV to escape the attention of the immune system. In addition, HAV inhibits the production of transforming growth factor β by

Treg cells, thereby blocking T-effector-mediated anti-HAV responses. Complementary, HAV stimulates IL-22 production that limits immune-mediated liver damage.⁸⁶

Taken together, by inhibiting Treg function, HAV creates an environment that favors viral replication and establishment of infection. These findings may also provide an explanation why HAV may elicit autoimmune hepatitis and extrahepatic manifestations including autoimmune hemolytic anemia, even though these complications are rare.⁸⁸

4. A LINK BETWEEN HEPATITIS A AND ATOPY

The hygiene hypothesis states that the uprising of atopic disease, including asthma, allergic rhinitis, and atopic dermatitis in industrialized countries is due to the increased hygiene.⁸⁹ As HAV serostatus can be regarded as a hygiene marker, its correlation with atopy was studied. It has been reported by several groups that HAV infection is negatively correlated with hay fever, asthma, and other atopic diseases.^{89,90} However, other studies were unable to replicate such effect.^{91,92} In addition, McIntire et al. described that previous HAV infection may protect against atopy in individuals carrying a six amino acid insertion (designated 157insMTTTVP) in the gene encoding HAVcr-1 (which is present in 46–64% of the population).⁹³ However, more recent studies failed to fully replicate these results.^{94,95} In addition, 157insMTTTVP appears to increase the susceptibility to severe HAV infection.⁹⁶ A double mechanistic basis was suggested to (partially) explain this observation: (i) HAV binds more efficiently to the 157insMTTTVP-carrying HAVcr-1, resulting in a more effective receptor and (ii) natural killer T cells expressing this long form of HAVcr-1 were more cytolytic against HAV-infected hepatocytes.⁹⁶ Thus, the 157insMTTTVP may protect from atopy, but predispose to severe HAV infections.

In conclusion, a possible relationship between a previous HAV infection, protection from atopic disease and HAVcr-1 polymorphisms is still subject of debate, but represents an interesting area of research. If HAV truly has a protecting effect, several questions should be addressed: does infection need to occur during childhood? Has vaccination a similar effect? Can this relationship be used in finding a therapeutic target for atopy? Insights into this relationship may prove crucial in combating the rise of atopic disease in industrialized countries.

5. CELL CULTURE

Unlike for most picornaviruses, culturing wild-type (wt) HAV in cell culture has proven to be quite a challenge as these strains replicate only very marginally in vitro (e.g., reference 97). HAV can be adapted to growth in cell culture through serial passaging; a process that introduces cell culture adapting mutations stepwise into the viral genome. Such mutations have been found in the P2 region (especially in 2B and 2C)^{98,99} and in the 5'-UTR,^{100,101} but also in other parts of the genome.⁹⁸ Intriguingly, these cell culture adapting mutations result in HAV strains that are highly attenuated in vivo (e.g., references 102, 103). A major advance in culturing HAV was the selection of a Huh7 cell line (designated Huh7-A-I) that allows genetically stable growth of wt HAV without the accumulation of cell culture adapting mutations.¹⁰⁴

Although wt HAV does not induce a cytopathic effect (CPE) in cell culture, cytopathic strains may arise during prolonged serial passaging.^{105,106} CPE appears to be cell type specific and is mostly found in monkey kidney cell lines like FRhK-4 and BS-C-1. These selected virus strains induce apoptosis through ribosomal RNA degradation by RNase L^{107,108} and consequent caspase activation.¹⁰⁹ Interestingly, it seems possible to induce CPE with cell culture adapted noncytopathic strains under specific conditions of lower temperature (<34°C) and decreased cell density, both in FRhK-4 and human A549 cells.¹¹⁰ Similarly, we observed CPE

for the PA21 strain (genotype IIIa) in FRhK-4 cells under these conditions (unpublished results).

6. THE NEED FOR ANTIVIRALS?

Despite the availability of an efficient vaccine, an antiviral drug against HAV would be of great use. First, since HAV growth *in vitro* is rather limited, the production of vaccines remains a painstaking process, which in part explains the high cost.¹⁵ This cost is particularly a problem in relatively poor regions with improved hygiene where infections occur at later age and consequently are more severe.⁴ Second, antivirals could shorten the period of illness and decrease symptoms and associated economic costs in infected unvaccinated patients. Early treatment of these infected unvaccinated persons may also prevent severe cases of fulminant hepatitis. Third, an antiviral could be a useful tool in rapidly containing epidemics. Lastly, the potential emergence of vaccine-escape variants has been reported recently;¹⁸ an anti-HAV drug could therefore be instrumental in halting the spread of such virus strains. In conclusion, there is certainly a need for antiviral drugs for hepatitis A, given that they are safe, efficacious, and preferably cheap.

Research into HAV antiviral drug discovery was mostly performed during the late eighties and early nineties. Afterwards, research efforts and funding waned due to the introduction of vaccines,¹⁵ although some interesting work has been done on amantadine and 3C^{pro} inhibitors in the last decade. An overview of the most relevant inhibitors is presented here (also see Table I).

A. Interferon

In 1984, IFN β was already demonstrated to efficiently inhibit the replication of HAV in human embryo fibroblasts.¹¹¹ HAV-infected cells were completely cleared following five passages with 1000 IU/mL of IFN β . Later, another study reported an effective concentration 50 (EC₅₀, minimal concentration required to reduce virus replication by 50%) of 90 IU/mL and a selectivity index (SI = 50% cytotoxic concentration (CC₅₀)/EC₅₀) of more than 100 for IFN α -2a.¹¹² In addition to the *in vitro* activity, a case study describes the *in vivo* efficacy of IFN β in three patients with fulminant hepatitis A and one patient with severe hepatic failure due to HAV. Following a treatment with 3 million units per day, liver functioning ameliorated and all four patients survived.¹¹³ However, in addition to its high price and parenteral administration, the clinical use of IFN is associated with severe side effects hampering usage in less developed regions.

B. Amantadine

Amantadine was initially thought to inhibit HAV by increasing the intravesicular pH. However, its antiviral effect required an extended incubation when compared to NH₄Cl and other lipophilic amines (e.g., methylamine, dansylcadaverine).²⁹ In line with these findings, inhibition of HAV IRES-dependent translation was later reported as a mechanism of action (MOA).¹¹⁴ A follow-up study confirmed the moderate activity of amantadine in cell-based assays.¹¹⁵ In addition, an increased effect was observed when amantadine was combined with IFN α or IL-29 (IFN λ 1).^{115,116} However, in addition to a fairly limited effect *in vitro* (EC₅₀ of 58 μ M and a SI around 5¹¹⁷), pharmacokinetic studies demonstrated maximum plasma concentrations (C_{\max}) following a multiple dosage scheme well below the reported EC₅₀,¹¹⁸ limiting its use in a clinical context.

Table I. Overview of Reported Inhibitors of HAV with (Putative) Targets and Calculated Values for 50% Effective Concentrations (EC₅₀), 50% Cytotoxic Concentrations (CC₅₀), and Selectivity Indices (SI) When Provided

	Antiviral activity	Toxicity	SI	(Putative) target	Reference
IFN α -2a	EC ₅₀ : 90 IU/mL	CC ₅₀ : >10,000 IU/mL	>100	Induction of antiviral state	112
Amantadine	EC ₅₀ : 58 μ M	CC ₅₀ : 310 μ M	5.3	HAV IRES-mediated translation	117
Guanidine HCl	Variable inhibition between 0.1 and 3 mM	ND	ND	Protein 2C	99, 107, 119–121
Iota-carrageenan	EC ₅₀ : 2.5 μ g/mL	CC ₅₀ : >1000 μ g/mL	>400	Attachment	122
Atropine	84% reduction in viral titer at 1.7 mM	CC ₅₀ : >3.5 mM	ND	Attachment and/or uncoating	123
Glycyrrhizin	EC ₅₀ : 325 μ M	CC ₅₀ : 5 mM	15	Membrane penetration	117
4,6'-dichloroflavan	EC ₅₀ : 6 nM	CC ₅₀ : 45 μ M	7500	Entry and/or uncoating	127
4,6'-dichloroisoflavan	EC ₅₀ : 6 nM	CC ₅₀ : 90 μ M	15,000	Entry and/or uncoating	127
Ribavirin	EC ₅₀ : 94 μ M	CC ₅₀ : 430 μ M	4.6	Inosine 5'-monophosphate dehydrogenase	117
Pyrazofurin	EC ₅₀ : 0.62 μ M	CC ₅₀ : 28 μ M	45	Orotidine 5'-monophosphate decarboxylase	117
Protamine	97% reduction in viral titer at 100 μ g/mL	CC ₅₀ : 200 μ g/mL	ND	RNA polymerase	123
Ac-LAAQ'-fluoromethylketone	96% reduction in viral titer at 5 μ M	ND	ND	Protein 3C ^{pro}	143
Halopyridinyl ester	EC ₅₀ : 53 nM	ND	ND	Protein 3C ^{pro}	151

ND, not determined.

C. The Curious Case of Guanidine HCl

For guanidine HCl, a protein 2C-targeting enterovirus inhibitor, mixed results were reported. The earliest publication detected no inhibition at 1 and 2 mM,¹¹⁹ but other groups found moderate inhibition (around 50%) following treatment with 3 mM¹²⁰ or 2.5 mM.¹⁰⁷ Cho and colleagues reported reversible inhibition at 2 mM but also reported enhancement of replication at 0.1–1 mM.¹²¹ Conversely to these results, Yi et al. reported strong inhibition of viral replication at concentrations as low as 0.1 mM.⁹⁹ Several factors may explain the inconsistency of these data: (i) mutations in the different viral genomes studied, specifically in the 2C region; (ii) the cell type used in the antiviral assay; (iii) the multiplicity of infection (MOI), which was found to be an important determinant for the antiviral activity¹⁰⁷; and (iv) the detection method employed.

D. Miscellaneous Small Molecule Inhibitors of the HAV Replication Cycle

Only limited information is available on other compounds with anti-HAV activity. Early steps of the replication cycle can be blocked by iota-carrageenan, which probably functions as an inhibitor of virus attachment and receptor binding.¹²² Atropine, at 1.7 mM, proved to moderately reduce HAV replication by targeting the early steps of the HAV replication cycle as well (attachment and possibly uncoating).¹²³ Due to toxicity and limited antiviral activity, the

clinical potential of both compounds is low. For glycyrrhizin, an aqueous extract of the licorice root, an EC_{50} of $325\mu\text{M}$ and a SI of 15 were reported.^{117,124} Subsequent studies suggested inhibition of membrane penetration as the putative MOA.¹²⁴ In addition to its anti-HAV activity, glycyrrhizin proved also to be active against a relatively large panel of other viruses, including HBV, HCV, HSV, and HIV, with several proposed mechanisms of action, as reviewed by Fiore et al.¹²⁵ However, glycyrrhizin may cause pseudohyperaldosteronism due to its aldosterone-like effects. In addition, the compound is readily metabolized following oral and intravenous administration and the attained plasma concentrations are considerably lower than the reported EC_{50} ,¹²⁶ hampering clinical use for treatment of HAV-infected patients.

Probably the most potent small molecules reported thus far are 4',6-dichloroflavan and 4',6-dichloroisoflavan (EC_{50} values of 6 nM).¹²⁷ Inhibition of cell entry or viral uncoating were posited as possible mechanisms of action for the antiviral activity against HAV. These drugs were also found to be effective against rhinoviruses and poliovirus in vitro.¹²⁷ However, 4',6-dichloroflavan failed to protect volunteers from experimental rhinovirus infection when administered both orally or intranasally,^{128,129} raising doubts concerning its in vivo efficacy against HAV as well.

Other early stage inhibitors include chlorpromazine and chloroquine.³¹ The former is thought to block endocytosis of nonclathrin-coated vesicles, while the latter inhibits endosome acidification.³¹ Monensin, another endosome acidification blocker, yielded minor to extensive inhibition of HAV replication in different studies.^{30,31,62}

In addition to early stage inhibitors, several other HAV inhibitors were identified to target different steps of the intracellular replication process including RNA synthesis, translation, and capsid assembly. However, the information on their precise MOA is very limited. For instance, ribavirin was demonstrated to have a moderate effect at $100\mu\text{M}$,¹³⁰ showing an EC_{50} of $94\mu\text{M}$ and a selectivity index of around 4.¹¹⁷ Improved anti-HAV activity was reported for pyrazofurin, an orotidine 5'-monophosphate decarboxylase inhibitor, but further development was halted due to toxicity (interference with nucleotide metabolism).¹¹⁷ Protamine reduced HAV replication with 97% at $100\mu\text{g/mL}$ and was suggested to function as a RNA polymerase inhibitor.¹²³ In addition, inhibitors of oxidative phosphorylation and 2-deoxy-D-glucose, a glycolysis inhibitor, were identified as inhibitors of HAV replication by unknown mechanisms.³⁰ Other inhibitors include brefeldin A^{62,107} and cycloheximide,¹⁰⁷ inhibitors of ER-to-golgi transport and protein synthesis, respectively. A moderate anti-HAV activity was also reported for amphotericin B¹³¹ and methisoprinol,¹³² drugs used against fungal infections and as an immunomodulator in the treatment of subacute sclerosing panencephalitis, respectively. However, no data are available on the MOA.

In recent years, several new anti-HAV compounds were synthesized, but the antiviral activity remained rather limited.^{133–135} Other papers reported highly potent inhibitors of HAV replication, but did not report toxicity data, making it impossible to judge whether or not these are selective inhibitors.^{136–138}

E. Rational Design of 3C^{pro} Inhibitors

Considerable efforts have been undertaken to rationally design and develop inhibitors for HAV and human enterovirus 3C^{pro} and the related coronavirus 3C-like protease (3CL^{pro}).¹³⁹ These enzymes have a topology comparable to that of the chymotrypsin-like serine proteases, but are in fact cysteine proteases.¹³⁹ Consequently, the general research strategy is to introduce thiol-reactive groups into the catalytic site. Several of such protease inhibitors have been developed over the years, both modified peptides and nonpeptidic analogs. Despite the fact that the modified peptides are easier to design (by merely mimicking the natural protease substrate), nonpeptidic and peptidomimetic inactivators are preferred, since they can be modified in order

to increase stability and cellular and gastrointestinal uptake, properties that are difficult to attain for peptides. A similar approach has already been applied to rhino- and enterovirus 3C^{pro} and resulted in the molecule rupintrivir that was halted after unsuccessful phase II clinical trials.¹⁴⁰

The first HAV 3C^{pro} inhibitors developed were modified peptides based on the acetyl-Leu-Ala-Ala sequence, serving as competitors for the natural peptide substrate Leu-Arg-Thr.¹⁴¹ Based on these findings, peptidyl aldehyde and monofluoromethylketone (FMK) inhibitors were developed.^{142,143} Resolving the crystal structure of HAV 3C^{pro} in complex with a peptidyl FMK inhibitor indicated that the inhibitor covalently binds the catalytic cysteine.¹⁴⁴ One of these peptidyl FMK inhibitors was also tested *ex vivo* and was shown to reduce HAV replication by 96%.¹⁴³ However, cellular uptake appeared to be a major problem. In addition, several azaglutamine derivatives were found to be irreversible peptidic inhibitors.¹⁴⁵

On the other hand, several nonpeptidic antagonists were reported as well. Asymmetric azodicarboxamides were found to be irreversible inhibitors of HAV and HRV 3C^{pro}.¹⁴⁶ Serine and threonine β -lactones also inhibited HAV 3C^{pro}.^{147,148} Other inhibitors include monophenyl pseudoxazolones of glycine (IC₅₀ of 4–6 μ M)¹⁴⁹ and cathepsin K inhibitor-based ketoglutamines.¹⁵⁰ Lastly, halopyridinyl esters were reported as the most potent nonpeptide HAV 3C^{pro} inhibitors thus far with IC₅₀'s as low as 53 nM.¹⁵¹

Although recent publications in this field seem to focus increasingly on HRV 3C^{pro} and coronavirus 3CL^{pro},¹⁵² it would be wise not to neglect possible anti-HAV activity for potential future (off-label) use of a marketed HRV or coronavirus protease inhibitor for severe HAV infections.

F. RNA Interference

RNA interference (RNAi) has been suggested as a future treatment of severe HAV infections.¹⁵³ Small interfering RNA (siRNA) targeting nonstructural genes can effectively block replication.^{153,154} Inhibition was also reported when targeting domains IIIC and V of the HAV IRES.¹⁵⁵ Three consecutive applications of siRNA over 14 days resulted in a 3 log₁₀ infectivity titer reduction in persistently infected cells. However, resistance emerged fairly quickly through mutation of the target site, necessitating combination of different siRNAs.¹⁵⁴ Once clinical RNAi therapy would be an established treatment for other (infectious) diseases, it would be interesting to further explore this for hepatitis A. However, such clinical applications of RNAi are currently still at the experimental stage.

G. Novel Antiviral Assays

We recently reported the development of three assays to screen for and identify small molecule inhibitors of HAV replication.¹⁵⁶ A CPE reduction assay based on the cell culture adapted HM175/18f strain (genotype IB) is amendable to high-throughput screening. RT-qPCR-based virus yield assays for HM175/18f and genotype IIIA strain PA21 were developed as well, allowing for confirmation and further characterization of the antiviral activity of selected molecules. The known inhibitors IFN α and amantadine were used to validate these assays. Using these assays, three enterovirus inhibitors with different targets were evaluated for anti-HAV activity. Pleconaril, a known capsid binder,¹⁴⁰ yielded (not unexpectedly) a very limited activity; inhibition by rupintrivir, a 3C^{pro} inhibitor, proved to be strain-dependent¹⁵⁶; and enviroxime, a direct inhibitor of phosphatidylinositol-4-kinase III β (PI4KIII β) that induces resistance mutations in enterovirus protein 3A,¹⁵⁷ was inactive in all three systems. Recently, PI4KIII β has been identified as an essential host factor in the replication strategy of enteroviruses. Following recruitment by enterovirus protein 3A to ER-derived organelle membranes, a phosphatidylinositol-4-phosphate-rich environment is created, promoting viral RNA replication. The observation that

HAV protein 3A is targeted toward the outer mitochondrial membrane instead of the ER,⁵⁰ together with the observed lack of activity for enviroxime in two different cell lines,¹⁵⁶ suggests that PI4KIII β kinase activity does not play a role in HAV replication.

H. Small Animal Models

A small animal model would be an additional prerequisite for the development and validation of anti-HAV compounds. The host range of HAV is restricted to humans and several nonhuman primates, for example, tamarins and chimpanzees.¹⁵⁸ Some efforts have been undertaken toward the development of small animal models, for example, virus adaptation to mouse cell lines or infection of guinea pigs.^{159,160} However, so far no robust model for clinical hepatitis A is available. Very recently, it has been found that the chimeric SCID/Alb-uPA mouse model, which supports the engraftment and proliferation of transplanted human hepatocytes, is susceptible to HAV infection (Pang D., personal communication). Despite the fact that these results still need to be published, they could represent a major leap forward for anti-HAV research.

7. CONCLUSION

Despite the fact that hepatitis A is a vaccine-preventable disease and infections are usually mild, continued attention for this pathogen is warranted. Outbreaks and occasional cases of fatal fulminant hepatitis A still occur and as the age of infection shifts upwards, the severity of the infection increases as well. Morbidity and mortality may also increase in patients with chronic liver disease, for example, due to HBV or HCV. Additionally, a recent report warned for the potential emergence of vaccine-escape variants.¹⁸ Taken together, these reasons warrant continuation and expansion of the current vaccination programs, but also the development of antiviral drugs against HAV, even though this may not be particularly interesting from a commercial point of view. So far no potent or selective inhibitors have been reported. We therefore propose to focus antiviral efforts on marketed drugs and antivirals that are currently in clinical development. Off-label use of such drug would be a much appreciated therapy. In fact, we are currently employing our CPE reduction assay for screening of a library of FDA-approved/marketed drugs and with promising results so far. Also on the fundamental biology level, HAV remains a largely understudied virus, despite many interesting features. Further investigation could provide unique insights into pathogenesis, tissue tropism determinants, and replication strategies for HAV and picornaviruses in general.

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