

Inhibitor-Based Therapeutics for Treatment of Viral Hepatitis

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Abstract

Viral hepatitis remains a significant worldwide threat, in spite of the availability of several successful therapeutic and vaccination strategies. Complications associated with acute and chronic infections, such as liver failure, cirrhosis and hepatocellular carcinoma, are the cause of considerable morbidity and mortality. Given the significant burden on the healthcare system caused by viral hepatitis, it is essential that novel, more effective therapeutics be developed. The present review attempts to summarize the current treatments against viral hepatitis, and provides an outline for upcoming, promising new therapeutics. Development of novel therapeutics requires an understanding of the viral life cycles and viral effectors in molecular detail. As such, this review also discusses virally-encoded effectors, found to be essential for virus survival and replication in the host milieu, which may be utilized as potential candidates for development of alternative therapies in the future.

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Introduction

Hepatitis is a medical condition wherein the liver undergoes inflammation due to a plethora of reasons, including drug abuse, excessive alcohol abuse, disease conditions etc.¹

Keywords: Viral hepatitis; Viroporin; Inhibitor.

Abbreviations: CMV, Cytomegalovirus; EBV, Epstein-Barr virus; HCC, Hepatocellular carcinoma; FHF, Fulminant hepatic failure; HBV, Hepatitis B virus; HAV, Hepatitis A virus; HCV, Hepatitis C virus; HEV, Hepatitis E virus; HDV, Hepatitis D virus; HAVcr-1/TIM-1, Hepatitis A virus cellular receptor 1/T-cell immunoglobulin and mucin domain 1; IRES, Internal ribosome entry site; 5'-UTR, 5'-Untranslated region; L-SIGN, Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin; DC-SIGN, Dendritic cell-specific intercellular adhesion molecule-3; GAG, glycosaminoglycan; HSC70, Heat shock cognate 70 protein; HSPG, Heparan sulfate proteoglycans; Grp78, Glucose-regulated protein 78; HSP90, Heat shock protein 90; ORF1, Open reading frame 1; Ntcp, sodium taurocholate cotransporting polypeptide; cccDNA, covalently closed circular DNA; PK, Protein kinase; NA, Nucleos(t)ide analog; IFN, Interferon; L-HBsAg, Large hepatitis B surface antigen; HDag, Hepatitis D antigen; PegIFN- α , Pegylated IFN- α ; SVR, Sustained virological response; HBeAg, Hepatitis B e antigen; HBIG, Hepatitis B immunoglobulin; CHB, Chronic hepatitis B; DAAs, Direct-acting antivirals; ZFNs, Zinc finger nucleases; TALENs, Transcription activator-like effector nucleases; CRISPR, Clustered regulatory interspaced short palindromic repeats; RNAi, RNA interference; HMA, Hexamethylene amilorides; NN-DNJ, N-nonyl deoxyjirimycin.

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When such inflammation, as manifested in symptoms such as jaundice, nausea, abdominal pain, malaise etc, is caused by viral infections, the condition is referred to as viral hepatitis.¹ Five hepatotropic viruses – named hepatitis A, B, C, D and E viruses – target liver cells in humans and cause acute and chronic hepatitis. In addition, other viruses such as the adenovirus, cytomegalovirus (CMV) and Epstein-Barr virus (EBV), occasionally cause symptoms of hepatitis.²

While an acute infection in healthy, immunocompetent individuals is cleared spontaneously, complications like cirrhosis, hepatocellular carcinoma (HCC) and fulminant hepatic failure (FHF) may arise in immunocompromised individuals, due to associated secondary reasons such as existing infections, alcohol abuse, or genetic predisposition.^{1,3} HCC, the third leading cause of cancer-related deaths worldwide,⁴ is closely associated with hepatitis B virus (HBV) infections. Even though the therapeutic strategies devised till date are targeted towards chronic infections, treatment options become severely limited for advanced stage patients.¹ In addition, current medications have significant side-effects, which poses an issue with disease management. Hence, there is an urgent requirement for safer and more potent drugs.

This review will focus on the therapeutics currently available for treating viral hepatitis of all forms. In addition, the potential of new therapeutics and targeted inhibitor-based therapies against viral membrane-penetrating peptides and viroporins, a group of virally encoded proteins involved in facilitating replication and other specific steps in the viral life cycle, are also discussed.

Life cycle of hepatotropic viruses

Life cycles of the known hepatotropic viruses – particularly A, B, C and E – have been studied in significant detail. Although the lack of appropriate cell culture systems, and the slow-growing nature of the virus, has hampered studies, considerable information is available on the entry, replication and exit mechanisms of these viruses, and on specific host-interacting partners for each virus. Hepatitis A virus (HAV) is thought to associate with a cell-surface protein receptor, HAVcr-1/TIM-1 (Hepatitis A virus cellular receptor 1/T-cell immunoglobulin and mucin domain 1), a member of the immunoglobulin superfamily, by which it gains entry into host cells.⁵ Post-entry, translation of the positive-sense RNA genome by the host ribosomal machinery, mediated via the presence of an internal ribosome entry site (IRES) encoded in the 5'-untranslated region (UTR) of viral RNA, results in the production of a large viral polyprotein. The polyprotein is cleaved into structural (VP1-2A, VP2, VP3, VP4) and non-structural proteins (2B, 2C, 3A, 3B, 3C, 3D) as well as intermediates (3CD, 2BC etc) by a virally-encoded protease 3C^{pro}. Following the cleavage event, the viral RNA replicase

synthesizes a minus-sense strand corresponding to the viral RNA genome, which serves as a template for the production of multiple plus-sense strands. Meanwhile, structural proteins assemble to form virions, which package a majority of the plus-sense RNA generated during replication. Two specific cleavage events result in generation of mature infectious virions - the VP1-2A precursor cleavage and the VP4/VP2 junctional cleavage.⁶⁻¹¹ Virions are subsequently released from the apical membrane of infected hepatocytes.¹¹

Entry of hepatitis C virus (HCV) into target host cells is a multistep event, requiring several host components. The cellular receptors and surface molecules, which are thought to be involved in this process, include the C-type lectins liver/lymph node-specific ICAM-3-grabbing integrin (L-SIGN) and dendritic cell (DC)-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN), glycosaminoglycans (GAGs), claudins 6 and 9 and CD-81.¹²⁻¹⁴ Binding to cellular receptor(s) is primarily mediated by the viral envelope proteins E1 and E2. Post-entry, the virus nucleocapsid disassembles releasing the plus-sense RNA genome in the cell cytosol, which acts as a template for 5'-IRES mediated translation. The single HCV polyprotein generated during translation, like the corresponding HAV polypeptide, also undergoes protease mediated co- and post-processing to yield the entire repertoire of viral structural and non-structural proteins.¹⁴ During HCV infection, membranous webs¹⁴⁻¹⁶ are formed, which are sites for RNA replication (NS5B mediated) and translational post-processing events. While the endoplasmic reticulum (ER) serves as the site for the formation of the viral nucleocapsid, envelope formation and final virus maturation occurs in the Golgi. Matured virions are exocytosed into the extracellular space.¹⁴

The life cycle of hepatitis E virus (HEV) is not well understood, primarily due to the non-availability of an appropriate cell culture system. Studies have shown the possible involvement of heat shock cognate 70 protein (HSC70) and heparan sulfate proteoglycans (HSPGs) in the initial attachment of HEV to its target cells, while the final entry is mediated by a yet unknown receptor.^{17,18} Several host factors, such as glucose-regulated protein 78 (Grp78) and heat shock protein 90 (HSP90), are also thought to be involved in viral entry.^{19,20} Capsid uncoating and RNA genome release in the cytosol is closely followed by translation of the viral open reading frame (ORF) 1, which encodes the entire non-structural cassette.^{21,22} Multiple copy synthesis of the positive-sense RNA genome is mediated by virus-encoded RNA-dependent RNA polymerase via a negative-sense RNA intermediate. The viral ORF2 encodes the capsid protein, which associates with the progeny viral genomes to form the nucleocapsids, followed by the intracellular transport and release of mature virions.^{21,22} It has been postulated that the viral ORF3 encodes a protein involved in intracellular trafficking process.^{21,22}

HBV, unlike other hepatotropic viruses, has a distinct mechanism for propagation within its host. Cellular entry of incoming particles via endocytosis is initiated through interactions with HSPGs,^{23,24} followed by that with the sodium taurocholate cotransporting polypeptide (NTCP).^{24,25} After gaining access to the cytosol, the virus travels to the nuclear pore complex, where the DNA genome is released into the nucleus. The partially double-stranded DNA genome is subsequently converted into a covalently closed circular DNA (cccDNA) form which associates with histones and other nuclear proteins. Within the nucleus, transcription events lead to the production of mRNA, encoding for the reverse

transcriptase and the nucleocapsid (pregenomic). In addition, transcripts encoding the surface antigens (subgenomic) are also produced. Both pre- and subgenomic transcripts are transferred to the cytosol. Surface protein synthesis, to generate core and subviral particles, occurs in the ER. Translation products (viral polymerase and core protein) of the pregenomic RNA associate with protein kinases (PKs),^{26,27} HSPs^{27,28} and pregenomic RNA to yield mature core particles, which are either released from infected cells, or are recirculated to the nucleus to maintain the cccDNA level.^{25,28} Interestingly, while subviral particles are released via the secretory pathway involving the Golgi and ER, mature core particles are released through multivesicular particles.²⁵

Although not much is known regarding the cellular entry process of hepatitis D virus (HDV), studies have suggested similarities with the HBV entry pathway involving interaction of HSPGs and NTCPs with the viral large hepatitis B surface antigen (L-HBsAg; pre-S1 domain).^{24,29} Post-entry, transfer of the RNA genome to the nucleus is facilitated by the virally-encoded hepatitis D antigen (HDAG).³⁰ Within the nucleus, host RNA polymerases initiate genome replication, which proceeds via a rolling circle mechanism.³⁰ Subsequent transcriptional and translational events lead to the production of the large delta antigen which undergoes prenylation prior to association with progeny viral genomes. Subsequent assembly and viral release requires assistance from HBV.³⁰

Current treatment scenario

HAV

Currently there are no specific treatment options available for HAV. Infected individuals usually receive symptomatic treatment, with liver transplantation being the only viable option in cases of FHF.¹¹ Vaccination, with inactivated HAV virions, is fairly effective for conferring protection against all prevalent strains of the virus found in the human population.^{31,32} Vaccination with attenuated virions has also been employed, but has met with limited success.³³ In spite of active vaccination efforts, however, the number of clinical cases of HAV reported is quite high, being ~1.5 million worldwide.¹¹

HCV

Due to the mostly asymptomatic nature of acute HCV infection, treatment options are limited. However, studies have reported that interferon-based treatment at an early stage might be helpful.³⁴ Individuals exhibiting HCV RNA and antibodies at detectable levels in their blood after more than 6 months post-infection are considered to be cases of chronic HCV infection. A combination therapy of ribavirin and Peg-IFN- α (pegylated interferon α), for a duration of either six months or one year, was the accepted treatment regimen for chronic cases of HCV infection up until 2011.³⁵ However, achievement of a sustained virological response (SVR), which is the key to cure, was observed to vary between genotypes and populations.³⁶ Western European patients infected with HCV genotype 1 reportedly achieve a higher SVR (50%) than the North American patient population (40%). The SVR achieved with this treatment in case of patients infected with genotypes 2, 3, 5 and 6 was observed to be higher in general compared to genotype 1-infected patients (SVR of up to 80%). In particular, genotype 2 was the found to be

most susceptible (SVR > 80%).³⁶ Direct-acting antivirals (DAAs) such as telaprevir and boceprevir (first-wave, first generation), which target the HCV NS3-4A serine protease, were introduced in 2011 for treating infections caused by HCV genotype 1.³⁷ Phase III clinical trials on treatment-naïve patients found that administration of telaprevir and boceprevir in conjunction with ribavirin and Peg-IFN- α (triple-combination) was effective in achieving a SVR in the range between 65% and 75%.³⁷ However, this treatment regimen comes with its own set of disadvantages, including significant side-effects and not being cost-effective in the case of patients with advanced fibrosis.³⁷ Since 2011, the following DAAs have been approved which can be used either independently or in combination with Peg-IFN- α and/or ribavirin. These DAAs are (a) sofosbuvir (nucleotide analogue), (b) simeprevir (NS3-4A protease inhibitor), (c) daclatasvir (NS3-4A protease inhibitor), (d) paritaprevir (NS3-4A protease inhibitor), (e) ombitasvir (NS5A inhibitor), (f) asunaprevir (NS3-4A protease inhibitor), (g) grazoprevir (NS3-4A protease inhibitor) and (h) elbasvir (NS5A replication complex inhibitor).³⁵ The rate of SVR achieved depends on the combination used, the genotype, the presence of resistant strains and the disease severity. Finally, attempts to create an effective vaccine against HCV have not been successful till date due to the extreme sequence variation of the virus genome.

HEV

Acute HEV infections in immunocompetent individuals usually culminate in spontaneous clearance of the virus after a certain time period. However, in severe cases leading to decreased liver functioning, administration of ribavirin was found to help in prompt clearance of the virus and inhibition of further liver damage.³⁸ It remains unclear if ribavirin intake can arrest the progress towards complete liver failure (fulminant hepatitis). Till date, liver transplantation from appropriate donors remains the only choice for such patients.³⁹ For treatment of transplantation patients suffering from chronic infection with HEV, the following course of action is usually undertaken post-transplantation. The first step involves the reduction of immunosuppression, which has been shown to have a 25% clearance rate.⁴⁰ If this is unsuccessful, the next step usually involves monotherapy with ribavirin for an initial period of 90 days.^{39,41,42} Administration of Peg-IFN- α is also an option; however, due to its many side-effects and complications in solid organ transplantation recipient patients, it is not preferred.³⁹ HEV infection in pregnant women is associated with significant complications like stillbirths, abortions, premature distribution and maternal or fetal death.⁴³ Interestingly, significant differences exist in the severity of complications caused by HEV infection in diverse endemic populations. For instance, while the infection typically has severe outcomes in pregnant women from northern India, it follows a fairly benign course in populations from Egypt and western countries.^{44,45} Studies suggest that poor prenatal care and nutrition may constitute major contributory factors for such severe outcomes in selected populations.⁴⁶ Efforts are ongoing to develop an effective vaccine against HEV, and currently a promising candidate (trade name: Hecolin) is in Phase IV of clinical trials (clinicaltrials.gov database).

HBV

As with other forms of hepatitis, acute infections with HBV are treated symptomatically^{47,48} and no specific treatment regimen exists. However, patients who develop acute liver failure resulting from severe HBV infection are administered oral anti-HBV medication.⁴⁹ The major emphasis so far is on treatment of chronic infection of HBV. The currently approved drugs are interferons, Peg-IFN-2a for adults and IFN- α -2b for children, and nucleotide/nucleoside analogues or NAs (nucleoside analogues: lamivudine, entecavir, emtricitabine, telbivudine; nucleotide analogues: adefovir, tenofovir).^{50,51} Due to their high barrier to development of resistance, tenofovir and entecavir are used as the first-line drugs for treatment.^{50,51} Other analogues like lamivudine, adefovir and telbivudine are only prescribed when the more potent drugs are either not appropriate or not available.^{50,51} This is due to fact that these drugs either have a low barrier to resistance (telbivudine), are less efficacious (adefovir) or have been shown to develop resistance over long-term usage (lamivudine).⁵⁰ Interferons are utilized as they enhance the host antiviral immune response.^{50,51} The therapeutic strategy usually involves two specific routes applicable to chronic hepatitis B (CHB): (i) a short treatment (1 year) with Peg-IFNs or NAs and (ii) treatment with NAs on a long-term basis.

- i. Peg-IFN-based therapy is prescribed for a period of 48 weeks for patients who have the best possibility for HBe seroconversion (absence of HBeAg in serum and presence of anti-HBe antibodies) in order to obtain an off-treatment response.^{50,52} Combinations of Peg-IFN with NAs like lamivudine or telbivudine are not recommended because of a low off-treatment response for the former and due to recipients developing polyneuropathy for the latter.⁵³ HBeAg-positive patients who undergo serotype conversion to anti-HBe can be treated with NAs for some time. However, it is not possible to define exactly the timespan before treatment initiation since it is based on the time taken to undergo serotype conversion and treatment continuation post-seroconversion.⁵⁰ A stable off-treatment response in (40–80)% of treated individuals has been observed⁵⁴ when treatment is continued for about 12 months post serotype conversion to anti-HBe.
- ii. The second strategy applies to those individuals who do not undergo seroconversion and have HBV-related liver cirrhosis.⁵⁰ Entecavir and tenofovir are used, however, the long term effects of the use of these drugs is not known. Current data shows that monotherapy for 3 years or more ensures remission in majority of treated patients.^{55–57}

Patients undergoing liver transplantation due to HBV-related HCC or decompensated liver cirrhosis undergo a pre-transplantation treatment regime with a potent NA having a high resistance barrier in order to reduce the viral DNA levels to a minimum before transplantation.^{58,59} Post-treatment, a combination of adefovir and/or lamivudine with hepatitis B immunoglobulin (HBIG) has been found to be effective (graft rejection rate of less than 10%).^{59,60} Given the treatment scenario and the complications associated, prevention of HBV infections is a priority. An effective vaccine against HBV exists and is administered 3–4 times over a 6 month period.⁵⁸

HDV

Till date, there are no known effective therapeutic strategies available for treatment of either the acute or chronic forms of HDV infection.⁶¹ Studies have shown that long-term treatment with Peg-IFN α helps in clearance of the virus in about 25% of the cases.⁶¹ The efficacy is similar when interferon is administered in combination with adefovir.⁶¹ Thus, in the absence of an effective antiviral therapy, liver transplantation is the only option for patients suffering from liver damage due to chronic infection. There are no commercially available vaccines against HDV; however, the vaccine available for HBV can be used to prevent co-infection of HBV and HDV.⁶¹

Viral inhibitors and their mechanism of action

Based on the current knowledge of the molecular details of viral life cycles and virus-host interactions, there can be several targets for designing inhibitors. The following classes of inhibitors are discussed below: (i) viral polymerase inhibitors, (ii) viral protease inhibitors, (iii) CyPA inhibitors and (iv) host lipid modulators.

- i. Viral polymerases play integral roles in the virus life cycle by carrying out replication and transcription of the viral genome and are therefore targets for inhibitor therapy. Inhibitors in current use against viral polymerases can be classified as (a) NAs and (b) non-NA polymerase inhibitors. NAs are used in the treatment of several viral diseases and are one of the first class of inhibitors identified.⁶² Upon their uptake by cells, nucleoside analogues undergo a set of three phosphorylation events, producing nucleoside triphosphates that lack a 3'-OH group on the deoxyribose sugar. These inhibit the replication process by chain termination and stalling replication-associated polymerases. NAs have a similar mechanism of action, with the distinction that they essentially bypass the phosphorylation steps.⁶³ Several such drugs, like emtricitabine, lamivudine, tenofovir, entecavir, stavudine, abacavir etc, are available for treatment of human immunodeficiency virus (HIV). Several analogues are also being used in treatment of chronic HBV and HCV infections, as mentioned in the previous sections. Other effective inhibitors include favipiravir, which can successfully inhibit both negative- and positive-sense RNA viruses and has been approved for the treatment of influenza virus in Japan, and BCX4430, which shows similar inhibitory effects.⁶² These compounds have significant toxicity issues and side-effects due to the replication inhibition being primarily non-specific. In addition, resistant phenotypes are often encountered, which decreases treatment efficacy.⁶² (b) Non-NA polymerase inhibitors, on the other hand, are targeted to bind to sites other than the active site on viral polymerases, and induce conformational changes, leading to down-regulation in activity. In this class, HIV-1 reverse transcriptase inhibitors like nevirapine, delavirdine and efavirenz are well known. These primarily inhibit HIV-1 replication by binding to the p66 subunit of the p66/p51 heterodimeric complex.⁶⁴ Four different classes of non-NA polymerase inhibitors are currently undergoing clinical trials for treatment of HCV genotype 1: Thumb I (BI-207127), Thumb II (GS-9669, filibuvir and VX-222), Palm I (ABT-333, ABT-072 and setrobuvir) and Palm II (nesbuvir, tegobuvir and IDX-375).

The nomenclature is based on the structural organization of the HCV NSSB.⁶⁵

- ii. Proteases play an essential role in the life cycle of many viruses, making them essential targets for inhibitor design. The HIV protease cleaves the viral polyproteins into individual components, and inhibiting the protease can effectively block viral replication.⁶⁶ Currently, the United States' Food and Drug Administration has approved several inhibitor drugs which belong in this category, namely saquinavir, atazanavir, darunavir, fosamprenavir, indinavir, nelfinavir, lopinavir, ritonavir and tipranavir. While inhibitors against the NS3-4A serine protease of HCV, such as boceprevir and telaprevir, were used initially, their usage has been discontinued since a more effective treatment regimen of simeprevir, asunaprevir and paritaprevir are now available, as mentioned earlier herein. Several 3C or 3C like proteases exist in picornaviruses, coronaviruses and noroviruses.⁶² In fact, rupintrivir (an irreversible 3C protease inhibitor), which was originally designed to treat rhinovirus infections, also displays inhibitory activity against the above mentioned virus families.⁶⁷ Lastly, treatment regimen usually involves combination therapy due to chances of emergence of resistant phenotypes.⁶²
- iii. Cyclophilin A (CyPA) belongs to a ubiquitous, host-encoded protein family (peptidyl-prolyl cis/transisomerases), which helps in folding and trafficking of other host proteins.⁶⁸ CyPA was found to interact with the non-structural protein NS5A^{69,70} of HCV and the capsid protein p24 of HIV.⁷¹ Presently, two drugs that inhibit CyPA, namely alisporivir and SCY-635, are in use. Both drugs are derivatives of the original immunosuppressive drugs sanglifehrins A and cyclosporine A.⁶²
- iv. Since host lipid metabolism is essential for viral replication,⁷² compounds which specifically alter this process have been found to inhibit viral infections. Perhaps the most notable of this class of inhibitors are the statins, which block cholesterol biosynthesis by obstructing the activity of 3-hydroxy-3-methyl-glutaryl coenzyme A.⁶² However, although this class of inhibitors have shown positive results *in vitro* against several pathogens like HIV and HCV, significant contradictions were encountered during *in vivo* studies.⁶² The indole derivative arbidol interferes with virus membrane fusion and entry of enveloped viruses. Although it has been approved in China and Russia for treating respiratory pathogens like influenza virus, and *in vitro* data on HBV, Chikungunya and HCV appears encouraging, data related to the *in vivo* efficacy of this drug is lacking.⁶² LJ001 is another lipid modulator that induces membrane lipid oxidation and interferes with virus membrane fusion. It was found to be fairly effective against enveloped viruses such as influenza, filoviruses and HIV,⁷³ but its poor physiological stability and requirement of light for optimal activity makes it rather unsuitable for further development. Derivatives of LJ001 have since been produced with improved characteristics.⁶² Squalamines are compounds that interact with the host membrane and alter the cellular microenvironment to make it unsuitable for virus propagation. This compound has also been successful in phase II trials for retinal vasculopathies and cancer, warranting its further development.⁶²

Several new targets have been identified in HBV against which inhibitors have been designed, and many are presently at different stages of development or in clinical trials. These include potent polymerase inhibitors, entry inhibitors, cccDNA inhibitors, nucleocapsid assembly inhibitors, inhibitors of viral gene expression and HBsAg release inhibitors. Equally effective but safer derivatives of tenofovir and entecavir (tenofovir alafenamide and besofovir) have shown promising results in randomized phase III trials with respect to bone and renal safety profiles.⁷⁴⁻⁷⁷ Besofovir is another drug which is being tested as an alternative to entecavir.^{78,79} Myrcludex-B is an engineered lipopeptide that effectively blocks NTCP receptors, the entry points for incoming HBV virions.⁸⁰ Mouse model studies with Myrcludex-B have shown a reduction in both cccDNA levels and virus spread, and phase II clinical trials are currently ongoing.⁸⁰ Three types of site-specific DNA binding proteins are being engineered to target cccDNA: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR)-Cas endonucleases.⁸¹ *In vitro* studies in cells have shown that all three systems can deactivate cccDNA by either site-specific cleavage,⁸² by introducing frameshifts in viral ORFs⁸³ or by specific disruption (CRISPR-Cas).^{84,85} Disubstituted sulphonamides (CCC-0975 and CCC-0346) have also been identified; these have been shown to prevent the formation of cccDNA from relaxed circular DNA *in vitro*.⁸⁶ Compounds that prevent the encapsidation of viral pregenomic RNA, such as sulfamoylbenzamide and phenylpropanamide derivatives, also constitute attractive options for therapy.^{87,88} These are effective against both NA-resistant and wild-type HBV.⁸⁹ Inhibition of viral gene expression using RNA interference (RNAi) is a very attractive option since low levels of viral antigens will allow the immune system to recover. ARC-520, one such drug under development, has shown promising results *in vitro*.^{90,91} A nucleic acid polymer, REP 2055, has been shown to be able to inhibit the release of HBsAg from infected hepatocytes in *in vivo* studies.⁹² Finally, recent studies have also shown that the HBV RnaseH, which is critical for virus replication, can also be an important drug target.^{93,94} As in the case of HBV, novel drug targets have been identified in other hepatotropic viruses as well (Table 1).

Viroporins as promising inhibitor targets

The virus life cycle begins when it infects a suitable host in order to make multiple copies of itself by utilizing the host machinery. The process from infection to generation of progeny virions is executed as a series of very well coordinated steps and involves attachment, entry, uncoating,

replication-transcription-translation of genetic material, assembly and egress. The virus genome encodes a limited number of proteins, all of which can be categorized primarily into structural and non-structural proteins. While structural proteins generate the virus capsid, non-structural proteins are produced after infection and are usually not a part of the capsid.^{74,95} Recently, a set of viral proteins have been identified which play essential roles in the virus life cycle, primarily through their ability to interact with and penetrate cellular membranes. This class of multifunctional proteins, designated viroporins, form pores or channels in cellular membranes, which directly or indirectly facilitate the intracellular survival of the virus.⁹⁶ It has been suggested that the ability of viroporins to alter ionic concentration and remodel host cell membranes, enhances viral uncoating, replication and egress.⁹⁶ Usually viroporins are small peptides (up to 100 amino acids) and contain one or more transmembrane domain(s). Formation of pores or channels in cellular membranes is facilitated by homo-oligomerization between viroporins. Oligomerization levels can range from dimeric association (e.g. in HAV 2B)⁹⁷ to large heptameric assemblies (e.g. in HCV p7).⁹⁶ Till date, several viroporins have been identified and functionally characterized for both enveloped and non-enveloped viruses (Fig. 1, Table 2). Some of the well-known examples are influenza A virus M2, HIV-1 Vpu, picornavirus 2B and HCV p7.⁹⁶

The central role of viroporins in facilitating several steps in the life cycle of viruses suggests that this class of viral proteins are promising candidates for development of inhibitor-based therapeutics. In fact, small molecule inhibitors have been successfully designed and tested against certain viroporins with encouraging outcomes; drugs such as amantadine and rimantadine have been shown to be effective in inhibiting the ion channel activity of M2.^{98,99} Interestingly, several influenza strains, with specific point mutations (Leu26Phe, Ser31Asn, Val27Ala, Ala30Thr, Leu28Phe Ser31Asn and Gly34Glu) which cause resistance to M2 inhibitors have been identified recently.⁷⁹ These mutations most likely affect the drug binding sites of the M2 channel by altering binding pocket hydrophobicity or the overall conformation of the channel. The most prevalent point mutation is Ser31Asn, which has been identified in the H1N1, H7N9 and H1N5 strains of the virus. Attempts are currently being made to synthesize inhibitors against the resistant strains using derivatives of M2 inhibitors. Although novel effective derivatives have been obtained against several of the mutations, those against point mutations at Asn31 have proven to be difficult to generate.⁹⁸

Hexamethylene amilorides (HMA), and its derivatives (e.g. BIT225), have been found to be effective in blocking Vpu

Table 1. List of potential inhibitors against viral proteins currently under investigation

Virus	Inhibitor	Target	Reference
HCV	Hydroxyanthraquinone	NS3 helicase	Furuta <i>et al</i> , 2015 ¹⁰⁰
HBV	Hydroxlate tropolones	Ribonuclease H	Hu <i>et al</i> , 2013 ⁹³ Lu <i>et al</i> , 2014 ⁹⁴
HAV	Niranthin	Not known	Liu <i>et al</i> , 2014 ¹⁰²
	AZD1480	JAK2	Jiang <i>et al</i> , 2015 ¹⁰³
	Sirtinol	Sirtuin	Kanda <i>et al</i> , 2015 ¹⁰⁴
HDV	Lonafarnib	Viral prenylation	Koh <i>et al</i> , 2015 ¹⁰⁵

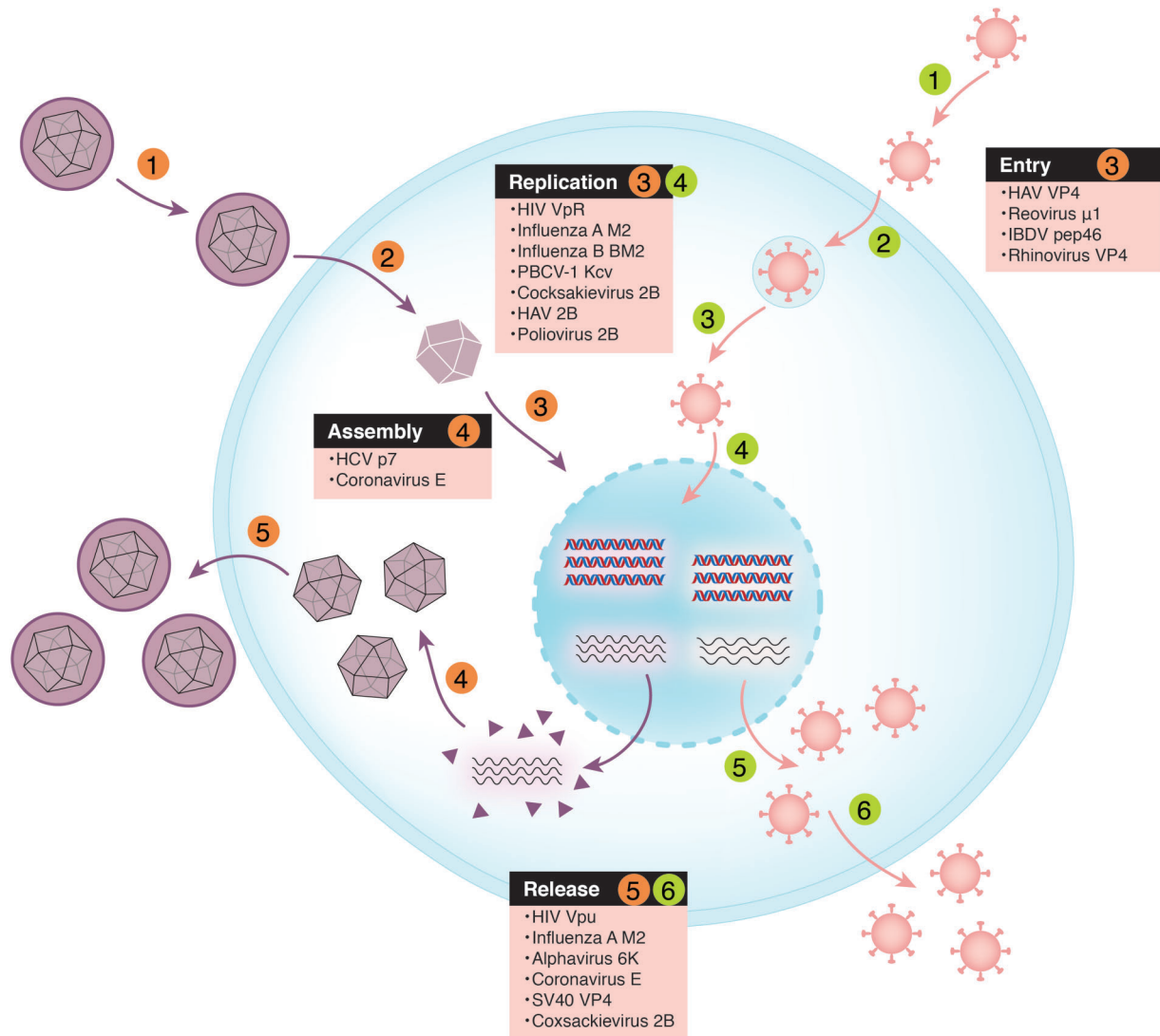


Fig. 1. General schematic showing various steps in the life cycle of enveloped and non-enveloped viruses and the involvement of viroporins or viral membrane penetrating peptides. In the case of enveloped viruses, steps 1, 2, 3, 4 and 5 represent attachment, entry, uncoating and replication, assembly and egress, respectively. For non-enveloped viruses, steps 1, 2, 3, 4, 5 and 6 represent attachment, entry, release from the intracellular vesicle, uncoating and replication, assembly and egress processes.

channel activity and is thought to bind in the channel lumen. The BIT225 HMA derivative is currently undergoing clinical trials.⁹⁸ This inhibitor is specific to HIV-1 and till date no resistant strains have been reported.

Coronavirus-encoded E peptides are also effectively blocked by HMA, as has been observed in electrophysiology studies using HEK-293T cells.⁹⁸ Amantadine is also capable of inhibiting activity, but the effect requires significantly higher concentrations.⁹⁸

Adamantanes, alkyl imino-sugars and HMA were identified as HCV p7 blockers based on protein based *in vitro* assays.^{98,100} However, studies reported inconsistency in efficacy data, which was preliminarily due to the genotypic/subtypic differences between the p7 channels, even though compounds like rimatadine and N-nonyl deoxyjirimycin (NN-DNJ) exhibited wide-spectrum activity amongst genotypes.⁹⁸ Availability of high resolution structural data and *in*

silico docking studies helped in unravelling the mechanism of action of these inhibitors.⁸⁰ NN-DNJ was found to interfere with p7 oligomerization via interaction with a specific phenylalanine residue at the 25th position. Interestingly, the inhibitory activity of adamantane was found to be due to binding of the inhibitor at a peripheral site (Leu20) that is exposed on the membrane, separate from the NN-DNJ binding site.⁹⁸ The disparate binding sites for inhibitors provides the possibility of using a combination therapy approach to treat resistant strains. Current drug development efforts typically use a combination of rational and high-throughput approaches. This has resulted in identification of compounds against resistant p7 phenotypes with higher potency at minimal concentrations.⁹⁸

Current interest and the pool of available data leads to the conclusion that viroporin-based therapeutic approaches will subsequently play an important role in treating clinically

Table 2. List of reported viral membrane penetrating peptides and their role in the virus life cycle

Name	Length (residue length)	Virus	Stage of life cycle
Vpu	77–86	HIV-1	Egress ¹⁰⁶
Vpr	96	HIV-1	Replication ¹¹⁶
M2	97	Influenza A	Replication and egress ¹⁰⁸
BM2	115	Influenza B	Replication ⁹⁵
E	76–109	Coronavirus	Assembly and egress ¹⁰⁹
3a	274	Coronavirus	Egress ¹¹⁰
Kcv	94	PBCV-1	Replication ¹¹¹
VP4	23	HAV	Entry ¹⁰¹
VP4	125	SV40	Egress ¹¹²
2B	251	HAV	Replication ⁹⁷
2B	97–99	Coxsackievirus	Replication and egress ¹¹³
2B	97	Poliovirus	Replication ¹¹⁴
6K	58–61	Alphavirus	Egress ¹¹⁵
p7	63	HCV	Assembly ¹⁰⁷
μ1	29	Reovirus	Entry ¹¹⁷
Pep46	46	IBDV	Entry ¹¹⁸

relevant human pathogens. The approaches towards designing inhibitors for treatment of HCV and its resistant phenotypes can also be extended to the other hepatotropic viruses. In fact, recent work from our laboratory has characterized the membrane activity of capsid component VP4 and non-structural protein 2B from HAV.^{97,101} Utilizing a combination of biophysical studies, MD simulation and electron microscopy, it was shown that VP4 forms discreet pores of 5–9 nm in artificial membranes. Our data thus indicates that VP4 is a membrane penetrating peptide which may allow HAV to escape from endosomes and gain access to the cellular cytosol. The potentially critical role played by VP4 in HAV entry makes it an important target for design of inhibitors.

We further investigated the non-structural 2B protein of HAV, which was found to contain a stretch of 60 residues in its C-terminus, and which has viroporin-like activity.⁹⁷ A combination of crosslinking studies, biophysical assays and simulation was employed to determine that 2B probably forms dimers and generates ~3 nm pores in membranes. The resulting alteration of membrane permeability is thought to be essential for HAV replication. It is probable that inhibition of the membrane activity of 2B would negatively impact the viral replication process. Thus, a treatment regimen against HAV, which includes a combination therapy targeting VP4 and 2B, could be a very promising and effective approach.

Conclusion and future perspectives

Given the essential roles played by viroporins and viral membrane penetrating peptides in enhancing several aspects of the virus life cycle, targeted inhibitor therapy holds promise for development of virus-specific drugs. Establishing the commonalities between the structures and functions of these membrane active components might make it easier to design small molecule inhibitors. Finally, given the promise of p7-based therapy against HCV, it is essential to pinpoint such potential candidates in other hepatotropic

viruses, in order to expand therapy options against chronic and acute viral hepatitis.

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Conflict of interest

None

Author contributions

Wrote the manuscript (DD, MB).

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