RESEARCH ARTICLE

Peptide Inhibitors of Viral Membrane Fusion

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Abstract

Lipid-enveloped viruses, including HIV-1 and SARS-CoV-2, infect their host cells by fusion either directly with the plasma membrane or with the endosome membrane following endocytosis. Biophysical insights into the conformational changes of viral fusion proteins have led to the development of peptide inhibitors of these changes, and hence of membrane fusion. The peptide T-20 (Enfuvirtide) inhibits HIV-1-cell fusion and is being used clinically. The cholesterol-conjugated C34 peptide has an IC_{50} of 4 pM for inhibiting HIV-1 infectivity, much lower than that of plain C34 and T-20. A peptide (P155-185-chol) corresponding to a segment of the post-fusion structure of influenza virus hemagglutinin, also coupled to cholesterol, inhibits infection by the A/H3N2 subtype with an IC_{50} of 0.4 μM. Myrcludex B, a myristoylated lipopeptide, inhibits the entry of hepatitis B virus and hepatitis D virus into hepatocytes with an IC_{50} of 80 pM. Dimer peptides (HRC2 and HRX4) derived from the measles virus F-protein and coupled to cholesterol inhibit measles virus infection at IC_{50} values of less than 1 nM to 2 nM. Peptides derived from the E protein of Japanese encephalitis virus inhibit infection at nanomolar IC_{50} values.

The COVID-19 pandemic has prompted numerous studies to design peptide inhibitors of the SARS-CoV-2 spike protein-mediated membrane fusion. The coupling of a lipidic anchor like cholesterol to some of these peptides enhances the antiviral effect of the peptides, lowering the IC_{50} to low nanomolar concentrations. It is highly likely that peptides against SARS-CoV-2 will soon be evaluated in clinical trials.

Infectious Entry of Lipid Enveloped Viruses by Membrane Fusion

Following the development and approval of the anti-HIV-1 peptide, Enfuvirtide, which inhibits the fusion of the virus with target cell membranes, and hence infection,[1] the COVID-19 pandemic has prompted much interest in the design of peptide inhibitors of the SARS-CoV-2 spike protein-mediated membrane fusion.[2] Lipid-enveloped viruses, including HIV-1 and SARS-CoV-2, infect their host cells by fusion either directly with the plasma membrane or with the endosome membrane following endocytosis. This step follows the initial binding of one of the viral envelope proteins to a receptor on the host cell membrane, which is one of the determinants of viral tropism. The receptor-binding protein may be the same protein that induces membrane fusion, as in the case of the influenza virus hemagglutinin (HA), which binds to sialic acid receptors on glycoproteins or glycolipids. In paramyxoviruses, such a measles virus and Newcastle disease virus, attachment proteins (termed HN, H or G) bind to sialic acid or protein receptors on host cells; this is followed by the interaction of the fusion protein, F, with the cell membrane.[3]

Proteins that mediate virus-cell fusion can be classified into Class I, Class II and Class III fusion proteins. Class I proteins are synthesized in host cells as precursor proteins that are subsequently activated by proteolytic cleavage, and form trimers in the viral membrane.[4] While mediating fusion, they fold into hairpin-like structures in which 2 alpha helices from each monomer interact with the dual alpha helices from the other 2 monomers that have also undergone the conformational change. This final structure is called the six-helix bundle, which appears to be an important intermediate stage in membrane fusion and is a target for antiviral peptides. Examples of Class I fusion proteins are the hemagglutinin, HA, of influenza virus, and the gp120/gp41 envelope protein of HIV-1.

Class II fusion proteins are formed via the proteolysis of a precursor that produces the E1 protein in alphaviruses and the E protein in flaviviruses.[5-7] Alphaviruses include the Western, Eastern, and Venezuelan equine encephalitis viruses, Semliki Forest virus and chikungunya virus. Their precursor polyprotein, p62-E1, forms dimers as it is incorporated into the budding viral particles; the p62 is then cleaved off to produce the E2 protein that seems to protect the E1 protein and bind to its receptor on the plasma membrane of the host cell.

Flaviviruses include yellow fever virus, Dengue virus, West Nile virus, tick-borne encephalitis virus and Zika virus. As immature virions, they have the precursor prM-E protein complexes as they bud into the endoplasmic reticulum. The prM protein is cleaved off of the E protein in the exocytotic pathway; the E protein is then expressed on the virus surface as a dimer. To initiate membrane fusion, these proteins change their oligomerization pattern, from an unstable dimer into a stable trimer that has transformed into a hairpin-like configuration. During this process, the fusion domain of the protein protrudes towards the endosome membrane, and the terminal hydrophobic fusion loop inserts into the membrane.

Class III glycoproteins are found in rhabdoviruses, such as rabies virus and vesicular stomatitis virus (the G protein), and herpesviruses (the gB protein), and do not require the cleavage of a precursor.[8-10] Similar to class II proteins, a stretched β-sheet fusion domain of class III proteins inserts hydrophobic fusion loops into the target membrane. In the case of the G protein,

the conformational change is triggered by the mildly acidic pH in endosomes.

Peptide Inhibitors of Viral Membrane Fusion

In perhaps the earliest observation of the inhibition of viral infection by a peptide, Richardson et al.[11] found that a peptide (carbobenzoxy-D-Phe-L-Phe-Gly) with an amino acid sequence similar to those at the N-termini of the paramyxovirus F1 or orthomyxovirus HA2 proteins inhibited the replication of these viruses. The peptide was shown to inhibit the fusion of Sendai virus with phospholipid vesicles.[12] Although this peptide inhibited syncytium formation between HIV-1-infected cells and CD4+ cells, its effect on HIV-1 infection was very limited.[13] A peptide corresponding to the heptad repeat of the Sendai virus fusion protein closest to the viral membrane inhibited virus fusion with erythrocytes.[14] A peptide corresponding to a potential alpha-helical region of the ectodomain of the HIV-1 transmembrane protein, gp41, was found to inhibit completely virus-induced cell-cell fusion.[1]

We review here the infection cycle of various lipid-enveloped viruses, with emphasis on the fusion between the viral and cellular membranes, and the peptide inhibitors of fusion that have been developed in the last three decades.

Peptide Inhibitors of Human Immunodeficiency Virus Type-1

Human immunodeficiency virus type-1 (HIV-1) belongs to the lentivirus subfamily of *Retroviridae*. It infects primarily CD4+ cells of the immune system, using this cell surface protein as the main receptor. This receptor, however, is not sufficient to mediate the fusion of the virus with the plasma membrane of the host cell. The chemokine receptors, CXCR4 and CCR5, have been identified as co-receptors that are necessary for viral entry (**Figure 1**). The Env protein of HIV-1 consists of two non-covalently interacting glycoproteins, the surface protein gp120 and the transmembrane protein gp41. Following the interaction of gp120 with CD4 and the co-receptor, the gp41 inserts its fusion peptide into the host cell membrane, initiating the fusion reaction.[15]

The Env protein is produced as a precursor (gp160) that is inserted into the membrane of the endoplasmic reticulum. It then oligomerizes, mostly into trimers, and is glycosylated by high-mannose oligosaccharides in the Golgi. Here, gp160 undergoes proteolysis into gp120 and gp41 by cellular furin, or furin-like proteases.[16] The gp120/gp41 trimer reaches the plasma membrane and is incorporated into the budding virions of HIV-1.

The tropism of different strains of HIV-1 is determined largely by the binding affinity between gp120 and the co-receptors. Macrophage-tropic viruses use the CCR5 co-receptor and are termed R5 viruses. Although these viruses do not infect CD4+ T cell lines, they may infect primary (i.e. patient- or donor-derived) CD4+ T cells. Virus strains that infect T cell lines and primary isolates utilize the CXCR4 co-receptor, and are designated as X4 viruses.[16]

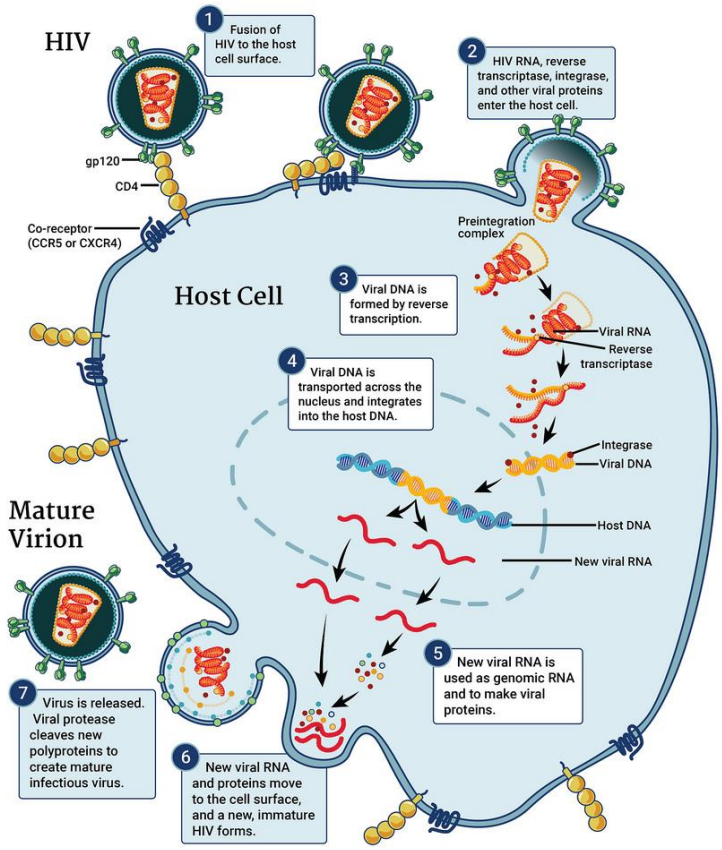

Figure 1. The HIV-1 life cycle
(Reproduced from https://www.niaid.nih.gov/sites/default/files/hivReplicationCycle2.jpg. Courtesy: National Institute of Allergy and Infectious Diseases)

Human infection is essentially caused by HIV-1 that uses CCR5. Person-to-person transmission is carried out primarily by such R5 viruses. During the late stage of infection, viral strains emerge that use CXCR4 for entry. Such X4 viruses, as well as dual-tropic R5X4 viruses, cause the rapid decline in CD4+ T cells, which translates into severe immunodeficiency.

Peptides derivatized by benzyl groups corresponding to residues 81–92 of CD4 inhibit HIV-1-induced cell-cell fusion and infection at micromolar concentrations,[17] indicating that the peptides can block the interaction of gp120 with the cellular receptor.

An early study in our laboratory showed that the hydrophobic tripeptide, Z-D-Phe-L-Phe-Gly, inhibits HIV-1-induced cell-cell fusion in several cell lines in a dose-dependent manner.[13] However, the effect of the peptide on viral infectivity was very limited, suggesting that the mechanisms of gp120/gp41-mediated membrane fusion may be different in virus-cell and cell-cell fusion. A synthetic peptide corresponding to the CD4 complementarity determining region 2 (CDR-2)-like domain binds specifically to HIV-1-infected cells.[18] When this peptide is coupled covalently to liposomes, it enables the binding of the liposomes to HIV-1-infected cells. A different peptide derived from the CD4 CDR-3-like domain inhibits

HIV-induced syncytia formation at micromolar concentrations.[18]

A series of peptides were synthesized corresponding to a region of gp41 that was predicted to form an alpha helix. The peptide designated DP-178 inhibited HIV-1-induced cell-cell fusion at an IC_{50} of 100 pM,[1] most likely via its interaction with a distal site of gp41 (i.e. a region further away from the viral membrane, towards the N-terminus) (**Figure 2**). This inhibition occurs after HIV-1 Env interacts with its receptors, suggesting that such peptides corresponding to the C-terminal heptad repeat region (CHR) bind to the N-terminal heptad repeat region (NHR) in an intermediate conformation of gp41.[19] There are some observations, however, that complicate this interpretation. For example, C-peptide-mediated inhibition cannot be blocked by N-peptides, which should theoretically block the binding the CHR peptide to the NHR domain.[20] Furthermore, complexes of C-peptides and N-peptides also have inhibitory activity, and the C-peptides also interact with the fusion peptide region of gp41.

Intravenous administration of DP178, later termed T-20, as the sole antiviral for 14 days to HIV-patients resulted in a dose-dependent, almost 2 $\log_{10}$ reduction in plasma HIV levels.[21] T-20 was approved by the U.S. Food and Drug Administration and the European Medicines Agency in 2003.

The specificity of viral strains for a particular co-receptor is defined by the gp120 V3 loop, which also dictates sensitivity to T-20, together with the N-terminal heptad repeat region of gp41.[22] Another peptide that includes part of the T-20 amino acid sequence, but is shifted towards the N terminus of gp41 by 10 amino acids, is T-649.[23] The sensitivity of HIV-1 to this peptide is via regions of gp41 different from those for T-20. The co- receptor specificity of the V3 loop in different strains appears to dictate the conformational changes of gp41, which in turn determines the sensitivity of that strain to both T-20 and T-649.

After the emergence of severe acute respiratory syndrome (SARS) in 2002-2003, caused by SARS-associated coronavirus (SARS-CoV), sequence analysis of the S2 spike glycoprotein showed that it has some sequence motifs that are similar to that of gp41. These motifs include the N-terminal leucine/isoleucine zipper and the C-terminal heptad repeat (the region similar to T-20), suggesting that T-20-like peptides could be utilized to treat SARS-CoV infections.[24] Such peptides have indeed been developed for SARS-CoV-2 which emerged in 2019-2020 (*vide infra*).

The half-life of T-20 in plasma is about 1.1 h, which is rather short. Since the conjugation of poly(ethylene glycol) (PEG) to proteins can increase their half-life, Wang et al.[25] examined the effect of PEGylation of the peptide C34, which corresponds to a region of the C-terminal heptad shifted towards the N-terminus from the T-20 region, on plasma survival. The plasma half-life of the peptides with 2 kD PEG was 2.6 h, and that of the peptide with 5 kD PEG was 5.1 h.[25] These peptides inhibited cell-cell fusion mediated by HIV-1 Env with a 50% effective concentration of 36 nM, indicating that PEGylation increased slightly the IC_{50} compared to C34 alone. However, this concentration was lower than that of T-20 in this experimental system.

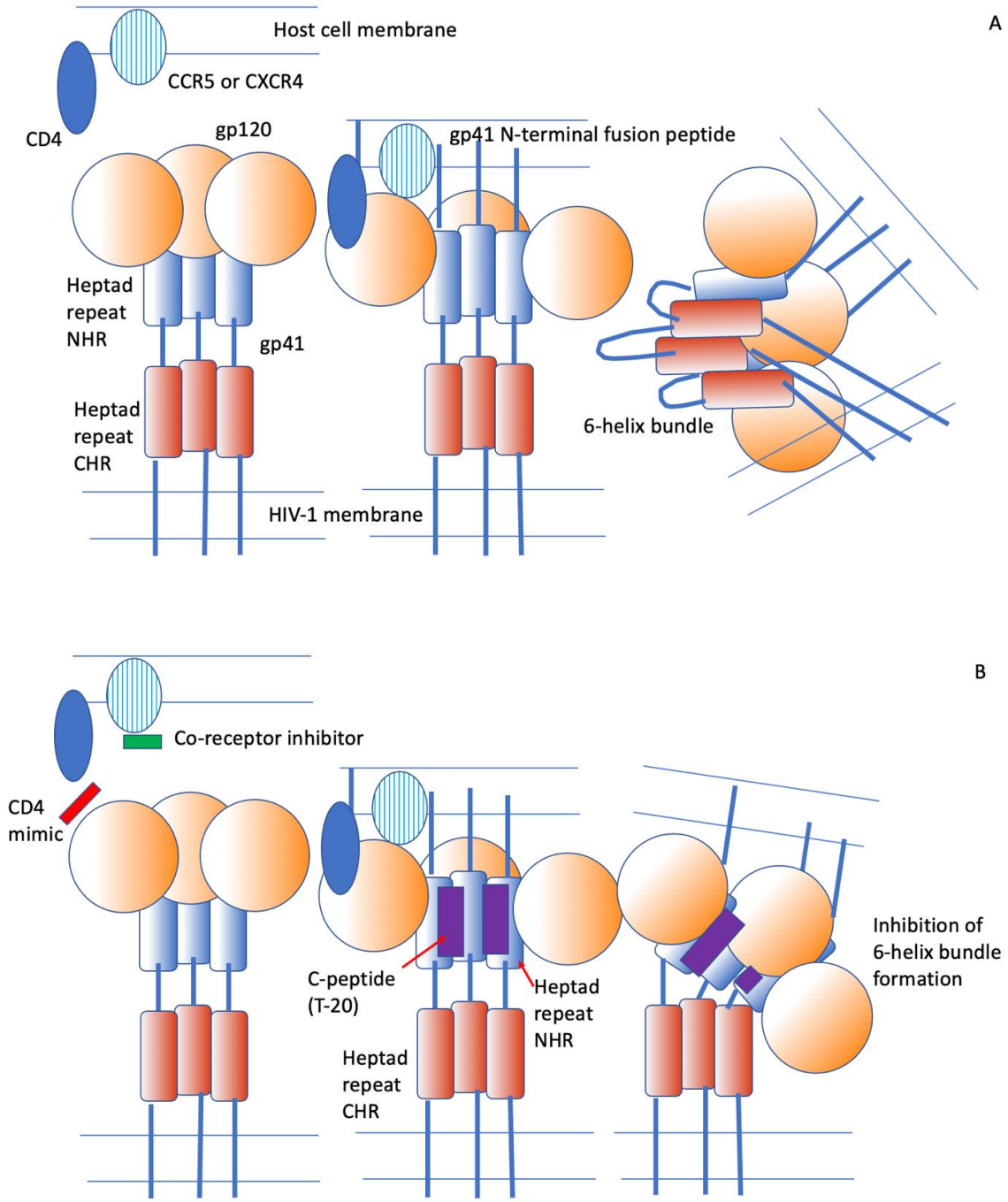

Figure 2. HIV-1 Env-mediated membrane fusion. **A.** Binding of gp120 to the CD4 receptor and the co-receptor (a chemokine receptor) and the subsequent conformational changes, leading to the insertion of the gp41 fusion peptide into the host cell membrane, and the formation of the 6-helix bundle comprising the three C-terminal heptad repeat domains and the three N-terminal heptad repeat domains. This conformational change brings the viral and cellular membranes in close proximity. The hydrophobic forces between the membrane-inserted fusion peptides and the membrane anchors of the gp41 is thought to cause membrane fusion. **B.** Inhibition of membrane fusion by various peptides. Red: An inhibitor of gp120-CD4 binding. Green: An inhibitor of gp120 binding to the co-receptor. Purple: A peptide derived from the C-terminal heptad repeat domain of gp41 (CHR) that interacts with the N-terminal heptad repeat (NHR), thereby preventing the normal interaction between the two HR regions.

The C34 peptide competes with the C-terminal heptad repeat region of gp41, thereby inhibiting the formation of the 6-helix bundle that normally incorporates both the N-terminal and C-terminal heptad repeat regions, and thus inhibiting membrane fusion between viral and target cell membranes.[26] Attachment of cholesterol to C34 appears to facilitate the partition of the lipidic peptide in the cell membrane, thereby concentrating it at the site where membrane fusion occurs. This modification results in an IC_{50} value of 4 pM, a remakably low concentration to inhibit HIV-1 infectivity,[27] particularly when compared to that of plain C34 (205 pM) and T-20 (692 pM). The IC_{90}s against different isolates of HIV-1 range from 0.4 nM to 1.8 nM. The cholesterol derivative also has an extended half-life following subcutaneous administration, with a plasma concentration more than 300-fold greater than the IC_{90}. However, the cholesterol-coupled T-20 has an IC_{50} of about 3726 pM [27] compared to 692 pM for the plain peptide, indicating that cholesterol coupling is not a universal method to enhance the inhibitory effects of peptides.

Peptide Inhibitors of Coronaviruses

The cell entry of coronaviruses is mediated by membrane fusion between the viral and cellular membranes. Recognition of the host receptors and the ensuing membrane fusion are mediated by the spike glycoprotein, S, a Class I fusion protein, which undergoes a significant conformational change after binding.[28,29] The S protein is highly glycosylated and assembles into trimers, forming the crown-like structures on the virus (**Figure 3**). The S protein can be functionally divided between the N-terminal S1 domain that has the receptor-binding domain (RBD) and the C-terminal S2 domain involved in membrane fusion (**Figure 4**). The severe acute respiratory syndrome coronavirus (SARS-CoV) can fuse with the plasma membrane but requires the presence of particular proteases that cleave the S protein into the S1 and S2 domains. The proteases of the trans-membrane protease/serine subfamily (TMPRSS) can cleave the S protein and facilitate the fusion of SARS-CoV with the cell membrane. Fusion at the plasma membrane appears to be 100–1000-fold more productive than entry via endocytosis and the endosomal pathway.[30]

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

SARS-CoV binds the angiotensin-converting enzyme 2 (ACE2) receptor on its host cells. Insights obtained from the mechanism of HIV-1 Env-protein-induced fusion involving the interaction of the heptad repeat regions (membrane-distal HR1 and membrane-proximal HR2) of the protein after binding to the cellular receptor, and the ability of various peptides, including T-20, to inhibit fusion inspired Liu et al.[32] to use a similar approach to identify peptide inhibitors of SARS-CoV fusion. They utilized peptides corresponding to these regions and studied their interaction by surface plasmon resonance and other biophysical techniques. A peptide (CP-1) derived from the HR2 region inhibits infection by the virus at micromolar concentrations and binds to a peptide derived from the HR1 domain, forming a six-helix bundle. Thus, during SARS-CoV fusion with the cell membrane, CP-1 may be binding to the HR1 region, thereby preventing the association between the HR2 and HR1 heptad repeat domains and thus preventing the fusion reaction. A study by Bosch et al.33 identified an HR2-derived peptide (sHR2-8) with an EC50 of 17 μM, based on SARS-CoV infection of Vero cells.

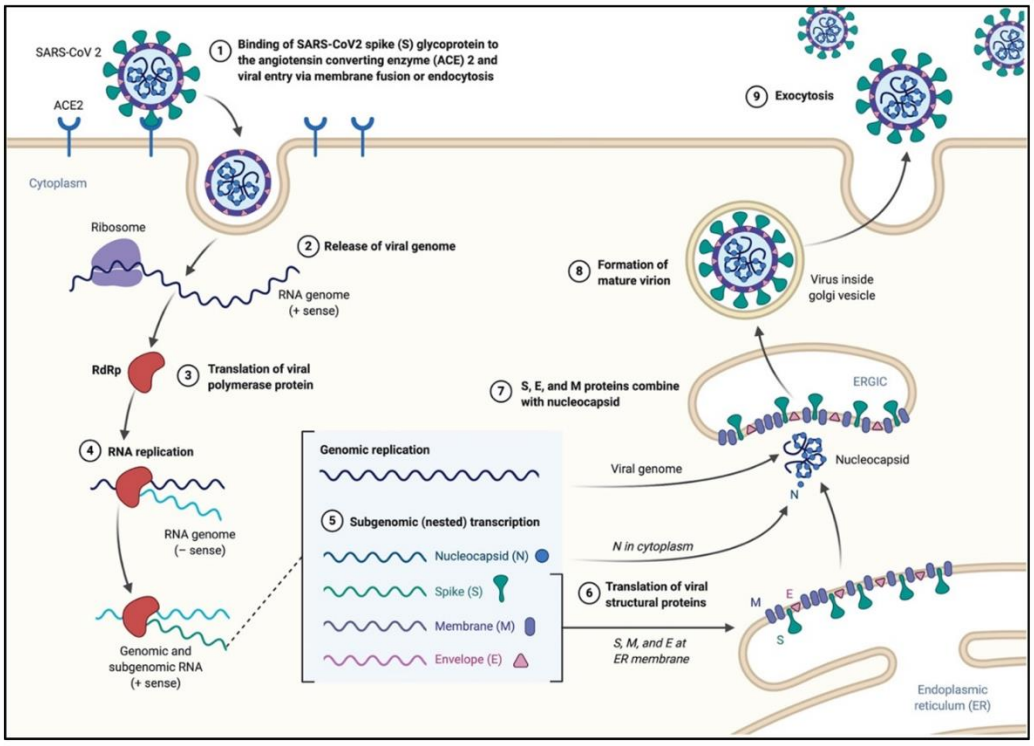

Figure 3. The life-cycle of SARS-CoV. The viral S protein binds the receptor ACE2, and may either fuse at the plasma membrane or with the endosome membrane after it is endocytosed. The viral RNA is translated into viral proteins and also transcribed via a negative-strand RNA back into positive-strand genomic RNA. The proteins and the RNA are assembled into virions that are transported to the plasma membrane inside vesicles (Reproduced with permission from Alanagreh et al.[31]).

Looking at the cytopathic effect caused by SARS-CoV in Vero cells, Zhu et al.[34] found that either a synthetic HR2 peptide or glutathione-S-transferase (GST)-fusion poly-peptides are inhibitory to viral infection. The EC_{50} for the HR2 peptide was 0.5–5 nM, a very impressive activity. Even for the GST-HR2, this range was 66–500 nM. However, no inhibitory effect was observed for HR1-derived peptides. Yuan et al.[35] used a pseudotyped virus, designated HIV-luc/SARS, to screen a series of peptides derived from HR1 and HR2. The EC_{50} for the HR1-1 peptide was 0.14 μM and that for HR2-18 was 1.19 μM. Wild-type SARS-CoV was also used to ascertain that the peptides were indeed inhibitory.

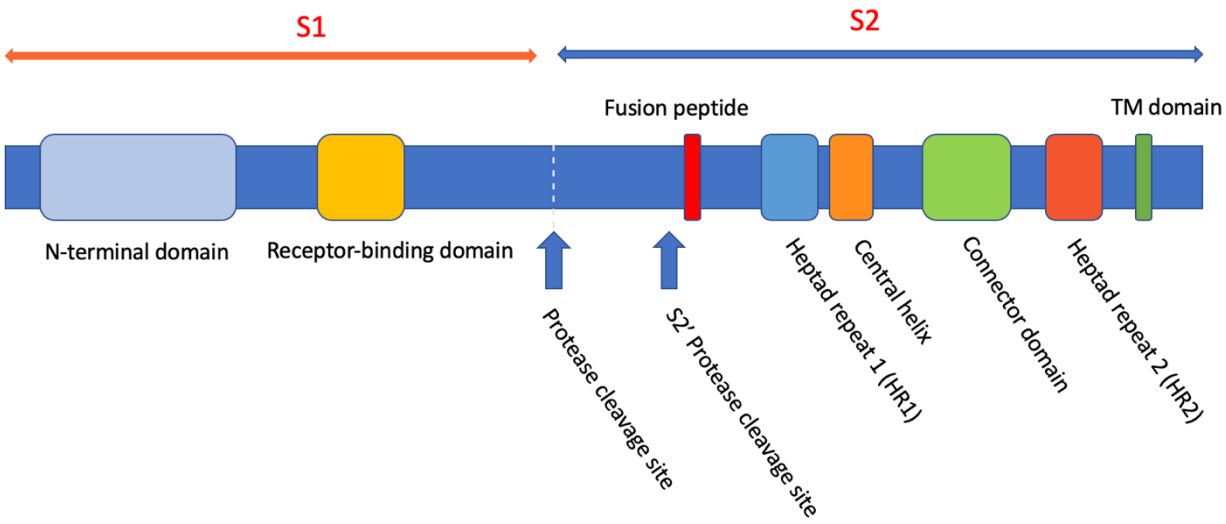

Figure 4. The domains of the SARS-CoV and SARS-CoV-2 spike protein, S.

A 23-mer peptide (P6) derived from the HR2 region inhibits membrane fusion between S-protein-expressing cells and cells expressing the angiotensin converting enzyme 2 (ACE2), which is the receptor for SARS-CoV, at an IC_{50} of 1 μM, suggesting that the peptide binds the deep groove of the HR1 trimer as a target for fusion inhibitors.[36] In contrast to previous studies, the combined use of a peptide derived from the HR1 domain, N46, and its mutant N46eg inhibits cell-cell fusion at an IC_{50} of 1.4 μM. Ujike et al.[37] showed that two HR2-derived peptides, SR9 and SR9EK13, inhibit entry of SARS-CoV via fusion with the plasma membrane at an EC_{50} of 4m-5 nM, following activation of the viral S protein by proteases, but are not effective for entry via the endosomal pathway. They suggested that such peptides would be effective in preventing infection in the lungs following activation by lung proteases.

Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012 as a zoonotic virus transmitted from dromedary camels to humans.[38] It has a very high fatality rate of 35%. It infects cells via the binding of its S protein to cellular receptors, followed by cleavage of the protein by cell membrane proteases, and fusion of the viral and cellular membranes.

X-ray diffraction of the crystallized S2 subunit indicated that the peptides, HR1P and HR2P, derived from the HR1 and HR2 regions of the S protein, respectively, forms a six-helix bundle.[39] HR2P inhibits virus replication and cell–cell fusion, and addition of hydrophilic amino acids to the peptide increases its alpha-helicity and enhances its anti-cell-cell fusion activity, with an IC_{50} of 0.56 μM. Another derivative, HR2P-M2, with improved pharmaceutical characteristics is effective in inhibiting MERS-CoV-S-protein-mediated cell-cell fusion (IC_{50} = 0.55 μM) and infection by a pseudovirus that expresses S (IC_{50} = 0.6 μM).[40] It is also effective in a murine animal model of MERS-CoV after intranasal delivery, reducing the viral titers in the lungs by more than 3 orders of magnitude.

An unusual fusion inhibitor comprising both peptides from the HR1 domain and two

peptides from the HR2 domain, constituting the MERS-five-helix-bundle, was found to interact strongly with an HR2 domain peptide, reflected by a K_D of 0.24 nM.[41] This construct (MERS-5HB) inhibits infection by pseudotyped MERS-CoV at an IC_{50} of approximately 1 μM, and S-protein-mediated cell-cell fusion at an IC_{50} of approximately 0.6 μM.

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2)

Unusual pneumonia cases emerged in Wuhan, China in late December, 2019, and were later attributed to a positive-strand RNA virus in the Coronavirus family.[42] The virus was designated as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and the disease it caused was termed coronavirus disease of 2019 (COVID-19) by the World Health Organization. The virus causes severe alveolar damage and respiratory failure, leading to death in about 4% of infected individuals. Like SARS-CoV, SARS-CoV-2 binds the ACE2 receptor via the S1 domain of its spike protein, S.[43]

SARS-CoV-2 appears to have a higher efficiency of membrane fusion with the host plasma membrane than its predecessor, SARS-CoV.[44] The peptide EK1 targets the HR1 domain of the S-protein and inhibits SARS-CoV and MERS-CoV infection (**Figure 5**). A lipopeptide derivative of EK1 (EK1C4) is a highly effective inhibitor of S-protein-mediated cell-cell fusion, with an IC_{50} of 1.3 nM, which is 241-fold more effective than the original peptide, EK1.[44] EK1C4 also inhibits infection by a pseudovirus expressing the spike protein, at an IC_{50} of 15.8 nM, 149-fold more effective than EK1. The lipopeptide EK1C4 is also effective against other human coronaviruses.

The HR1 domain of SARS-CoV-2 has higher α-helicity and binding affinity to the HR2 domain than the HR1 region of SARS-CoV.[43] A lipopeptide, IPB02, based on the HR2 sequence and derivatized by coupling cholesterol to its C-terminus, is highly effective in inhibiting SARS-CoV-2 S-mediated cell-cell fusion (IC_{50} = 25 nM) and infection by an S-expressing pseudovirus (EC_{50} = 80 nM).

Peptide Inhibitors of Influenza Virus

Influenza virus, including all three types, A, B and C, belongs to the family *Orthomyxoviridae*. Influenza A virus subtypes are designated by the antigenicity characteristics of its hemagglutinin (HA) molecule and its neuraminidase (NA) enzyme, both embedded in the viral membrane. Currently there are 18 H subtypes and 11 N subtypes. The H1N1 and H3N2 subtypes are prevalent currently in human population. Approximately 250,000 deaths per year occur around the world and on average 36,000 fatalities per year in the United States. The original H1N1 subtype caused the 1918 pandemic that caused an estimated 50m-100 million deaths.

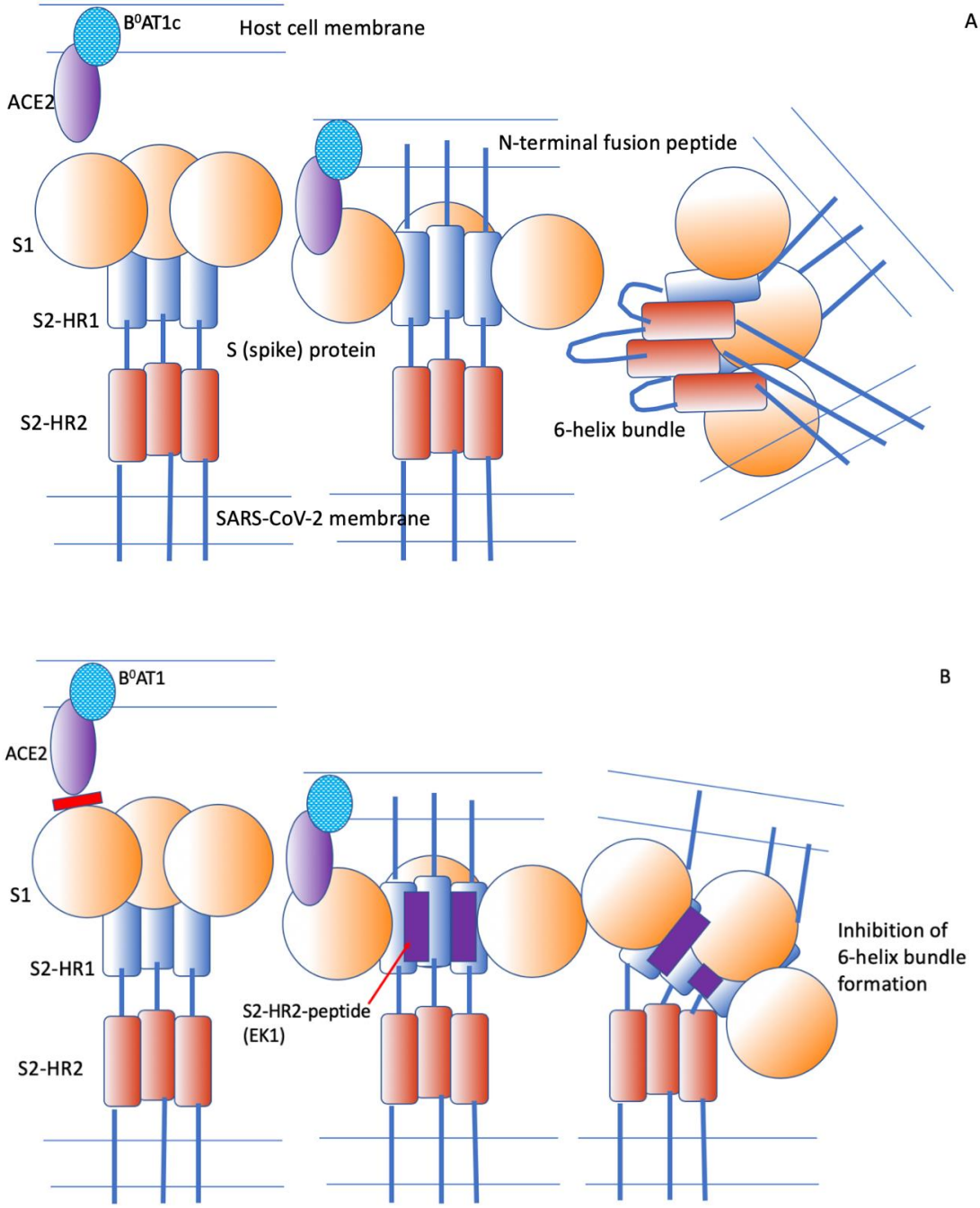

Figure 5. SARS-CoV-2-mediate membrane fusion and its inhibition. **A.** The S1 segment of the spike protein, S, bind to its receptors on host cells. This leads to the insertion of the fusion peptide into the host cell membrane and to the conformational change of the now separate S2 domain of S, resulting in the formation of the 6-helix bundle and the close approach of the two membranes. The hydrophobic interactions between the fusion peptide and the transmembrane domains of the S protein leads to membrane destabilization and fusion. B. Inhibition of membrane fusion by various peptides. Red: A peptide that binds to the receptor binding domain of S1. Purple: A peptide derived from the S2-HR2 region that binds with high affinity to the HR1 region and inhibits the interaction between the S2-HR1 and S2-HR2 domains, thus preventing 6-helix bundle formation and membrane fusion.

Influenza virus infects its host cells by the binding of its HA to sialic acid containing glycoproteins or glycolipids in the host membrane, followed by endocytosis in clathrin-coated or other endocytotic vesicles, and caveolae, or by micropinocytosis (**Figure 6**).[45,46] Following endosome maturation and acidification of the endosome lumen, the HA molecule undergoes an irreversible conformational change [47–50] that mediates the fusion of the viral and endosomal membranes. Fusion enables the viral ribonucleoproteins to enter the cytoplasm and reach the nucleus.

Fusion-capable HA is generated during viral biosynthesis by the proteolytic cleavage of the protein into HA1 and HA2 subunits. In the intact virion, HA1 functions as the receptor binding domain (RBD), and the exposed N-terminus of the HA2 (the fusion peptide) facilitates membrane fusion by inserting into the host cell membrane following the low pH-induced conformational change of the HA. We have proposed that it is the process of conformational change of the HA that mediates fusion, and not the low pH conformation itself.[51,52] It is also important to note that exposure of influenza virus to low pH in the absence of a target membrane leads to an irreversible conformational change of the HA and inactivation of the ability of the virus to fuse.[49, 50]

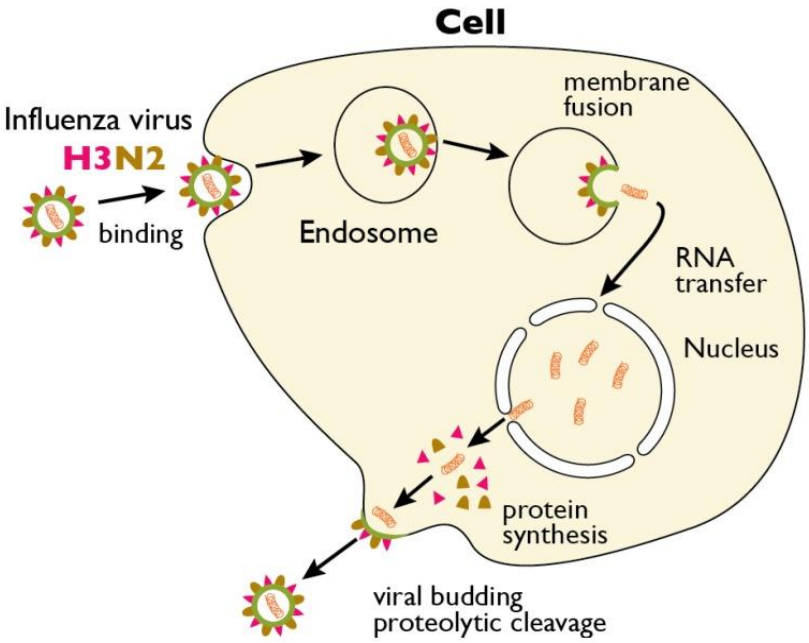

Figure 6. The replication cycle of influenza virus, here an H3N2 subtype. The virus binds to sialic acid residues on glycoproteins and glycolipids, and is endocytosed. During endosome maturation the pH in the lumen is acidified via the action of H+ pumps in the membrane (gray), the conformational change of the HA (red) causes the fusion of the viral membrane (green) with the endosome membrane, and the microinjection into the cytoplasm of the viral RNA (orange) and proteins. The negative-strand RNA localizes in the nucleus, is replicated and transported back to the cytoplasm. The positive-strand RNA is translated into viral proteins, which assemble together with negative-strand RNA at the plasma membrane, and the virus buds off. The NA (brown) enables the virions to be released from the plasma membrane by cleaving off the sialic acids to which the HA has bound. (Reproduced with permission from Hamilton et al.[45])

Competitive inhibitors of the receptor binding domain of HA could be used as inhibitors of HA-mediated membrane fusion. A phage display peptide library was used by Matsubara et al.[53] to identify peptides that could bind the receptor binding domain of HA (H1 and H3 subtypes). The stearic acid-coupled peptide C18-s2 inhibited infection of MDCK cells by an H1N1 influenza at an IC_{50} of 11 μM, and by an H3N2 virus at 15 μM.

A 20-amino-acid peptide, termed EB (entry blocker) inhibits the attachment of various strains of influenza virus to the cellular receptor by binding to HA. Virus-induced death of MDCK cells was inhibited by the peptide with an IC_{50} of about 4.5 μM.[54] The EB peptide was inhibitory to H5N1, H5N9 and H1N1 subtypes of influenza virus.

Peptides that could bind a "protein cavity" in the HA2 subunit of HA were designed by Perrier et al.,[55] based on molecular dynamics and quantum chemistry calculations. Two pentapeptides were identified that interacted with HA2 with high stability at both pH 7 and 5. The fusion inhibitory activity of these peptides has not yet been examined.

A region of HA right outside the viral membrane attaches to the internal coiled-coil of the protein that has been identified within the low pH-induced, post-fusion structure of HA [56]. A peptide (P155-185-chol) corresponding to a part of this region, coupled to cholesterol at its C-terminus, inhibited infection by the A/H3N2 subtype with an IC_{50} of 0.4 μM. The peptide, P155-181-chol, missing 4 C-terminal amino acids from the P155-185-chol had a higher IC_{50} of 2 μM. These peptides lacking the cholesterol did not have a significant inhibitory effect [56], indicating the crucial contribution of a lipid moiety coupled to the peptides. The authors attribute this effect to the insertion of the cholesterol into the plasma membrane and internalization of the coupled peptide in endosomes, together with influenza virus. The acidification of the endosome lumen then causes the conformational change of the HA, exposing the region recognized by the peptide. The peptide then prevents the full conformational change of the HA that might otherwise induce membrane fusion.[56]

Figueira et al.[57] generated fusion inhibitors by adding a cell penetrating peptide to a fusion inhibitory peptide of 43 amino acids (derived from a helical region of the HA2 subunit involved in helix-helix interactions) to facilitate the cellular entry of the inhibitor. The cell penetrating peptide was derived from the HIV-1 Tat protein. These constructs formed nanoparticles of 15–30 nm in diameter, and inhibited virus fusion with liposomes composed of a zwitterionic lipid (dioleoylphosphatidylcholine). However, the peptide concentration used was relatively high (10 μM). Nevertheless, intranasal administration of the peptide Tat-HA2Ec, reduced significantly the viral titer in the nose of infected rats.

Peptide Inhibitors of Hepatitis Viruses

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is one of the smallest enveloped DNA viruses. The virus particle (termed the "Dane particle"), consists of a lipid envelope and an icosahedral nucleocapsid enclosing a small genome of 3.2 kb of partially double-stranded DNA. The genome encodes the viral polymerase, HBV surface proteins, the structural core protein and the non-structural pre-core protein (secreted e-antigen; HBeAg), and the X protein. The genome is surrounded by the hepatitis B core (c) antigen (HBcAg) and an envelope containing the glycoprotein surface (s) antigen (HBsAg). Although it is a DNA virus, HBV replicates through an RNA

intermediate. The HBV polymerase is a multifunctional enzyme with RNA- and DNA-dependent polymerase and ribonuclease H activities. The tissue tropism of HBV is determined by specific interactions between the envelope proteins and cellular factors.[58–60] Hepatitis delta virus (delta agent) (HDV) replicates only in the presence of HBV, which provides an envelope for the delta virus RNA and its antigen(s).[61–63]

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The HBV envelope consists of the large (L), middle (M) and small (S) membrane-spanning glycoproteins. The proteins share the same C-terminal S-domain (226 amino acids), anchored in the lipid bilayer by four membrane-spanning helices, but differ by the size of their N-terminal ectodomain. The L domain contains N-terminal pre-S1 (108 or 119 amino acids), central pre-S2 (55 amino acids), and C-terminal S domains. M is shorter than L, lacking pre-S1, while S consists of the S domain only (**Figure 7**).[64] Envelope components are glycosylated, and type II transmembrane proteins that form multimers are stabilized by disulfide bridges. The HBV surface proteins are embedded in an endoplasmic reticulum-derived lipid bilayer.[65, 66] The mechanism of HDV entry is similar to that of HBV, and HDV has been used as a substitute for studying HBV infection at the entry level.[67–69]

The L protein and the integrity of S protein are critical for HBV and HDV infection. The pre-S1 domain of L is a major determinant for the entry of viruses and their interaction with the

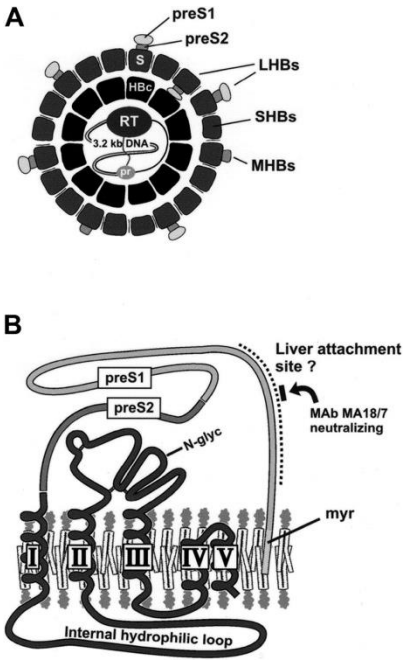

Figure 7. Hepatitis B virus. **(A)** Double line: Double stranded DNA. Single line: Single stranded DNA. pr: The priming domain of the viral DNA polymerase. RT: Viral reverse transcriptase. HBc: Nucleocapsid. S-HBs, M-HBs, L-HBs: Surface proteins with the S, preS2 and preS1 domains. **(B)** Schematic structure of L-HBs (the large hepatitis B surface protein). I–V: Intra-membrane helices. N-glyc: N-linked glycan. myr: site of myristic acid attachment (reproduced with permission from Glebe et al.[64]).

Hepatocyte receptor(s).[70–72] During synthesis and prior to translocation to the endoplasmic reticulum, the pre-S1 domain of L is post-translationally myristoylated at glycine 2 by the cellular *N*-myristoyltransferase.[73] Myristoylation is necessary for infectivity of the virus.[70] Studies on HBV mutants with deletions in the pre-S1 of L indicated that the first 77 pre-S1 amino acids are critical for HBV infectivity.[74] An N-myristoylated peptide containing the pre-S1 amino acids 2-78 (HBVpreS/2-78^{myr}), and shorter acylated peptides comprising the N-terminal 48 amino acids, inhibit HBV infection in primary human hepatocytes, *Tupaia belangeri*

hepatocytes, and HepaRG cells.[59,64,75–77] A second determinant of infectivity was identified in the surface-exposed antigenic loop (AGL), a polypeptide present in the S domain of all envelope proteins. The immunodominant "a" determinant is characterized by a specific conformation of the AGL polypeptide located between the transmembrane domain II (amino acids 80 to 100) and the hydrophobic carboxyl terminus (amino acids 165 to 226) of the S domain.[78–80]

Attachment of HBV particles to host cells proceeds via at least two steps. The virus initially interacts with heparan sulfate proteoglycans (HSPGs) on the hepatocyte surface. Binding to HSPGs is mediated by interactions between the negatively charged HSPG and two positively charged residues (Arg122 and Lys141) in the AGL region of the S domain.[81,82] Afterwards, the myristoylated N-terminal of the pre-S1 domain binds to the high-affinity receptor, the sodium-taurocholate co-transporting polypeptide (NTCP/ SLC10A1). NTCP is responsible for the uptake of conjugated bile acids from the blood.[83,84] After binding to NTCP, HBV enters hepatocytes by clathrin-mediated endocytosis. The epidermal growth factor receptor (EGFR) mediates the internalization of NTCP-bound HBV via its endocytosis/sorting pathway.[85,86]

The identification of NTCP as a hepatocyte receptor has revealed a target for HBV entry inhibition. Myrcludex B, a myristoylated lipopeptide, inhibits the entry of HBV and HDV in hepatocytes. Its sequence corresponds to the N- terminal amino acids (2–48) of the pre-S1 of L- and inhibits viral entry by binding with high efficacy to NTCP (IC_{50} 80 pM).[87–89] At low concentrations, Myrcludex B binding to NTCP inhibits HBV and HDV infection, but at saturating concentrations (IC_{50} 47 nM) it also blocks the uptake of bile salts. Myrcludex B passed acute and long-term toxicity studies, and is currently in phase II clinical trials in chronically HBV and HDV-infected patients.[63,66,83,84]

Hepatitis C virus (HCV) was discovered in 1989 and classified in the Hepacivirus genus of the *Flaviviridae* family. HCV is a small, enveloped virus containing a positive-sense, single-stranded RNA. The genome encodes a single polyprotein of approximately 3100 amino acids, which is processed into three viral structural and seven non-structural proteins. The structural proteins consist of the membrane-anchored envelope glycoproteins E1 and E2 and the icosahedral core that is 33 to 40 nm in diameter. The nonstructural proteins comprise the p7 viroporins and the NS2, NS3, NS4A, NS4B, NS5A and NS5B protease. E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a short C-terminal trans-membrane domain. Virion-associated E1-E2 envelope glycol-proteins form large covalent heterodimers stabilized by disulfide bridges. HCV mutates rapidly due to the high error rate of HCV polymerase and high rate of virion production. The mutation rate produces so many variants of the virus that it is considered a quasi-species rather than a conventional virus species.[90–92]

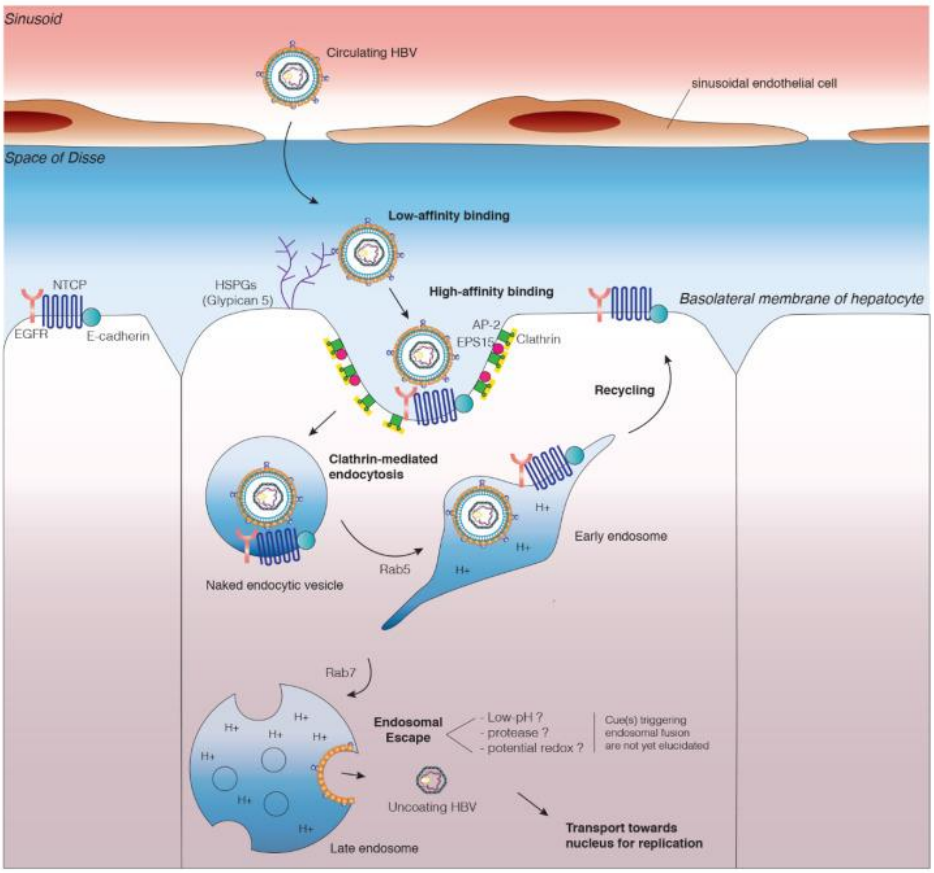

Figure 8. Early events in the life cycle of hepatitis B virus. The virus interacts with heparan sulfate proteoglycans on hepatocytes. It then binds to the receptor sodium taurocholate co-transporting polypeptide (NTCP) and the co-receptor epidermal growth factor receptor (EGFR) (reproduced with permission from Herrscher et al.[86]).

All steps of the HCV life cycle are dependent on the interaction with lipoproteins and apolipoproteins. HCV infectious particles are associated with very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). The interaction of HCV with lipoproteins leads to the formation of lipo-viro-particles (LVP) that are critical for HCV infectivity and evasion from neutralizing antibodies. Infectious LVP have a low density (between 1.03 and 1.10 g/ml), are rich in cholesterol and triglycerides, and contain apolipoproteins ApoB, ApoA1, ApoE, and ApoC1,2,3. These properties have also been confirmed for particles derived from cell culture (HCVcc). Lipoproteins and their receptors participate in HCV entry. HDL enhances HCV infectivity, probably by utilizing the lipid transfer function of scavenger receptor class B type I (SR-BI), a receptor for both HDL and HCV.[92–99]

The entry of HCV into hepatocytes requires interactions between the viral envelope proteins and several host cell surface receptors/co-receptors/factors: the tetraspanin CD81, SR-B1, the tight junction (TJ) proteins claudin-1 (CLDN1) and occludin (OCLN), transferrin receptor 1 (TfR1), the receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor

(Figure 9).[100,101] E2 is the major HCV envelope protein that directly interacts with the receptors/co-receptors. The first step of HCV entry involves the interplay between LVP-associated ApoE, cell surface heparan sulfate proteoglycans (HSPGs) (particularly syndecans 1 and 4) and the LDL receptor (LDLR). Subsequently, the LVP interacts with SR-BI through ApoE and ApoB-100. The cholesterol transfer activity of SR-BI allows for E2 exposure and binding to SR-BI and the tetraspanin, CD81. Binding of CD81 activates the EGFR signaling pathway and interaction between CD81 and CLDN1 that triggers HCV entry.[95–97,102–107]

Both E1 and E2 of HCV are type I transmembrane proteins containing an N-terminal ecto-domain (160 residues for E1 and 330 residues for E2) and a well-conserved C-terminal trans- membrane domain of about 30 amino acids. Virion-associated E1-E2 envelope glycoproteins form large covalent heterodimers stabilized by disulfide bridges. E1-E2-mediated interaction of HCV with entry factors instigates the binding to host cell receptors and internalization of the virus via clathrin-mediated endocytosis, followed by fusion of the viral membrane with the early endosome membrane at low pH. E2 (residues 384-746) is a major HCV envelope protein that interacts with the receptors/co-receptors. Based on computational models, E2 was predicted to be a class II fusion protein characterized by a highly extended conformation (~110–130 Å) of three predominantly β-sheet domains.[108,109]

In 2013, the globular structure of the E2 core with amino acid residues 412 to 645 was resolved.[110] This structure contains immunoglobulin-fold β sandwich flanked by two additional protein layers: a front sheet composed of a front layer and a central Ig-fold domain, and a back sheet (or back layer). The black layer domain was not described previously within a fusion protein. The neutralizing antibody AR3C binds to a large part of the front layer, which consists of the loops, short helices, and β sheets. The CD81 receptor-binding site identified by electron microscopy and by site-directed mutagenesis, overlaps with the AR3C epitope. The central Ig-fold domain represents a common structure among class-II fusion proteins; however, an original structure of the back layer reduces the possibility that HCV E2 is a classical fusion protein. The X-ray and electron microscopy E2 structures differ noticeably from predictions of an extended, three-domain, class II fusion protein fold.[106,110]

The E1 envelope glycoprotein (residues 192–383) is much smaller than E2, but both are type I transmembrane proteins, with the N-terminal ectodomain in the endoplasmic reticulum lumen and the C-terminus anchoring on the endoplasmic reticulum membrane, during biosynthesis. Bioinformatics analysis of E1 sequences reveals a conserved protein domain organization, including the N-terminal domain (NTD, 192–239), a putative fusion peptide (pFP, 272–285), the conserved region (CR, 302–329), and the C-terminal transmembrane domain (TMD, 350–381). Nevertheless, the crystal structure of E1 ectodomain NTD does not have the expected truncated class-II fusion protein fold. Therefore, rather than being mediated by a single glycoprotein, HCV fusion may be mediated by complex intra- and intermolecular E1-E2 interactions. It was proposed, by combining computational analysis and wet-lab data that E1 co-evolves with BL of E2, and this genetic association is critical for membrane fusion. A soluble back-layer derived polypeptide (71 amino acids; BLd-H77) inhibits fusogenic rearrangements and HCV infection, suggesting that E1 and

E2, bzck-layer stem regions control HCV fusion in a combined mode.[111,112]

Chi et al.[101] identified a peptide from the E2 stem domain, named E27 (671m-705), which blocks E1-E2-mediated cell-cell fusion, and inhibits cell entry of HCV pseudo-particles and infection of HCVcc, at nanomolar concentrations. The authors suggested that the interaction between E1 and E2 might be an appropriate target for designing HCV fusion inhibitors, as mutations in a specific region of E1 result in resistance to E27 and because E27 interfered with E1-E2 dimerization. Better activity and lower cost were achieved by shortening and optimizing this fusion inhibitory peptide from 35 to 29 amino acids. E27 may prevent fusion by (i) interfering with E1 and E2 dimerization and (ii) inducing conformational changes of the E1-E2 dimer. To affect virus-cell fusion an E27 pairing region should be present in either E1 or E2. Further studies are necessary to untangle all intricacies of HCV entry into target cells, especially the fusion step.

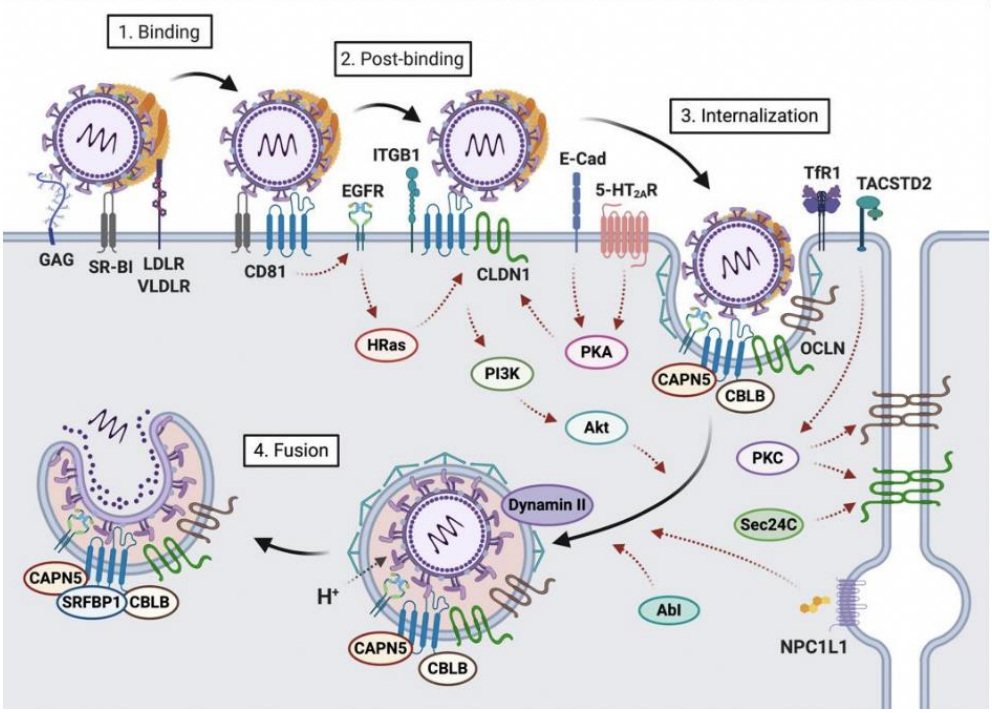

Figure 9. Cell-free hepatitis C virus (HCV) entry pathway into hepatocytes. The initial binding results in virus accumulation at the cell surface, and the exposure of envelope protein domains that interact with SR-BI, CD81, and CLDN1. The virus is internalized by clathrin-mediated, dynamin-dependent endocytosis. Endocytotic vesicles become endosomes with an acidic interior, which in turn mediates membrane fusion (reproduced with permission from Colpitts et al.[107]).

Peptide Inhibitors of Paramyxoviruses

Paramyxoviruses are lipid-enveloped, negative-strand RNA viruses and include the medically important viruses, measles virus, mumps virus and respiratory syncytial virus. The single strand of RNA forms a helical nucleocapsid with the nucleoproteins.[113] The negative-strand RNA is transcribed into mRNA by the RNA-dependent RNA polymerase. The lipid envelope, derived from the plasma membrane of the host cell, is pleomorphic and incorporates hemagglutinin (H), neuraminidase (N), and the fusion

protein (F). The virus also has the nucleoprotein (N), large protein (L), P protein and the matrix protein (M).[114]

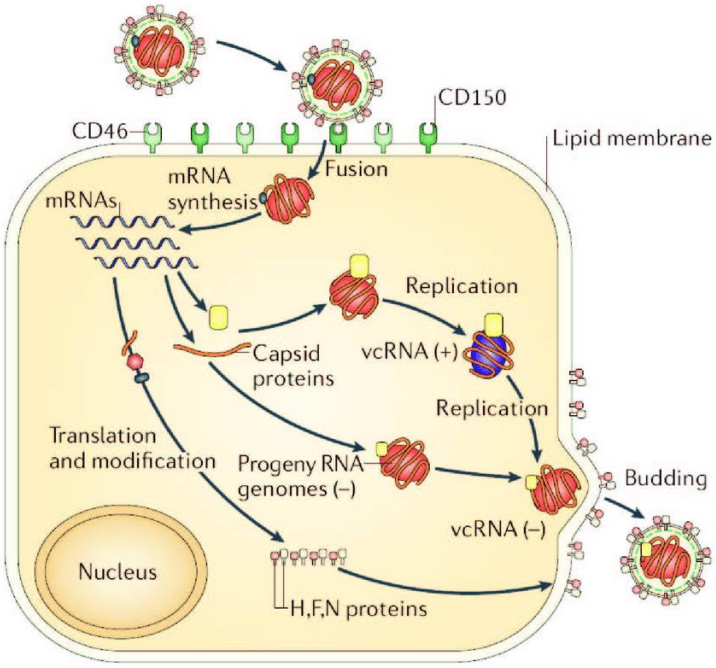

Figure 10. The replication cycle of measles virus, a paramyxovirus (Reproduced with permission from Moss & Griffin,[114]).

Host cell surface receptors for paramyxoviruses include sialic acid, and the CD46 and CD 150 proteins. Parainfluenza virus type 3 and mumps virus use α2,3-linked sialic acids in oligosaccharides on lipids or glycoproteins.[115] Hendra and Nipah viruses bind ephrin-B2, present in capillaries, arteries and arterioles, and ephrin-B3, present in the nervous system and the vasculature.[116] Depending on the strain, measles virus binds CD150, CD46 and the adherens-junction protein, nectin-4.[115]

Binding to host cells is achieved by the viral H protein, and fusion is mediated by the F protein. The active F protein is produced by the cleavage of a precursor, and the exposed fusion peptide is inserted into the host cell membrane,[52] providing an intermediate structure that initiates the pulling of the viral and cellular membranes. The two heptad-repeat domains on the F protein fold onto each other and the neighboring F proteins in an anti-parallel fashion, forming a six-helix bundle that further brings the membranes closer. As in the cases of the other viruses described above, this structure can be targeted by complementary peptides.[117]

The subsequent release of the nucleocapsid into the cytoplasm is followed by transcription, translation, and replication. The P protein is involved in regulating transcription and replication, as well as assisting in the association of the nucleoproteins to form a nucleocapsid.[114] All viral components gather at the plasma membrane, and the assembled virus buds off from the cell surface, taking with it part of the host cell membrane lipid bilayer.

Peptides derived from the paramyxovirus F1 protein, with amino acid sequences similar to that of peptide inhibitors of HIV-1 (DP-107 and DP-178 (T-20) have antiviral activity, blocking syncytium formation by respiratory syncytial virus, human parainfluenza virus type 3 and measles virus, with EC_{50} between 15 and 250 nM.[118]

Thirty four-amino-acid synthetic peptides corresponding to the heptad repeat domains of the F proteins of human parainfluenza virus type 2 and type 3 close to the transmembrane segment of the protein inhibit cell-cell fusion induced by these viruses at EC_{50} values of 2.1 μM and 1.2 μM, respectively.[119] They also inhibit viral entry. Shorter peptides are less effective. Dimer peptides derived from the measles virus F-protein C-terminal heptad repeat region, and coupled to cholesterol for membrane localization, inhibit measles virus infection at IC_{50} values of 2 nM (for peptide HRC2) and less than 1 nM (for peptide HRC4). The latter peptide construct, administered intranasally at a dose of 6 mg/kg 24 h before infection, prevented measles virus-induced death in a murine model.[120]

Peptides coupled to lipids can self-assemble, forming micellar nanoparticles. When they interact with cells, they can partition into cell membranes, where they can more efficiently interact with and inhibit the viral fusion mechanism, compared to plain peptides.[121] In an animal model of measles virus infection, the fusion inhibitory peptides were administered intranasally 24 h and 12 h before infection with the virus. Cholesterol-coupled peptides derived from the measles virus fusion protein C-terminal heptad repeat domain, termed HRC2, HRC3, HRC4, and HRC6, inhibited completely virus production in the lungs.[121]

Entry of parainfluenza virus type 3 infection of CV1 cells is inhibited to a much greater extent by cholesterol-derivatized HRC peptides than by the plain peptides, the IC_{50} for the former being about 7 nM, and for the latter, about 700 nM. The cholesterol-coupled peptides can inhibit completely viral infection, whereas the plain peptides do not achieve complete inhibition.[122] The same cholesterol-coupled peptide is effective against Hendra virus and Nipah virus infections that can cause severe central nervous system diseases, at an IC_{50} of about 10 nM, whereas the plain peptide has a 15 to 20-fold higher IC_{50}. It should be noted that the peptide terminal at which the cholesterol is attached is important for the enhanced activity, the derivative being much more active if the cholesterol is coupled at the C-terminus.

Peptide Inhibitors of Flaviviruses

The *Flaviridae* family includes the pathogenic viruses, Dengue, Japanese encephalitis, yellow fever, Zika and West Nile viruses that infect a very high number of humans every year.[123,124] Flavivirus infections can cause fatal encephalitis and hemorrhagic fever, but can also lead to mild symptoms. Among the flaviviruses, Dengue virus, causes the largest outbreaks and mortality. The flaviviral genome encodes three structural proteins, C, M and E, and seven non-structural proteins. The surface of the viruses is covered with the E and M proteins, and the core contains the capsid (C) protein and the genome.[125]

Flaviviruses are enveloped, positive-strand RNA viruses, and infect their host cells via receptor-mediated endocytosis. The E protein binds to attachment factors such as glycosaminoglycans, thereby increasing the local concentration of the virus on the cell membrane.[125] Unfortunately, the actual

identity of bone fide cellular receptors is poorly understood.[126] A possible receptor is the molecule, T-cell immunoglobulin and mucin domain (TIM), which recognizes the negatively charged phospholipid, phosphatidylserine, in the viral membrane, which is thought to be acquired while the virus is being processed in the endoplasmic reticulum.[126] Other possible receptors are the heat-shock proteins (e.g. GRP78), and C-type lectins (e.g. mannose receptor, DC-SIGN) among others.

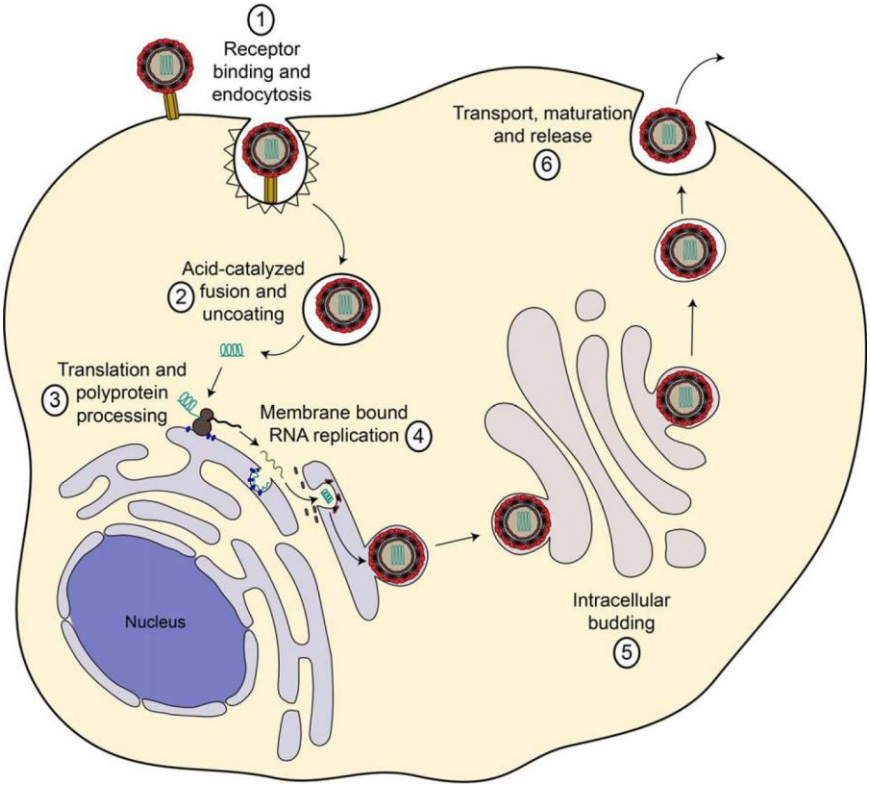

Figure 11. The flavivirus life-cycle. 1. Viral particles are endocytosed after binding to cell surface receptors. 2. Following endosome acidification, they fuse with the endosome membrane, microinjecting the RNA genome into the cytoplasm. 3. The viral RNA is translated into polyproteins at the endoplasmic reticulum. 4. The viral RNA is transcribed by virus-encoded RNA-dependent RNA polymerase in membrane compartments derived from the endoplasmic reticulum. 5. The nucleocapsid buds into the endoplasmic reticulum, and is transported to the Golgi network, where the host protease furin modifies the viral envelope protein. 6. The virus is exocytosed after processing and maturation. The flavivirus *HCV* is not processed proteolytically, but it goes through the host lipoprotein release pathway (reproduced with permission from Gerold et al.[132]).

The E protein is a class II fusion protein that undergoes conformational changes at low pH that mediate the interaction of its stem region and its domain II, and a transition from dimers to trimmers, [127–129] leading to membrane fusion with the endosome membrane, and microinjection of the genome into the cytoplasm. The RNA is translated into a polyprotein that is cleaved into individual proteins by proteases. The structural proteins and the nascent RNA bud into the endoplasmic reticulum lumen. The immature viral and sub-viral particles go through the trans-Golgi network and are processed by the host furin enzyme. The

resulting mature virions and sub-viral particles are then exocytosed.[124]

Using high-resolution structural information for the dimeric form of the E protein of dengue virus 2, as well as computational optimization of binding, Costin et al.[130] designed peptides that could interact with the protein, and inhibit viral infection in LLC-MK2 cells. The peptide DN57opt had an IC_{50} of 8 µM, and 1OAN1 had an IC_{50} of 7 µM. These peptides interfered with virus binding to host cells, bound directly to the E protein, and altered the virus surface. These peptides were not toxic to the cells up to 25 µM.

Chen et al.[129] used peptides derived from the E protein stem helix 2 of Japanese encephalitis virus to investigate their ability to block infection by both the same virus and Zika virus. These peptides inhibited Japanese encephalitis virus infection at nanomolar IC_{50} values. The peptide P5 decreased viral load in an animal model, prevented tissue pathology, and inhibited lethality. This peptide also inhibited Zika virus infection at an IC_{50} in the micromolar range, as well as histopathological damages in the brains of infected mice.

Concluding Remarks

The reports we have outlined above indicate unequivocally that peptides can be utilized to inhibit infection by lipid-enveloped viruses. The coupling of a lipidic anchor like cholesterol to some of these peptides enhances the antiviral effect of the peptides, possibly by inserting into the cellular or viral membranes and localizing the peptide to the vicinity of the membrane fusion reaction. Although the only clinically approved viral inhibitor is the anti-HIV peptide T-20, it is highly likely that peptides against other viruses will be evaluated in clinical trials, particularly in the case of the highly pathogenic SARS-CoV-2. The results of Xia et al.,[44] showing inhibition of membrane fusion by cholesterol-coupled peptides at low nanomolar concentrations are especially encouraging. It is also likely that peptide inhibitors of HIV-1 fusion with improved potency and stability over T-20 will be evaluated clinically.[131] As these peptide drugs are developed, it will be important to also evaluate the development of mutant viruses resistant to these novel drugs.

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