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Shibo Jiang
Lu Lu *Editors*

Virus Entry Inhibitors

Stopping the Enemy at the Gate

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Preface

Virus entry into the host target cell is the first step of the viral replication cycle. It is initiated by the attachment of the viral particle to the target cell through specific interaction between the viral surface protein(s) and receptor(s) on the host cell. This interaction triggers the conformation changes of the viral surface protein(s), resulting in entry, via membrane fusion or endocytosis, of the viral genetic materials into the host cell for replication.

Virus entry inhibitors are antiviral agents that block viral entry into the host cell, including those that bind viral surface proteins, interfere with interaction between viral and host proteins, inhibit viral protein-mediated plasma or endosomal membrane fusion, or inactivate cell-free virions.

In the early 1990s, Jiang and colleagues identified the first highly potent human immunodeficiency virus (HIV) entry inhibitor derived from the HIV-1 gp41 C-terminal heptad repeat (CHR) domain and the related patent was licensed to Trimeris, Inc., which jointly with Roche Pharmaceuticals developed the first virus entry inhibitor-based anti-HIV peptide drug, enfuvirtide (Fuzeon), approved by the US FDA in 2003. Since then, several series of the small molecule-, peptide-, and protein-based virus entry inhibitors have been or are being developed against enveloped viruses, including human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Ebola virus (EBOV), Hendra virus (HeV), Nipah virus (NiV), influenza virus (IFV), parainfluenza virus (PIV), Zika virus (ZIKV), Dengue virus (DENV), hepatitis C virus (HCV), and hepatitis B virus (HBV), and non-enveloped viruses, such as human papillomavirus (HPV). Since many of these viruses, such as SARS-CoV, SARS-CoV-2, EBOV, ZIKV, HeV, and NiV, can cause outbreaks of highly pathogenic emerging infectious diseases, virus entry inhibitors against these viruses have great potential to be used for the prevention and treatment of these infectious diseases.

In this book, we will discuss the advances in the research and development of the small molecule-, peptide-, and protein-based virus entry inhibitors as a novel class of antiviral drugs for the treatment and prevention of viral infection.

Theoretically, virus neutralizing antibodies should belong to the protein-based virus entry inhibitors since they can neutralize viral infection by blocking virus attachment to the receptor on the host cell, interfering with

virus-cell fusion or inhibiting virus entry into the host cell for replication. Since several books about the viral neutralizing antibodies have been published by Springer, this book contains no chapter mainly discussing the viral neutralizing antibodies.

Many authors for this book are in the top list of the experts worldwide in the fields of HIV fusion inhibitors (https://expertscape.com/ex/hiv+fusion+inhibitors) and viral fusion proteins (http://expertscape.com/ex/viral+fusion+proteins).

We heartily appreciate all the authors for their contributions to this book.

Shanghai, China — Shibo Jiang
Shanghai, China — Lu Lu

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Virus Entry Inhibitors: Past, Present, and Future

1

Shan Su, Wei Xu, and Shibo Jiang

Abstract

The approval of enfuvirtide marked a milestone for the development of virus entry inhibitor-based antiviral therapeutics. Since then, more peptide-, small-molecule-, and protein-based entry inhibitors have been identified and approved for viral diseases. Here we reviewed the development of virus entry inhibitors and the advantages and disadvantages of peptide-, small-molecule-, and protein-based entry inhibitors, herein summarizing the future trend of these antivirals. Virus entry inhibitors take effect outside the host cell, making them good candidates for development as pre- and post-exposure prophylaxis, microbicides, and therapeutics. This chapter, as well as this book, provides more information on the development and modification of peptide-, small-molecule-, and protein-based virus entry inhibitors.

Keywords

Virus · Therapeutics · Entry inhibitor · Peptide · Small molecule

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1.1 Introduction

In the early 1990s, Wild et al. and Jiang et al. independently found that the peptides DP-107 and SJ-2176, derived from the N-terminal heptad repeat (NHR, or HR1) and the C-terminal heptad repeat (CHR, or HR2), respectively, of the human immunodeficiency virus-1 (HIV-1) envelope protein (Env), could inhibit HIV-1 infection in vitro (Jiang et al. 1993; Wild et al. 1992). Later, another CHR-derived peptide, DP-178, with more than 60% sequence identity to that of SJ-2176, also showed potent inhibitory activity against HIV-1 infection. The group at Duke University that identified DP-178 then established a company, Trimeris Inc., and licensed the US patent #5444044 from Jiang's group at the New York Blood Center for further development of the HIV-1 CHR-derived peptide DP-178 (later named T20). In 2003, T20 (brand name: Fuzeon, generic name: enfuvirtide) was approved by the U.S. FDA as the first HIV entry inhibitor for the clinical treatment of HIV-infected patients who had failed to respond to other antivirals. The licensure of T20 is a milestone that marks the use of virus entry inhibitors and peptide-based antivirals to treat HIV-1 infection.

Viral entry is the first step of the viral life cycle, and viral entry of enveloped viruses is mediated by fusion proteins located on the viral surface. In history, most pathogenic viruses are enveloped with class I fusion protein, such as HIV, severe acute respiratory syndrome

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coronavirus-2 (SARS-CoV-2) and SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), influenza A virus (IAV), and Ebola virus (EBOV). Class I fusion protein is a heterodimer of a surface subunit (SfS) and a transmembrane subunit (TmS), forming trimers on the surface of the viral membrane (Fig. 1.1a). Viral entry is initiated by the binding of SfS to its specific receptor, or simultaneous binding to primary and secondary receptor, on the host cell. For entry via the surface fusion pathway, receptor binding triggers the conformational change of fusion protein directly. For entry via endocytosis, the viral particle enters the endosome after receptor binding, and the conformational change of fusion protein is triggered by low pH. Viral fusion protein then transforms into a pre-hairpin intermediate state in which the fusion peptide (FP) region of TmS is exposed and inserts into the membrane of host cell or endosome. Then, the heptad repeat-1 (HR1) of TmS interacts with homologous heptad repeat-1 (HR2), forming a stable six-helix bundle (6-HB) in which three HR2 helices pack into hydrophobic grooves on the HR2-trimer core in an antiparallel way (Chan et al. 1997; Lu and Kim 1997; Weissenhorn et al. 1997). The formation of 6-HB is the driving force that pulls viral and target cell membranes into close proximity (Fig. 1.1b). Then the viral membrane fuses with cell or endosome membrane, and the viral genome is released into the cytoplasm of host cell. The viral structural and nonstructural proteins will then be synthesized, according to the viral genome, by the host protein production machinery. The viral fusion protein is synthesized as a precursor and requires cleavage by host protease, such as furin, transmembrane protease serine 2 (TMPRSS2), or cathepsin L, during bio-synthesis or viral entry to release functional proteins. Antivirals that interfere with any step of the above process can block viral entry and are classified as virus entry inhibitors.

1.2 Peptide-Based Virus Entry Inhibitors

1.2.1 Viral Fusion Protein Targeting

The first approved viral entry inhibitor, T20, and its analogs SJ-2176 and C34 are peptides derived from the HR2 domain of HIV-1 Env. Structural and mechanistic studies revealed that HR2-peptides bind to the exposed HR1 trimer in the prehairpin intermediate state of HIV-1 Env, thus competitively interfering with the association between homologous HR1 and HR2 (Fig. 1.1b) (Chan et al. 1997; Lu and Kim 1997; Weissenhorn et al. 1997). The latest reports demonstrated that T20 can also bind to FP proximal region, except for HR1 (Xu et al. 2019). However, T20 lacks the pocket binding domain (PBD) which is crucial for the interaction between HR1 and HR2, thus showing ~10-fold less potency in inhibiting HIV-1 infection than C34 and SJ-2176 (Liu et al. 2005). Peptides derived from HR2 of other viruses with class I fusion protein, also showed homologous antiviral activity. For example, soon after the identification of SARS-CoV, MERS-CoV, and SARS-CoV-2 in 2003, 2012, and 2019, respectively, Jiang's group, which designed SJ-2176, immediately identified the HR2 region of these three coronaviruses and found that SARS-CoV, MERS-CoV, and SARS-CoV-2 HR2-derived peptides had potent inhibitory activities against SARS-CoV, MERS-CoV, and SARS-CoV-2, respectively (Liu et al. 2004; Lu et al. 2014; Xia et al. 2020b). A peptide derived from HR2 of respiratory syncytial virus (RSV) inhibits RSV entry and infection in BALB/c mice (Gaillard et al. 2017). Similar to HR2-derived peptides, HR1-derived peptides also have antiviral activity by interfering with 6-HB formation. However, HR1 peptides are less effective than HR2 peptides because they are highly hydrophobic and prone to aggregate in neutral buffer.

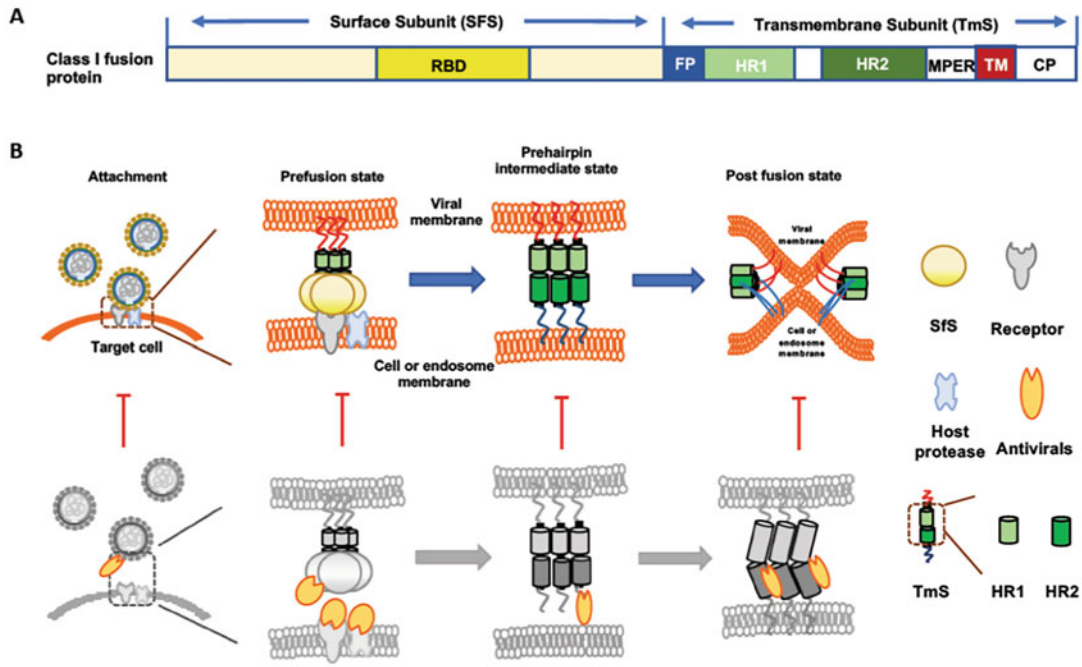

Fig. 1.1 Fusion process of viruses with class I fusion protein. (**a**) The schematic diagram of class I fusion protein. *RBD* receptor binding domain, *FP* fusion peptide, *HR1* heptad repeat-1, *HR2* heptad repeat-2, *MPER* membrane proximal external region, *Tm* transmembrane domain, *CP* cytoplasmic tail. (**b**) Schematic diagram of viral fusion mediated by class I fusion protein. Antivirals specific to fusion protein, receptor, and host protease can block the viral entry process

Many approaches have been developed to improve the potency and breadth of HR2 peptides. For example, T1249 (Otaka et al. 2002) with hybrid sequences from HIV-1, HIV-2, and SIV has broader effects; sifuvirtide (He et al. 2008) with introduction of E-K salt bridge to stabilize the helical structure showed improved solubility and affinity to HR1 of HIV-1; addition of MT-hook and IDL-anchor to the N-terminus and C-terminus of HR2 peptides strengthened their binding to the pockets on the HR1 trimer of HIV-1 (Su et al. 2017). Linking a lipid group (e.g., fatty acids, cholesterol, sphingolipids) to HR2 peptides can significantly extend their potency and half-life by condensing peptide concentration on the cell surface where HIV particles fuse into host (Su et al. 2019). For example, Xie et al. conjugated a maleimidopropionic acid (MPA) group to a C34 variant. The resulting lipopeptide, FB006M (albuvirtide), could bind to human serum albumin, and it displayed a half-life of more than 100 h in rhesus monkeys (Xie et al. 2010). In 2018, albuvirtide was approved by the China Food and Drug Administration for weekly application in HIV-1 patients. Other approaches, such as conjugation of an albumin-binding domain or IgG Fc-binding motif, have also proved effective in prolonging the half-life of HR2 peptide in vivo (Bi et al. 2019). Since the HR2 region is usually conserved among species of the same viral genus, Jiang's group modified a HCoV-OC43 HR2-derived peptide with E-K salt bridge. The resulting peptide, EK1, inhibited the infection of all circulating human coronaviruses tested by targeting the HR1 domains of human coronaviruses (HCoVs) (Xia et al. 2019). Similarly, modification of EK1 by adding a lipid group (EK1C4) further

improved its potency. EK1C4 showed prophylactic and therapeutic efficacy against HCoV-OC43 and SARS-CoV-2 infection in susceptible mouse models (Xia et al. 2020a). Several α-helical peptides/lipopeptides from de novo design also showed antiviral activity by binding to viral HR1/HR2 (Wang et al. 2018; Zhu et al. 2015).

Aside from HR1/HR2 domain, other regions of TmS can also serve as antiviral targets. For example, a natural C-proximal segment of α1-antitrypsin, designated virus-inhibitory peptide (VIRIP), can inhibit HIV-1 infection by targeting FP (Munch et al. 2007). Monotherapy of a VIRIP variant, VIR-576, in treatment-naive, HIV-1-infected individuals reduced the plasma viral load without causing severe adverse effects in a clinical trial (Forssmann et al. 2010). A natural virucidal peptide, urumin, can physically destroy influenza virions by specifically targeting the conserved region of IAV TmS (Holthausen et al. 2017).

The SfS of viral fusion protein is responsible for binding between virus and receptor/co-receptor. Therefore, many peptides that mimic the conformation of viral receptor/co-receptor can bind to the viral SfS and thus block viral entry. For example, constructing residues of CD4, the receptor for HIV, to a scorpion toxin scaffold can produce a CD4 mimetic which can target the conserved CD4-binding pocket of HIV-1 SfS and thus inhibit HIV-1 infection (Van Herrewege et al. 2008). Aside from CD4-mimic peptides, peptides able to simulate HIV co-receptor, namely, CC-chemokine receptor 5 (CCR5), also showed inhibitory activity against R5 HIV-1 in vitro (Dogo-Isonagie et al. 2012). Peptides derived from angiotensin converting enzyme 2 (ACE2), the receptor for SARS-CoV and SARS-CoV-2, showed cross-reactivity against both viruses in vitro (Curreli et al. 2020b). Except for the artificial design, a series of peptide libraries or display platforms have also been applied for the screening of SfS-specific inhibitors (Mann et al. 2013; Schutz et al. 2020).

1.2.2 Host Protein or Pathway Targeting

Viral receptors/co-receptors are host proteins with physiological functions and natural ligands. Therefore, natural ligands themselves, or peptides derived from natural ligands of these receptors/co-receptors, are also able to inhibit viral entry (Heon Lee et al. 2019; Zhang et al. 2020). In addition, peptides that interfere with endocytosis showed broad antiviral activity. For instance, peptides derived from mouse β-defensin-4, P9, and P9R exhibited broad-spectrum antiviral effects against multiple respiratory viruses in vitro and in vivo, including SARS-CoV-2, SARS-CoV, MERS-CoV, as well as IAV H1N1, H3N2, H5N1, H7N7, and H7N9 (Zhao et al. 2020). The antiviral activity of P9R results from the high-affinity binding to viral glycoproteins and the inhibition of host endosomal acidification because of its abundance of basic amino acids. As mentioned above, viral fusion protein requires activation by proteolytic cleavage. The peptide aprotinin, a broad-spectrum inhibitor of serine proteases that inhibits TMPRSS2, can inhibit infection of SARS-CoV-2, IAVs, and Sendai virus in vitro and in vivo (Schutz et al. 2020). Generally, host protein-targeting antivirals are more broadly effective because different viruses may use a common host pathway. However, this kind of inhibitor interferes with the host's physiological function, thus requiring more comprehensive evaluation.

1.2.3 The Future of Peptide-Based Virus Entry Inhibitors

Peptides and proteins both consist of amino acids. Peptides usually contain 2–50 amino acids, whereas proteins are made up of 50 or more amino acids. Because they are larger in size than small molecules, typically less than 900 Da, they can precisely and specifically bind to the target proteins, resulting in higher potency and fewer

side effects. In addition, the metabolites of peptides and proteins are amino acids which are generally safe. Meanwhile, the shorter length of peptides allows them to be chemically synthesized, as opposed to proteins that are generally expressed in yeast or mammalian cells, which can save the cost and time involved in the manufacturing of peptide-based drugs. Besides, the smaller size of peptides compared to proteins reduced their potential to induce host immune responses against exotic agents.

However, the instability of peptides is a major difficulty in the development of peptide-based drugs. First, peptides can degrade rapidly in vivo, thus, dramatically reducing their plasma half-life. Second, peptides are rarely orally available because they can be digested into amino acids by pepsin or other host proteases easily. Furthermore, even though peptides can resist proteolysis, the high polarity and molecular weight of peptides limit intestinal permeability, which will, in turn, significantly limit their accessibility to the circulatory system and the target tissue (Schutz et al. 2020).

As previously observed, many approaches have been applied to improve the potency and breadth of peptide-based antivirals, including amino acid modification and conjugation of functional moieties. Some of these modifications, such as addition of lipid group, albumin-binding domain, or Fc-binding motif, are able to prolong the half-life of peptides. Formulation of staple peptides, cyclic peptides, or incorporation of non-natural amino acids, can lower the degradation speed and may render the peptides resistant to digestive enzymes, thus improving the in vivo stability and oral availability of peptides (Lau and Dunn 2018). The manufacturing of nanoparticles containing peptides or conjugation to carrier molecules can increase intestinal permeability and ability to enter the central nervous system. The formulation of novel strategies that can increase the half-life of peptides is the future trend of peptide-based entry inhibitors. Long-acting peptides that reduce injection frequency make injection a more acceptable route of administration, making oral unavailability moot.

1.3 Small-Molecule-Based Virus Entry Inhibitors

1.3.1 Viral Fusion Protein Targeting

Small-molecule drugs are cheap, and most are available orally; therefore, they still account for most drugs approved in the past three decades. Many scientists have tried to use small molecules to mimic the function of peptides or protein drugs. Viral SfS is a widely used bait to screen small-molecule-based antivirals. Based on a prescreening method measuring competitive inhibition against the attachment of antibodies specific for HIV-1 SfS, Jiang's group found that porphyrin derivatives could block HIV-1 entry (Neurath et al. 1992). From then on, this and other groups screened out a series of small-molecule-based HIV-1 entry inhibitors, the inhibitory activity of which was modestly improved (Curreli et al. 2020a). More importantly, they were able to stabilize the CD4-induced structure of HIV-1 Env, resulting in better recognition by many anti-HIV-1 antibodies (Ding et al. 2019; Kobayakawa et al. 2019). The Bristol-Myers Squibb Company has been one of the leading forces in developing small-molecule-based entry inhibitors. Fostemsavir (Rukobia, BMS-663068) reduced the viral load in patients with multidrug-resistant HIV-1 infection in a clinical trial (Kozal et al. 2020). Therefore, in 2020, fostemsavir became the first SfS-targeting HIV-1 drug approved by the U.S. FDA. Except for HIV, infection of IAV and EBOV can also be inhibited by SfS-targeting small-molecule compounds (Anantpadma et al. 2016; Yu et al. 2014).

TmS is also a popular target for the design of small-molecule-based entry inhibitors. For example, mechanistic studies revealed that a deep hydrophobic pocket on the surface of the HR1-trimer of HIV-1 is vital for HR2 binding, thus making it an ideal target for compound screening. Debnath et al. used a computational tool to screen a database of 20,000 organic compounds and then tested the inhibitory activity of the high-scoring candidates against the formation of 6-HB, Env-mediated cell-cell fusion, as

well as HIV-1 replication. A disulfonic acid derivative, ADS-J1, was identified as effective in inhibiting HIV-1 infection at micromolar concentration (Debnath et al. 1999). Later on, more 6-HB-targeting compounds with higher potency against infection of HIV-1 and other viruses, such as EBOV and IAV, were identified (Jiang et al. 2011; Si et al. 2018; Yin et al. 2018). An approved influenza drug, Umifenovir (arbidol), was reported to bind to a specific hydrophobic pocket in the upper region of IAV TmS to block viral entry (Kadam and Wilson 2017). FP of TmS can also be targeted by small-molecule-based entry inhibitors against HIV-1 (Murray et al. 2010) and EBOV (Zhao et al. 2016). The development of antibody against the membrane proximal external region (MPER) of viral TmS has advanced, whereas MPER-specific small-molecule-based antivirals are rare. Very recently, Xiao et al. screened a library of 162,106 compounds through an in vitro high-throughput assay seeking compounds that compete with antibody binding to MPER. They identified dequalinium, an FDA-approved antimicrobial drug, which specifically inhibited HIV-1 Env-mediated cell–cell fusion by partially inserting into a hydrophobic pocket formed exclusively by the MPER residues, thereby blocking the conformational change of Env (Xiao et al. 2020).

1.3.2 Host Protein or Pathway Targeting

Maraviroc (Selzentry, UK427857), a derivative of an imidazopyridine CCR5 ligand, is the first FDA-approved co-receptor-targeting antiviral for the treatment of HIV infection. Notably, except for inhibitory activity against HIV-1, the blockade function of CCR5 by these CCR5 antagonists may also be beneficial for use against other immunological diseases, such as acute graft versus host disease (Khandelwal et al. 2020) and autoimmune encephalitis (Karampoor et al. 2020). Another CCR5 antagonist, cenicriviroc (TBR-652, TAK-652), with good bioavailability and potency against HIV-1 infection, recently entered a phase II clinical trial to evaluate its effects on arterial inflammation in people living with HIV (NCT04334915). The additional effects of receptor/co-receptor antagonists do not always bring benefits and may also affect the safety of the drug. The first identified CXC-chemokine receptor 4 (CXCR4)-specific compound for HIV treatment, AMD3100, is an approved drug for the mobilization of hematopoietic stem cells for cancer patients. However, the clinical development of AMD3100 as an HIV drug was suspended because of weak efficacy and the development of cardiac abnormalities (Hendrix et al. 2004).

When an emerging virus suddenly causes an epidemic, it is a widely adopted practice to identify specific antivirals from various libraries containing approved small-molecule-based drugs. Empirically, many of the effective drugs that have been screened out are inhibitors against endocytosis or host protease. An endocytosis modulator, apilimod, and a clinical drug, colchicine, are effective in inhibiting infection of SARS-CoV-2 and EBOV (Anantpadma et al. 2016; Ou et al. 2020). Furin inhibitors have shown broad-spectrum antiviral activity against SARS-CoV-2, HIV, IAV, MERS-CoV, and EBOV (Bestle et al. 2020; Millet and Whittaker 2014). TMPRESS2 and cathepsin L inhibitors, camostat and teicoplanin, both of which are clinically approved drugs, showed potent anti-coronavirus activity (Hoffmann et al. 2020; Zhou et al. 2016).

1.3.3 The Future of Small-Molecule-Based Virus Entry Inhibitors

Small-molecule drugs are easy to manufacture and transport, cheap, and more suitable for oral delivery, making them ideal candidates for application in developing countries that always see the most devastation in global viral pandemics. However, because of the size of small molecules, the in vivo half-life is short, and specificity is poorer than that of peptide and protein-based entry inhibitors.

Currently, the identification of small-molecule-based entry inhibitors mainly depends on the large-scale screening of compound libraries. Different laboratories usually have different screening systems based on different viruses. To cope with more emerging viruses that may suddenly appear in the future, it is better to establish a rapid, stable, and large-scale system that can be applied immediately to screen antivirals against any virus. Lead compounds that have been screened out need further modifications based on structure-activity relationship analysis to improve their potency and other pharmacokinetic features. Therefore, modifications of small-molecule-based entry inhibitors need the assistance of progress in structural biology. Since most clinical drugs are based on small molecules, the repurposing of approved drugs may be a faster way to identify entry inhibitors against emerging viruses. However, many approved drugs showed antiviral activity in an in vitro screening system, but failed in clinical trials (Boulware et al. 2020). Meanwhile, multifunctionality of repurposed drugs also increases the risk of side effects. Therefore, the effectiveness and safety of repurposing drugs for the treatment of emerging viruses also need to be evaluated comprehensively in standardized clinical trials.

1.4 Protein-Based Virus Entry Inhibitors

1.4.1 Viral Fusion Protein Targeting

Viral receptors have been long considered promising antivirals resistant to viral escape since viral-receptor binding is essential for viral infectivity. When CD4 was identified as the receptor for HIV, scientists thought soluble CD4 (sCD4) could be a potential anti-HIV drug by competitively blocking HIV Env-binding to cellular CD4. However, sCD4 demonstrated dual effects on HIV-1 infectivity: inhibitory or stimulative, depending on different sCD4 concentrations, HIV strains, and target cell types (i.e., CD4+ or CD4−), limiting the clinical application of sCD4 in treating HIV infection (Schutten et al. 1995; Sullivan et al. 1998). Later on, more modified, or truncated, CD4, such as PRO-542, showed sole inhibitory activity against HIV-1 (Chen et al. 2011). The receptor for SARS-CoV-2, ACE2-related protein, also showed potent inhibitory activity against SARS-CoV-2 (Guo et al. 2021).

Bifunctional multivalent proteins can be constructed by combining two groups specific for different domains of viral fusion proteins. For example, bifunctional proteins, such as 4Dm2m, consisting of a CD4-binding site-specific domain and a co-receptor binding site-specific domain, showed broadly cross-reactive and exceptionally potent neutralizing activity against different subtypes of HIV-1 (Chen et al. 2014). Lu et al. generated a bivalent chimeric protein, 2DLT, by fusing T1144, a HR2-peptide, with truncated CD4. The 2DLT is highly effective in neutralizing HIV-1, and, more importantly, it can be produced in large quantities in an E. coli system, which can markedly reduce its manufacturing cost (Lu et al. 2012). These bispecific proteins simultaneously recognize two epitopes, making them more resilient to viral resistance than antivirals specific for a single target.

Several proteins originated from natural products have broad-spectrum antiviral activity. Back in the 1990s, Jiang et al. identified that a casein (isolated from milk) modified by aromatic acid anhydrides (AAA), 3-HP-β-LG, could block the interaction between CD4 and HIV Env, thus inhibiting HIV-1 infection (Neurath et al. 1995). Later on, a large number of AAA-modified proteins was found to be effective in inhibiting IAV and other viral infections (Li et al. 2017). The mechanism that underlies the broad effectiveness of AAA-modified proteins remains elusive, but it could be attributed to the abundance of surface negative side chains. Some plant lectins, such as Cyanovirin-N (CV-N), isolated from the cyanobacterium Nostoc ellipsosporum (Boyd et al. 1997), and Grithsin, isolated from red algae (Mori et al. 2005), also showed antiviral activity against many enveloped viruses, including HIV, IAV, and EBOV (McFadden et al.

2007). The above proteins are prepared from cheap resources and have broad antiviral activity, prompting extensive investigation for development as microbicides for prophylaxis and treatment of viral diseases in underdeveloped countries. However, the above proteins, as well as the related peptides, face challenges driven by the emergence of drug-resistant mutations and rapid clearance in vivo. In addition, exogenous proteins are likely to induce specific antibodies in vivo, which would counteract inhibitory activity and shorten half-life, limiting clinical use as therapeutic agents.

In the 1940s, serum and plasma from convalescent patients were found to be effective in treating communicable diseases (Rambar 1946). The main effective constituent in convalescent serum and plasma is pathogen-specific antibodies. Antibody is also a special group of antiviral proteins produced by the host immune system and able to specifically bind to the viral fusion protein and disrupt viral entry. In the past decade, different antibody isolation platforms have been well established, and as a result, more and more potent antiviral antibodies have been identified. Except for direct blocking of viral entry, antibodies can mediate many other immune effects, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADPC), to eradicate virus and virus-infected cells. Therefore, antibodies, which can be strictly classified as viral entry inhibitors, will not be comprehensively discussed, either here or in the chapters ahead.

1.4.2 Host Protein or Pathway Targeting

In 1992, Moore et al. identified a murine antibody 5A8 targeting CD4. It inhibited HIV-1 infection by blocking sCD4-induced conformational changes, while not interfering with CD4-HIV Env interaction. In a phase III clinical trial, mono-administration of a humanized antibody based on 5A8, ibalizumab (TMB-355 or TNX-355), reduced viral load significantly in 83% of the tested patients with multidrug-resistant HIV-1 infection who had advanced disease and limited treatment options (Emu et al. 2018). Ibalizumab was approved by the U.S. FDA in 2018 for biweekly intravenous administration in combination with other ARTs in heavily treatment-experienced adults failing their current antiretroviral regimen. Similarly, co-receptor-targeting proteins are effective in inhibiting viral entry (Cocchi et al. 1995). Another group of proteins inhibit viral entry by regulating viral receptor expression, rather than direct binding, as exemplified by the downregulation of ACE2 expression by selective estrogen receptor modulators (Zhou et al. 2020).

1.4.3 The Future of Protein-Based Virus Entry Inhibitors

Protein-based antivirals, similar to peptide-based antivirals, are able to mimic natural proteins vital for viral infection and, hence, somehow resilient to viral resistance. Because of the larger size of proteins, compared with peptides or small-molecule compounds, they can bind to target protein with higher specificity, potentially reducing the risk of side effects. However, the large size of proteins also increases the risk of inducing drug-specific host immune response, which will significantly impair drug effectiveness. Besides, the manufacture of proteins, especially antibodies, is cost- and time-consuming. Also, cold-chain transport is often needed, further increasing cost. Another disadvantage is digestion of protein by pepsin, or other host protein, thus limiting protein-based drugs to parenteral use. Because of the high cost of the protein-based antiviral drugs, approval is rare. On the other hand, the relatively long in vivo half-life of antibodies, compared with peptide and small-molecule compounds, has encouraged researchers to search for long-acting antivirals for the treatment of persistent infection. Therefore, the trendline in the development of protein-based viral entry inhibitors depends on further extending half-life and, thus, reducing the frequency of medication.

1.5 Conclusion and Prospect

In 2003, the approval of enfuvirtide for the treatment of HIV-1 marked the beginning of viral entry inhibitors as clinical antiviral drugs. Virus entry inhibitors can halt the first step of the viral life cycle and defeat viruses outside host cells, thus avoiding maximal damage to host cells. Viral fusion proteins, including TmS and SfS, are the most investigated targets for the development of virus entry inhibitors. Generally, viral TmS is much more conserved than SfS and thus a more desirable target for broad-spectrum entry inhibitors. Nevertheless, viral SfS is more exposed on the cell surface, whereas most epitopes in TmS are sheltered before receptor binding (Kwon et al. 2015). Therefore, SfS-targeting antibodies that can neutralize free virions and infected cells have been extensively investigated. Construction of bispecific proteins or antibodies consisting of both SfS- and TmS-reactive agents exploits the advantages of both. Host proteins, including viral receptor/co-receptor, host protease, and endocytosis modulator, are vital for virus entry and can be targeted to inhibit a broad range of viruses. However, the physiological function of these host proteins may be activated or impaired by host protein-targeting entry inhibitors.

Except for antibodies authorized for emergency use during times of epidemic, such as SARS-CoV-2 and EBOV, the virus entry inhibitors in current clinical use are mainly HIV drugs. Five approved HIV-1-specific entry inhibitors, including HIV TmS-targeting enfuvirtide and albuvirtide, HIV SfS-targeting fostemsavir, CD4-reactive ibalizumab, and CCR5-associated maraviroc, happen to cover all three categories, peptide, protein (antibody), and small molecule, of virus entry inhibitors that have been introduced in this chapter. Small-molecule-based entry inhibitors are cheap, but less safe because of their relatively poor specificity and ability to enter cells. Protein-based entry inhibitors are safe, but much more expensive. Peptide-based entry inhibitors are more balanced between cost and safety. Therefore, it is hard to tell which category is most ideal for treatment of viral disease, and these three formulations are simultaneously under extensive development. A major strength of small-molecule-based entry inhibitors is their oral availability, whereas peptides and proteins currently need parenteral injection. Therefore, multiple approaches to facilitate oral delivery of peptides and proteins are being pursued, including nanoparticle carriers, permeation enhancers, intestinal patches, methods to combat enzymatic degradation and microneedle delivery devices (reviewed in (Drucker 2020)).

Extending the in vivo half-life of virus entry inhibitors can reduce the frequency of medication and thus ease the inconvenience caused by the parenteral injection, and efforts in this direction are proceeding in order to improve drug adherence. As noted above, addition of lipid- and Fc-binding group to HR2-peptides and introduction of mutations to antibodies substantially extended their in vivo half-life (Ko et al. 2014). More methods, such as increasing hydrodynamic volume and implementing neonatal Fc-receptor-mediated recycling processes, have also been adopted (Kontermann 2009). However, RNA viruses mutate easily and account for most emerging viruses that have historically caused significant damage. Therefore, another trend is the development of broad-spectrum antiviral entry inhibitors. Identification of conserved target and combination of inhibitors for multiple targets are the main two approaches toward achieving broad-spectrum virus entry inhibitors.

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Peptide-Based HIV Entry Inhibitors

2

Jing Pu, Qian Wang, and Shibo Jiang

Abstract

The development of peptide-based HIV entry inhibitors has made an important contribution to the stock of anti-HIV drugs. In particular, the peptide-based anti-HIV drugs enfuvirtide and albuvirtide were approved for clinical use by the U.S. FDA and CFDA in 2003 and 2018, respectively. Peptide-based HIV entry inhibitors exert antiviral activity by targeting the early stage of viral infection, i.e., binding of a viral surface protein to the receptor(s) on the host cell and the subsequent fusion between the viral and host cell membranes. Therefore, they are particularly useful for HIV-infected patients who have failed to respond to the highly active antiretroviral drugs (ARD) targeting the late stage of HIV replication, such as reverse transcriptase inhibitors and protease inhibitors. In this chapter, we will focus on the past, current, and future trends in research and development of peptide-based HIV entry inhibitors.

Keywords

Peptide · Lipopeptide · HIV · Entry inhibitor · Membrane fusion · 6-HB

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2.1 Introduction

The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) is composed of a heterodimer of receptor binding protein gp120 and fusion protein gp41, which form a trimer and distribute on the surface of the virus membrane. Gp41 consists of fusion peptide (FP), N-terminal heptad repeat (NHR), C-terminal heptad repeat (CHR), membrane-proximal external region (MPER), transmembrane region (TM), and cytoplasmic region (CP). In the process of HIV-1 infecting a target cell, gp120 binds to CD4 receptor and CXCR4 or CCR5 co-receptor on the cell surface, triggering conformational changes of gp41. FP inserts into the target cell membrane. NHR and CHR fold each other in an anti-parallel manner to form a six-helix bundle (6-HB) structure with NHR trimer as the inner core and CHR trimer outside. This reverse folding shortens the distance between viral membrane and cell membrane, leading to the formation of a fusion hole, and then HIV-1 releases the genome into target cell, opening the subsequent life cycle phase. At present, the development of effective HIV-1 inhibitors is still one of the daunting challenges facing the scientific world. According to UNAIDS statistics, 37.7 million people were living with HIV in 2020 in the world (https://www.unaids.org). However, the pace of progress in reducing new HIV infections, increasing access to treatment, and ending AIDS-related deaths is slowing down. Moreover, mutants with resistance

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to reverse transcriptase inhibitors and protease inhibitors, which are the main components of the highly active antiretroviral drugs (ARDs) therapeutic regimen, continue to appear (Ávila-Ríos et al. 2016). These facts call for the urgent development of inhibitors that act on different targets. Viral entry inhibitors do not target reverse transcriptase or protease, but rather interact with viral surface proteins or cellular receptors to block virus fusion with or entry into the host cells (Fig. 2.1). T20 is the first peptide-based HIV-1 entry inhibitor approved by the U.S. FDA for treatment of patients who have failed to respond to ARD therapy (Lalezari et al. 2003). However, its clinical application is limited by low potency and short half-life. Therefore, T20 must be injected twice a day at 90 mg per injection, causing severe injection site reactions and high cost to patients (Maggi et al. 2004). To overcome these weaknesses, several groups of scientists have optimized the sequence and structure of T20 to generate peptides with improved antiviral activity, longer half-life, and higher genetic barrier to resistance (Chong et al. 2018b; Ding et al. 2017; Xu et al. 2019; Zhu et al. 2019). Here, we summarize the progress in research and development of peptide-based HIV entry inhibitors either directly targeting the HIV-1 gp41 and gp120 or receptors on the host cell.

2.2 Peptides Targeting Cellular Receptors

Some peptides derived from the HIV-1 gp120 V3 loop were found to have anti-HIV-1 activity in early studies (Nehete et al. 1993; Yahi et al. 1994). Later, Sakaida et al. found that cyclized V3 peptide, V3-BH10, V3-ELI, and V3-89.6, derived from the gp120 V3 region of the T-tropic HIV-1 IIIB and ELI strains and the dual tropic HIV-1 89.6 strain, respectively, could inhibit T-tropic HIV-1 infection. These peptides have been shown to function by directly binding to the CXCR4 co-receptor (Sakaida et al. 1998). However, Rossi et al. found that some peptides derived from the V3 region of gp120 did not inhibit HIV-1 infection, but rather enhanced it, possibly by promoting the expression of CD4 and inhibiting the downregulation of CD4 receptor (De Rossi et al. 1991). Similarly, Zanotto et al. found that the DB3 peptide derived from the V3 region in gp120 of HIV-1 MN strain and its mutant peptide DB3-N19 could increase the expression of CD4 and enhance the binding of gp120 to CD4, leading to the promotion of HIV-1 infection (Zanotto et al. 1995). Therefore, the development of peptides targeting the cellular receptor has been discontinued.

2.3 Peptides Targeting Virus

2.3.1 Peptides Targeting CD4-Binding Site (CD4bs) or Co-Receptor-Binding Site (CoRbs) in the HIV-1 gp120

Since HIV-1 infection begins with the binding of gp120 to the receptor and then co-receptor on the host cell, gp120 is considered to be the primary target for the development of HIV entry inhibitors. Some CD4-mimetic peptides are effective HIV entry inhibitors by targeting the CD4bs in gp120. Vita et al. transferred the side chains of nine amino acids in the gp120-binding site in the CDR2-like loop of CD4 to the structural homologous region of the scorpion toxin scaffold to construct a CD4-mimic peptide, CD4M3, and then its mutant peptide CD4M9. They found that CD4M9 could bind to CD4bs in gp120 with nanomolar affinity to inhibit HIV-1 infection at submicromolar to low micromolar levels (Vita et al. 1999). According to structural characteristics of the CD4-gp120-17b complex, this group further optimized CD4M9 to generate its mutant peptide CD4M33 with much improved anti-HIV-1 activity at low nanomolar level (Martin et al. 2003). After analyzing the crystal structures of CD4M33 and gp120 complex, Huang et al. replaced the thiopropionic acid in CD4M33 with biphenylalanine to generate a new derivative, F23. They found F23 to be more similar to CD4 than CD4M33 in terms of structural and thermodynamic analyses. While it showed broader spectrum of HIV-1, HIV-2, and SIVcpz

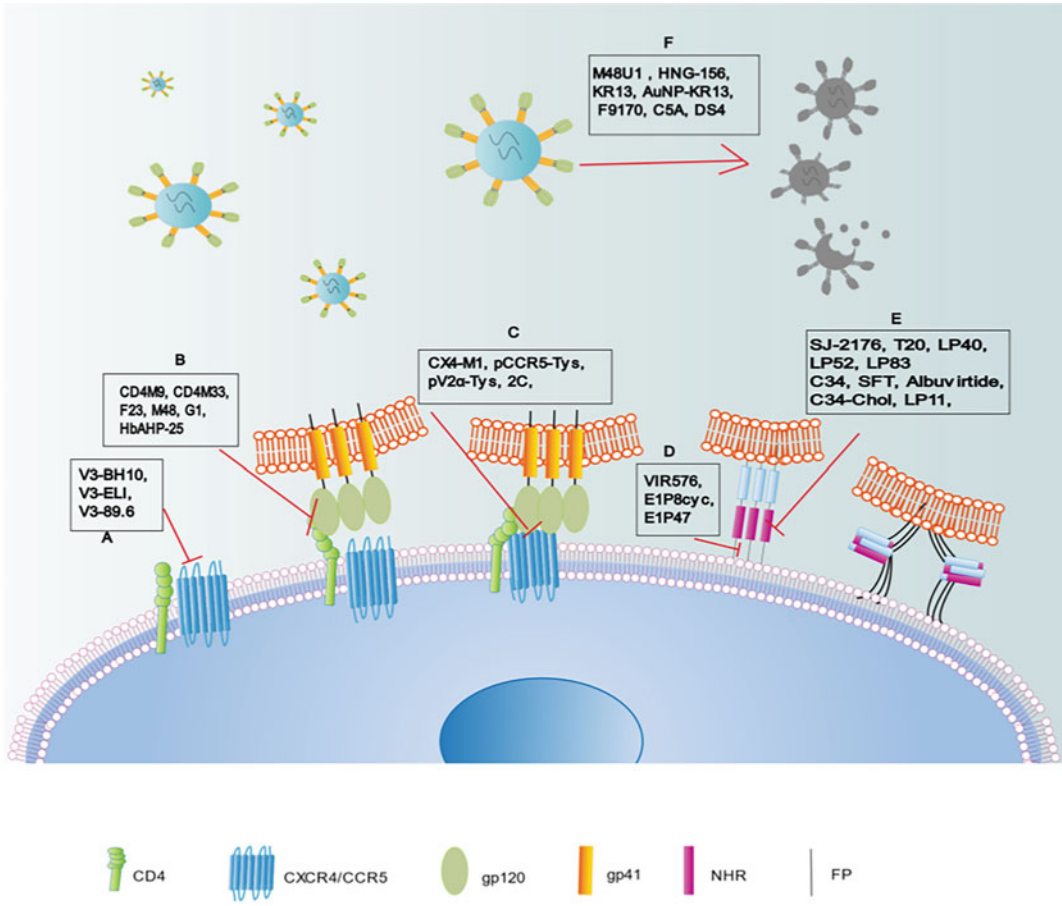

Fig. 2.1 Peptide-based HIV entry inhibitors targeting different sites in the viral surface proteins or cellular receptors. (**a**) Peptides targeting co-receptor. (**b**) Peptides targeting CD4-binding site in gp120. (**c**) Peptides targeting the co-receptor-binding site in gp120. (**d**) Peptides targeting FP. (**e**) CHR-peptides targeting NHR in gp41. (**f**) Peptides inactivating cell-free virions

inhibitory activity, it also had reduced inhibitory potency (Huang et al. 2005). Taking the affinity between CD4M33 and gp120 as a reference, Van Herrewege et al. identified two analog peptides, M47 and M48, from a synthetic peptide library and found that their binding affinity to gp120 was 2- to 10-fold higher than that of CD4M33 (Van Herrewege et al. 2008). Using phage display screening technology, Choi et al. identified a G1 peptide and its derivatives with specific binding to gp120, thereby blocking the interaction between gp120 and CD4. However, their anti-HIV-1 activity has not been reported (Choi et al. 2001).

Groß et al. designed a CXCR4-mimic peptide, CX4-M1, which presents the three extracellular loops (ECL) of the CXCR4 co-receptor (Möbius et al. 2012). CX4-M1 selectively binds to the gp120 of the X4-tropic HIV-1 strains and inhibits infection of X4-tropic HIV-1 strains NL4-3 and Hxbc2 at low micromolar concentration (Groß et al. 2015; Möbius et al. 2012).

Cormier et al. found that some tyrosine-sulfated peptides derived from the N-terminal (Nt) domain of the co-receptor CCR5 exhibited antiviral activity against HIV-1 R5 isolates (Cormier et al. 2000). pCCR5-Tys, a tyrosine-sulfated peptide derived from the N-terminus of

CCR5, inhibits interaction of the gp120/sCD4 complex with CCR5 and has inhibitory activity against R5-tropic HIV-1 infection (Farzan et al. 2000). pV2α-Tys, which is an 18-mer tyrosine-sulfated peptide derived from the V2 region in gp120 of R5-tropic HIV-1 strain Bal, can bind to the CCR5 N-terminal binding site in gp120 and block binding of gp120 to CCR5 co-receptors. Unlike CCR5 N-terminal peptides (e.g., pCCR5-Tys) that inhibit infection of only R5-tropic viruses, pV2α-Tys has broad-spectrum antiviral activity against divergent HIV-1 strains, regardless of their usage of the co-receptor (Cimbro et al. 2016). Dogo-Isonagie et al. found that peptides derived from the C-terminus of CCR5 ECL2 bound to the conserved sites in gp120 of both X4- and R5-tropic HIV-1 strains in a CD4-independent manner, while the binding of CCR5 N-terminus to gp120 requires CD4 activation. The 14-mer peptide 2C derived from the C-terminus of CCR5 ECL2 inhibits the entry of R5- and X4-tropic HIV-1 strains into the target cell at low micromolar concentration (Dogo-Isonagie et al. 2012).

In addition to receptor- and co-receptor-mimetic peptides, some other peptides, such as HbAHP-25, an analog of human hemoglobin (Hb)-α-derived peptide, can also directly act on gp120 to inhibit its binding to CD4. This peptide can inhibit HIV-1 infection at the micromolar level (Bashir et al. 2015). Natural cationic peptides, alpha-defensin-1 and -2, inhibit gp120-CD4 interaction by targeting the D1 region of CD4 and a surface contiguous with the CD4bs and CoRbs sites in gp120, thus having broad-spectrum anti-HIV-1 activity at low micromolar concentrations (Furci et al. 2007).

2.3.2 Peptides Inactivating Cell-Free Virions

In order to increase the binding affinity of a peptide with the Phe-43 cavity on the HIV-1 gp120, a compound that can specifically bind to Phe-43 was introduced to the peptide M48 to generate its derivatives, M48U1, M48U2, and M48U3. These derivative peptides make up for the insufficient inhibitory activity of the parent peptide CD4M33 and/or M48 on the HIV-1 clade C strains (Van Herrewege et al. 2008). Further studies have shown that the coincubation of M48U1 with pseudovirions can cause the shedding of gp120, resulting in the inactivation of the viral particles (Selhorst et al. 2013). Peptide 12p1, which was identified through screening a phage display peptide library, can inhibit the binding of gp120 to sCD4 and to antibodies targeting the CoRbs in gp120 (Ferrer and Harrison 1999). Furthermore, it can directly bind to gp120 and allosterically block gp120-CD4 interactions, thus inhibiting the ability of gp120 to interact with CD4 and co-receptors (Biorn et al. 2004). Although the anti-HIV-1 activity of 12p1 is at the micromolar level, it may function as an HIV-1 inactivator, making it uniquely attractive. Based on its characteristics, many peptide triazoles (PTs) have been studied. For instance, Gopi et al. designed a class of PTs in which the anti-HIV-1 activity of peptide 4 reaches the nanomolar level, about 1000-fold more potent than 12p1 (Gopi et al. 2008). They also reported another 12p1 derivative, HNG-156, which contains ferrocenyl triazole-substituted conjugate able to cause gp120 shedding, thus showing a stronger broad-spectrum HIV-1 inhibitory activity than 12p1 (Gopi et al. 2009; McFadden et al. 2012). Umashankara et al. minimized the structure of HNG-156 and found a 5-amino acid peptide (aa3-aa7) that retains the characteristics of the parent peptide's high gp120 affinity, high antiviral activity, dual receptor antagonism, and induction of gp120 allostery (Umashankara et al. 2010). Aneja et al. also designed some sequence-minimized peptides containing a ferrocenyl-triazolePro amino acid residue. They found that these peptide triazole inactivators used a two-site PT-gp120 binding model that could inhibit the binding of CD4 receptors and co-receptors to gp120 and destabilize the conformation of gp120 (Aneja et al. 2015). Bastian et al. added a cys-SH (sulfhydryl) group to the C-terminus of HNG-156 to generate HNG156 analogs, KR13 and AuNP-KR13, which are multivalent gold nanoparticle (AuNP) conjugates of KR13. AuNP-KR13 is about 20-fold more potent than

KR13 in inhibiting HIV-1 Bal pseudovirus infection (Bastian et al. 2011). Different from HNG-156 that can induce gp120 shedding, KR13 and AuNP-KR13 can also specifically disrupt the cell-free HIV-1 particles, resulting in the release of p24 protein in a dose-dependent manner. AuNP-KR13 has stronger virolytic activity than that of KR13 (Bastian et al. 2011, 2013). Furthermore, the antiviral effect of AuNP-KR13 is significantly enhanced by increasing the diameter of AuNP-KR13 and/or peptide density on the surface of AuNP, reaching low pM levels. Therefore, the enhanced antiviral efficacy of AuNP-KR13 can be attributed to the multivalent contact between the virus and the surface of the nanoparticle (Rosemary Bastian et al. 2015). In sum, these PT-based HIV entry inhibitors can induce viral inactivation by inducing gp120 shedding and/or lysing the viral membrane. However, other peptides that target both CD4bs and CoRbs may also act as HIV-1 inactivators. Baleux et al. conjugated heparan sulfate (HS) to a CD4 mimetic peptide, mCD4 (sequence identical to CD4M33), to generate the peptide conjugate mCD4-HS12 able to bind gp120 through its mCD4 component, inducing exposure of the CoRbs accessible by HS. Although mCD4-HS12 has low nanomolar inhibitory activity against HIV-1 R5, X4, and R5/X4 strains, its inactivating activity has not been tested (Baleux et al. 2009). Next, this group coupled mCD4 with heparan sulfate mimicking tridecapeptides to design a series of peptides, among which mCD4-P3YSO3 inhibited infection by HIV-1 clinical strains at picomolar to low nanomolar levels (Connell et al. 2012). By screening a library of synthetic peptides derived from HIV-1 Env, Wang et al. have identified a 15-mer amphipathic peptide F9170, which partially overlaps the sequence of HIV-1 cytoplasmic region LLP3. F9170 specifically targets the LLP1 intracellular region of the transmembrane protein gp41 and inactivates cell-free HIV-1 virions by disrupting the integrity of the viral membrane (Wang et al. 2020) The broad-spectrum virus inactivator C5A is an amphipathic α-helical peptide derived from hepatitis C virus (HCV) NS5A. It inactivates HIV-1 by destroying the integrity of the viral membrane and capsid core, resulting in the release of nucleic acid, but does not affect the integrity of the host cell membrane (Bobardt et al. 2008). Some peptides derived from amphibians, such as DS4 peptide and its mutant peptide K4-S4 (Lorin et al. 2005), as well as caerin 1 family peptides and synthetic analogs, caerin 1.9 and caerin 1.1 mod 9, can also disrupt the HIV envelope and release p24 protein (VanCompernolle et al. 2005, 2015).

2.4 Peptides Blocking Virus-Cell or Cell-Cell Fusion

2.4.1 Peptides Targeting Fusion Peptide (FP) in gp41

VIRIP, a 20-mer peptide derived from C-proximal region of α-antitrypsin, exerts anti-HIV-1 activity by targeting FP in gp41 to inhibit its insertion into cell membrane. Its derivative VIR-576 has much higher anti-HIV-1 activity than VIRIP, possibly because of the additional hydrophobic residues that enhance its interaction with FP and a cysteine bridge that stabilizes the active conformation (Munch et al. 2007). Sánchez-Martínhepatitis et al. found that some peptides derived from the E1 protein of GB virus C (GBV-C), such as P7 and P8, can bind to the gp41 FP and inhibit virus-cell membrane fusion (Sánchez-Martín et al. 2011a, b). E1P8cyc peptide, the cyclized form of P8 peptide, can change the secondary structure conformation of FP, resulting in anti-HIV-1 activity with about 20-fold higher potency than that of the parental linear peptide (Gómara et al. 2014; Galatola et al. 2014). E1 (139-156), namely E1P47, an 18-mer peptide derived from the E1 protein of GBV-C with a helix-turn-helix structure, has broad-spectrum inhibitory activity against HIV-1 infection at low micromolar levels by targeting FP (Gómara et al. 2016; Pérez et al. 2017). In addition, some E2 protein-derived peptides could inhibit HIV-1 infection at the micromolar

concentration by suppressing HIV-1 FP-mediated membrane fusion (Herrera et al. 2010).

2.4.2 Peptide Blocking Six-Helix Bundle (6-HB) Formation

Formation of 6-HB between NHR and CHR of the HIV-1 gp41 is an important process for fusion between viral and cell membrane or between infected and uninfected cells. Therefore, both NHR and CHR can serve as important targets for the development of peptide-based HIV fusion/entry inhibitors. Theoretically, the peptides derived from the NHR and CHR of gp41 are expected to interact with the HIV-1 gp41 CHR and NHR, respectively, to form 6-HB and block HIV-1 gp41 6-HB formation, thus inhibiting virus-cell fusion. However, several studies have shown that NHR-peptides have a tendency to aggregate in physiological solution, making them less effective than CHR-peptides in suppressing 6-HB formation and virus-cell fusion.

In 1993, Jiang et al. reported the first CHR-peptide, SJ-2176, with highly potent anti-HIV-1 activity (Jiang et al. 1993). In 1994, Wild et al. reported the second CHR-peptide, DP-178, that also has potent inhibitory activity against HIV-1 infection (Wild et al. PNAS 1994). Subsequently, Trimeris, Inc. began its development of DP-178 (renamed as T20) after licensing US patent #5444044 from Jiang's group at the New York Blood Center because T20 has 22 amino acids identical to those in SJ-2176. T20 (generic name: enfuvirtide, brand name: Fuseon) was approved by the U.S. FDA in 2003 for use in patients who have failed to respond to highly active antiretroviral drugs.

In 1995, Lu et al. reported that the CHR-peptide C34, which overlaps 100% and 67% of SJ-2176 and T20 sequence, respectively, was highly effective in inhibiting HIV-1 infection by blocking virus-cell fusion (Lu et al. 1995). X-ray crystallographic analysis has demonstrated that C34 binds to the NHR-peptide N36 through interaction between C34's pocket-binding domain (PBD) and N36's pocket-forming domain (PFD) to form a highly stable 6-HB (Chan et al. 1997). Therefore, C34 and SJ-2176 inhibit gp41-mediated membrane fusion by binding to viral gp41 NHR and blocking 6-HB formation between viral gp41 NHR and CHR. Unlike C34 and SJ-2176, T20 does not have PBD at its N-terminal region, but has a tryptophan-rich motif (TRM) at its C-terminal region. Thus, T20 binds to the NHR and the fusion peptide proximal region (FPPR) of gp41 through its N-terminal fragment and C-terminal TRM, respectively, and weakly blocks 6-HB formation in the early stage of virus-cell membrane fusion (Xu et al. 2019). These findings may explain why T20 has much lower anti-HIV-1 activity than C34. Xu et al. added another TRM to the C-terminus of T20 to generate the peptide T20-SF, which targets NHR, FPPR, and FP. It has better anti-HIV-1 activity than T20 against divergent HIV-1 strains, including those with resistance to T20 (Xu et al. 2019).

Because of the weakness of T20, Trimeris tried to develop a second-generation HIV fusion inhibitor, T1249, a 39-mer peptide composed of a chimeric partial sequence of CHR in HIV-1, HIV-2, and SIV (De Clercq 2002). It contains the PBD of C34 and the FPPR-binding motif of T20 (Xu et al. 2019). Compared with T20, T1249 has significantly improved anti-HIV-1 activity and is effective against T20-resistant strains (Chinnadurai et al. 2007). However, the clinical trial of T1249 was terminated in 2004 (Martin-Carbonero 2004). Subsequently, Trimeris also tried to develop a third-generation HIV fusion inhibitor, T1144, a peptide analogous to the 38-mer CHR-peptide T-651. Its HIV-1 fusion inhibitory activity is significantly more potent than T20 and T1249 against divergent HIV-1 strains, including those with resistance to T20 and T1249 because T1144 has a higher genetic barrier to resistance and better pharmacokinetic properties (Dwyer et al. 2007). Currently, however, no information is available about its further development. Sifuvirtide (SFT) is a 36-mer peptide designed by modification of the CHR-peptide C34. Like C34, SFT could also bind to N36 to form stable 6-HB and has higher HIV-1 fusion inhibitory activity than T20 (He et al. 2008; Wang et al. 2009; Yao et al. 2012). Its phase I and II

clinical trials have shown promising results, but the phase III clinical trial of SFT has not started yet. Albuvirtide (ABT) is a chemically modified CHR-peptide with 3-maleimidopropionic acid (MPA), which allows quick and irreversible conjugation with serum albumin. Because ABT's half-life is more than 100 h (Xie et al. 2010), its once-a-week injection can significantly improve patient compliance and avoid side effects at the injection site, a limitation of T20 that was injected twice a day. ABT was approved for clinical use by CFDA in 2018.

CHR-peptides, such as C34, have been optimized with different approaches. Chong et al. have found that the two amino acids upstream of the CHR-peptide pocket-binding region, Met-626 and Thr-627, can form a hook-like structure, named M-T hook, which plays an important role in stabilizing the interaction between NHR and CHR and 6-HB conformation (Chong et al. 2012). Subsequently, they added the M-T structure to the N-termini of different CHR-peptides to produce a series of new peptides, such as MT-SC22EK and HP23, with improved anti-HIV-1 activity (Chong et al. 2013, 2015). Su et al. reported that adding an IDL anchor to the C-terminus of the CHR-peptide WQ, or MT-WQ, could effectively enhance antiviral activity of WQ, or MT-WQ (Su et al. 2017b). They extended the sequence of HP23, a short CHR-peptide, by six amino acids at its C-terminus and added an IDL anchor to the C-terminus of HP23-E6 to generate HP23-E6-IDL. They found HP23-E6-IDL to be about 2- to 16-fold more potent than HP23 against a broad spectrum of HIV-1 strains, including those with resistance to HP23 (Su et al. 2017a).

Based on the sequence of T20, a variety of optimized peptides have been developed, especially some lipopeptides modified with fatty acids showing improved anti-HIV-1 activity and prolonged half-life. He's group has developed a series of T20-based lipopeptide inhibitors. For example, they first generated the T20-based lipopeptide LP40 by replacement of T20's C-terminal TRM sequence with the fatty acid C16 (Ding et al. 2017). The fatty acid molecule in LP40 can anchor the peptide in the cell membrane without changing the original binding mode of T20. Then, they generated the T1249-based lipopeptide LP-46 by replacing T1249's C-terminal TRM sequence with fatty acid (Zhu et al. 2018). Based on the structural characteristics of LP40, they introduced multiple pairs of E-K motifs (Otaka et al. 2002) into LP40 to produce LP50 and then further introduced some HIV-2 and SIV sequences into LP50 to get LP51 (Chong et al. 2018a). LP52 was designed by mutating three residues at the N-terminal of LP51 (Chong et al. 2018b). They also found that the length of the fatty acid carbon chain is associated with the antiviral activity of the lipopeptides. LP80, which was designed by replacing C16 in LP52 with C18, exhibits higher anti-HIV-1 activity than that of LP52 (Chong et al. 2019). The lipopeptides LP83 and LP86 were constructed by replacing C16 of LP52 and LP51 with cholesterol, respectively, showing better HIV-1 fusion inhibitory activity than LP52 and LP51 (Zhu et al. 2019). The above T20-based lipopeptides have broad-spectrum anti-HIV-1 activity at picomolar level. They can bind human serum albumin, thus having prolonged half-life. Among them, LP83 inhibits infection of divergent HIV-1 strains with an average IC50 as low as 3 pM, having the potential for once-a-week use in clinics to treat HIV-1-infected patients (Zhu et al. 2019).

A series of C34-based lipopeptides have also been developed. Ingallinella et al. designed C34-chol by coupling cholesterol to C34 through a GSG linker (Ingallinella et al. 2009). Chong et al. designed the lipopeptide LP11 by connecting C16 to the short peptide HP23 through a PEG8 linker (Chong et al. 2016). Su et al. introduced the T639I mutation in HP23-E6-IDL and used GSG and PEG4 linkers to conjugate C16 to the C-terminus of the CHR-peptide YIK in order to finally produce lipopeptide YIK-C16 (Su et al. 2019). All these lipopeptides exhibited significantly improved antiviral activity, prolonged half-life, and enhanced genetic barrier to resistance.

2.4.3 Peptides Targeting the Disulfide Loop Region of gp41

In addition to FP, NHR and CHR on the HIV-1 gp41 as important targets for developing HIV entry inhibitors, the immunodominant disulfide loop located between NHR and CHR of gp41 may also serve as a target. Eissmann et al. found that peptides P4-7 and P6-2 derived from the N-terminus of the GBV-C E2 protein inhibited HIV-1 infection at low micromolar concentration by targeting this region (Eissmann et al. 2013; Koedel et al. 2011).

2.5 Conclusion

HIV entry inhibitors block HIV infection by targeting the viral surface proteins or host cell receptors to interrupt virus fusion with or entry into the host cell. Reverse transcriptase inhibitors or protease inhibitors act at the late stage of virus infection of cells. In contrast, HIV entry inhibitors can act outside the cell, thereby having less negative impact on the viability and biological functions of the host cell. In addition to the peptide-based HIV-1 entry inhibitors targeting the HIV-1 gp41, enfuvirtide and albuvirtide, approved for clinical use by the U.S. FDA and the CFDA, respectively, several small molecule-based HIV entry inhibitors, such as fostemsavir targeting the HIV-1 gp120 and maraviroc targeting the cellular co-receptor CCR5, as well as the antibody-based HIV entry inhibitor targeting the cellular receptor CD4, ibalizumab, have also approved for clinical use by the U.S. FDA. Therefore, peptide-based viral entry inhibitors targeting the HIV-1 gp120 and the cellular receptor and co-receptor are expected to be further developed in the future.

Peptide-based HIV entry inhibitors are lower in cost than antibodies and have higher specificity than small-molecule compounds, making them more attractive to drug developers. The approval of T20 (enfuvirtide) for clinical application advanced the research and development of peptide-based viral entry inhibitors. Its clinical application proved to be limited by its short half-life and low potency. However, further elucidation of its mechanism of action and characterization of its structure and pharmaceutical properties have resulted in the optimization of T20 or the design of new antiviral peptide drugs. After introducing amino acid mutations, M-T hook, IDL anchor, and coupling fatty acids, CHR-peptide-based HIV entry inhibitors have been substantially improved, resulting in the production of new drug candidates with greater antiviral potency and genetic barrier to resistance, as well as extended half-life, which is expected to reduce injection frequency and side effects at the injection site and, thus, its cost. Therefore, more and more anti-HIV peptide drugs with significantly improved potency and pharmaceutical profiles are expected to be approved for clinical use in the future.

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Small-Molecule HIV Entry Inhibitors Targeting gp120 and gp41

3

Fei Yu and Shibo Jiang

Abstract

Interrupting early events in the virus life cycle, such as those prior to the formation of provirus, could effectively prevent HIV-1 infection. HIV-1 entry into host cells is mediated by the envelope glycoprotein (Env) trimer, which is composed of three gp120 exterior glycoproteins and three gp41 transmembrane glycoproteins. Hence, the development of novel inhibitors targeting these two glycoproteins could hold the key to early inhibition of HIV-1 infection. Small-molecule entry inhibitors targeting early events in the virus life cycle comprise a well-established class of useful drugs. Many libraries of small-molecule inhibitors have been established to screen potential drug candidates for a variety of targets based on computer docking, FRET, or peptide-linked assay. This chapter reviews the mechanisms of some small-molecule inhibitors targeting HIV-1 gp120 and gp41 and corresponding high-efficiency screening strategies for potential small-molecule inhibitors.

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Keywords

Virus · Small-molecule inhibitors · Mechanisms of action · Screening strategies

3.1 Structure and Function of gp120 and gp41

HIV-1 infects host cells through membrane fusion triggered by binding between trimeric envelope glycoprotein and CD4 receptor on the cell surface with the help of co-receptor CXCR4 or CCR5. The binding of trimeric gp120 to CD4 sets in motion the rotation of each of three gp120 monomers, resulting in a rearrangement in which the V1V2 loops are relocated to the periphery of the trimer. Such rearrangement provides a model for receptor-triggered entry, and impacts Env sensitivity to antibody- and drug-mediated neutralization (Pancera et al. 2010).

The envelope glycoprotein (Env) of HIV-1 is composed of three gp120 envelope glycoproteins attached to three gp41 transmembrane polyproteins noncovalently. Then, by a single proteolytic cleavage, the stable association between gp120 and gp41 is processed by the polyprotein precursor gp160, corresponding to residues 1–511 and 512–856 of the HIV-1_{HXB2} gp160, respectively. The gp120/gp41 trimer can then be prompted to an irreversible conformational change.

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The HIV-1 gp120 includes five conserved constant regions, C1–C5, and five variable loop regions, V1–V5 (Starcich et al. 1986). The unliganded gp120 appears in a state of conformational equilibrium with its core showing a strong inherent propensity to fit into the CD4-bound conformation (Kwon et al. 2012). This propensity is furthered by gp41 noncovalent interaction and the V1/V2 and V3 variable loops (Huang et al. 2005).

According to the data of X-ray crystallography, Kwong et al. crystallized the overall architecture of gp120 binding to CD4 and the coreceptor mimic 17b by using the deglycosylated truncated core, describing ~60% of the polypeptide (Kwon et al. 2012). The discovery of this structure improved the potency and breadth of small-molecule mimics of CD4 (LaLonde et al. 2012; Madani et al. 2008) and the isolation of several broadly neutralizing antibodies.

The HIV-1 gp41 consists of an ectodomain, a transmembrane domain, and a cytoplasmic domain. The ectodomain contains three important functional regions: N-terminal glycine-rich fusion sequence and two helical regions containing hydrophobic 4-3 heptad (abcdefg) repeats named as the N- and C-helical regions (NHR and CHR where N and C refer to regions situated towards the N- and C-terminus, respectively). Three NHRs form an inner core, and then associate with three CHRs in reverse parallel (Chan et al. 1997). Crystallographic analysis identified that every grooves on the NHR trimer includes a deep hydrophobic pocket (~16-Å long, ~7-Å wide, and 5–6 Å deep), which can accommodate with three conserved hydrophobic residues, W628, W631, and I635, localized to the CHR domain (Chan et al. 1997). The antiparallel 6-HB core plays important roles in membrane fusion (Chan et al. 1998; Ji et al. 1999) and stabilization of gp41 6-HB formation (Dwyer et al. 2003). Hence, this pocket is an attractive target for small-molecule HIV fusion inhibitors (Chan et al. 1997; Ji et al. 1999).

CD4 is a 433 amino acid chain on cell-surface glycoprotein, which is composed of four immunoglobulin-like extracellular domains, a membrane-spanning region, and a cytoplasmic, carboxy-terminal segment (McDougal et al. 1991). HIV-1 Env generates a series of substantial conformational changes triggered by the binding of gp120 and CD4, inducing high-affinity interaction with co-receptor CCR5 or CXCR5 and the formation of a gp41 prehairpin intermediate (McDougal et al. 1991). Upon binding target cell-surface receptors, gp41 changes its conformation, exposing the hydrophobic N-helical regions and allowing the fusion peptides to insert into the host cell membrane (Chan and Kim 1998).

3.2 Small-Molecule HIV-1 Entry Inhibitors Targeting gp120

3.2.1 NBD-556 and Analogs

Using database screening techniques, Debnath et al. have identified two *N*-phenyl-*N*′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide analogs, namely *N*-(4-Chlorophenyl)-*N*′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide (NBD-556, MW = 337.8 Da) and *N*-(4-Bromophenyl)-*N*′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide (NBD-557, MW = 382.3 Da), that block gp120-CD4 interaction by binding with a conserved pocket in gp120, termed as the "Phe43 cavity," at the node of inner domain, outer domain, and bridging sheet minidomain of gp120 (Zhao et al. 2005). These compounds were equally potent inhibitors of X4 and R5 viruses tested in CXCR4- and CCR5-expressing cell lines, respectively, and, hence, independent of viral coreceptor tropism (Zhao et al. 2005). These two compounds were found to inhibit HIV-1 infection in the low micromolar range. Analysis of molecular dynamics trajectories demonstrated that binding of NBD-556 in the Phe 43 cavity increases the overall maneuverability of gp120, especially in the outer domain (Shrivastava and LaLonde 2011). The thermodynamic change of NBD-556 binding to gp120 is similar to that induced by CD4 (Kwon et al. 2012). Therefore, NBD-556 can be used as a structure-specific probe to identify the

CD4-bound state of gp120 and to evaluate the conformation of gp120 in the case of the functional viral spike (Kwon et al. 2012). NBD-556 was also shown to be a CD4-agonist that can strengthen HIV-1 infection in CD4-CCR5+ cells (Madani et al. 2008).

To investigate changing the phenyl group and oxalamide linker of NBD-556, Curreli et al. found that a series of compounds in which the tetramethyl piperidine ring was replaced with 208 commercially available amines showed modest inhibitory activity against HIV-1 replication, and NBD-09027, NBD-10007, NBD-11009, and NBD-11018 exhibited improved anti-HIV-1-activity and stronger binding affinity to the Phe43 cavity in gp120 (Curreli et al. 2012, 2014). NBD-11009 and NBD-11018 are the most active compounds containing 4-Cl with 3-F substituents in the phenyl rings (Curreli et al. 2014). According to GLIDE-based docking simulations, the 4-Cl-3-F-phenyl ring was surrounded by hydrophobic residues. While the positively charged piperidine nitrogen of the top-scoring (−8.22) 27 conformations of NBD-11009 did not form any H-bond/salt-bridge with Asp368, the next best-scoring (−7.89) conformation did, indeed, form an H-bond/salt-bridge with Asp368 (Curreli et al. 2014). For NBD-11018, the positively charged piperidine nitrogen in both top-scoring (−7.96) and next best-scoring (−7.81) conformations formed H-bond/salt-bridge with Asp368 (Curreli et al. 2014). These structural optimization analogs of NBD-556 improved the activity of several analogs by ∼6-fold compared to NBD-556 (Curreli et al. 2014).

In 2015, Asim K. Debnath et al. demonstrated the successful transformation of a full CD4-agonist (NBD-556) through a partial CD4-agonist (NBD-09027) to a full CD4-antagonist (NBD-11021) by structure-based modification of the critical oxalamide midregion (Curreli et al. 2015). NBD-11021 and NBD-09027 showed efficient antiviral activity against a large panel of clinical isolates pseudoviruses from a diverse set of Envs representing a wide variety of HIV-1 subtypes with IC50 as low as 270 nM and 800 nM, respectively (Curreli et al. 2012, 2015). Similar to NBD-11021, several isomers of NBD-11021 also showed anti-HIV infection activity in low micromolar. The cocrystal structure of NBD-11021 combined to a monomeric HIV-1 gp120 core of clade A/E 93TH057 H375S revealed that the chlorophenyl ring and the linker region of the compound NBD-11021 could penetrate the Phe43 cavity, while region III of the compound resides in the vestibule of the cavity (Curreli et al. 2015). More specifically, residues Val255 and Thr257 interact with the phenyl ring of hydrophobic region I, Trp427 interacts with the linker region (region II), and Gln428 and Gly429 hydrophobically interact with the five-membered thiazole ring of region III of NBD-11021 (Curreli et al. 2015). The nitrogen atom (N13) in the pyrrole ring of the linker makes a hydrogen bond with Asn425, and the new linker of NBD-11021 allowed its piperidine ring to site near the Asp368 of gp120 and make a hydrogen bond with the nitrogen atom of the piperidine ring not found in the NBD-09027-bound gp120. Hence, this hydrogen bond interaction may conduce to the improved affinity between NBD-11021 and gp120 and, hence, its antiviral efficacy. Then, researchers continued to optimize NBD-11021, and they synthesized 60 new analogs. The data showed that the ADMET profiles of these 60 compounds resulted in the discovery of a potent attachment inhibitor, BMS-626529, in vitro, a prodrug currently undergoing Phase III clinical trials (Curreli et al. 2017). Two of these compounds, NBD-14088 and NBD-14107, showed improvement activity compared to NBD-11021 and also showed broad-spectrum potency against a large panel of clinical isolates of a wide variety of subtypes with IC50 of 0.95–19 μM (Curreli et al. 2017). Among these clinical isolates, except for the CD4-antagonist activity targeting gp120, **89** (NBD-11021) inhibited HIV-1 RT with an IC50 of 47 μM, while **83B** and **84A** had an IC50 of 7.2 and 8.4 μM, respectively (Curreli et al. 2017).

In 2017, 25 new analogs were designed based on our earlier reported viral entry antagonist, NBD-11021. Two of the best compounds, **45A** (NBD-14009) and **46A** (NBD-14010), showed

broad-spectrum antiviral activity with IC50 as low as 150 nM against 51 Env-pseudotyped HIV-1 describing diverse subtypes of clinical isolates (Curreli et al. 2017). Compared to NBD-11021, **46A** also showed noticeable improvement in activity with IC_{50} of 3 ± 0.4 and 1.6 ± 0.2 μM in single-cycle (TZM-bl cells) and multi-cycle (MT-2 cells) assays, respectively (Curreli et al. 2017). According to structure-activity relationship analysis, compound 21A-28B and **45A-47B** with the thiazole ring showed the most potency when only CH_2OH substituent is present in the thiazole ring. But, when the thiazole ring was changed with an imidazole ring, the activity decreased, even though cytotoxicity was improved (Curreli et al. 2017). When the entire piperidine ring was replaced by a simple primary amine (CH_2NH_2), the evident improvement in antiviral activity and cytotoxicity was observed (Curreli et al. 2017).

In 2018, Curreli et al. found 29 new compounds that showed broad-spectrum antiviral activity against HIV-1 with IC_{50} of 0.1–9.3 μM (Curreli et al. 2018). ADMET data indicate that some of these 29 inhibitors have ADMET properties comparable to those of BMS-626529 (Curreli et al. 2018). Among these, NBD-14189 optimized by NBD-11021 showed antiviral activity against HIV-1HXB2 with a low IC_{50} of 89 nM, but also showed high cytotoxicity (Curreli et al. 2020). For this class of inhibitors, according to X-ray crystallography data, the phenyl ring represents the important moiety for antiviral activity cause this ring is anchored deep inside the narrow hydrophobic cavity (Curreli et al. 2018). Then, they used mutant pseudoviruses to determine if amino acid substitutions would introduce HIV-1 resistance to the compounds. The L116P substitutions did confer high resistance to BMS-626529, but did not affect the antiviral activity of the three NBD compounds against WT HIV-1_{HXB2} (Curreli et al. 2017). The A204D substitution located in the V2 induced a sixfold and fivefold increase in the IC50 of compound **39** and compound **55**, respectively (Curreli et al. 2018). I424F and N425K located in the CD4-binding site increased the sensitivity of compounds **39**, **48**, and **55** with IC_{50} of several-fold (Curreli et al. 2017). The increasing sensitivity to mutated HIV-1 may indicate that these compounds inhibit HIV-1 infection by targeting gp120/CD4 interaction.

In 2020, to improve the quality of ADME, 48 novel compounds were designed by replacing the phenyl ring in NBD-14189 with a pyridine ring, a bioisostere of phenyl (Curreli et al. 2020). Among these 48 compounds, NBD-14270 (compound **8**) and NBD-14235 with higher SI value of 683 and 243, respectively, showed broad-spectrum activity against all tested clinical isolates and drug-resistant viruses, regardless of viral subtypes (A–D) (Curreli et al. 2020). NBD-14270 showed an obvious improvement in selectivity index (SI) of 607 and 198 against 50 diverse clinical isolates in comparison to that of NBD-14189. In addition, the data of ADMET in vitro clearly showed enhanced aqueous solubility and performance compared to NBD-14189 (Curreli et al. 2020). The data of NBD-14270 showed that the pyridine scaffold is a good bioisostere for phenyl (Curreli et al. 2020). In sum, the pyridine substitution of the phenyl ring was effective in identifying a clinically relevant gp120 antagonist. NBD-14235 has close structural similarity to NBD-14270, which showed higher antiviral activities against HIV-1 subtype C and D (mean IC_{50} values of 0.37 ± 0.03 and 0.35 ± 0.07 μM, respectively). The overall mean of various strains IC_{50} value was 0.43 ± 0.02 μM (IC_{50} values ranged from 0.13 to 0.99 μM) (Curreli et al. 2020). NBD-14107 removed the CH3 from NBD-14010 and showed significant improvement in antiviral potency with IC_{50} of 0.27–0.96 μM for clade A, A2/D, A/D, A/E, A/G, B, C, and D (Curreli et al. 2018). NBD-14189 showed an IC_{50} as low as 63 nM against some clinical isolates by targeting HIV-1 gp120 (Curreli et al. 2018).

3.2.2 CD4 Mimics Binding Competitively with gp120 Suppress the Binding of gp120 to CD4

Env in the CD4-bound conformation presented HIV-1-infected cells on the Env surface preferentially recognized by antibody-dependent cell-mediated cytotoxicity (ADCC). Hence, CD4i epitopes are exposed for recognition by ADCC-mediating Abs and sera from infected individuals when agents promote the CD4-bound Env conformation (Richard et al. 2014; Veillette et al. 2014, 2015). The most attractive feature of CD4 mimics involves eliciting immune responses specific to epitopes that become exposed on the surface on Env upon the binding of CD4 mimics. With such prominent properties, CD4 mimics not only serve as entry inhibitors, but also as envelope protein openers and, putatively, agonists combined with anti-gp120 neutralizing antibodies. A highly conserved ~150-Å3 Phe-43 cavity pocket in the gp120 located at the boundary interface of the inner domain, outer domain, bridging sheet, and CD4 receptor is a key target for inhibitors of CD4 mimics (Kwong et al. 1998; Madani et al. 2008; Yoshimura et al. 2010). Based on computational, thermodynamic, and crystallographic data, a series of small-molecule ligands target the Phe43 cavity of the envelope glycoprotein gp120 have been designed.

JP-III-48 and DMJ-I-228 are two rationally designed CD4 mimetics that interact with gp120 within the Phe-43 cavity and also can act as CD4 agonists, triggering thermodynamic changes in the Env trimer. They are able to significantly enhance recognition of infected cells and ADCC-mediated killing of HIV-1-infected primary CD4 T cells with the four primary viruses by HIV-1^+ sera (Richard et al. 2015). JP-III-48 interacts with monomeric gp120 of the YU2 strain of HIV-1 with higher affinity than that of DMJ-I-228 in micromolar. JP-III-48 showed more potent inhibitory activity against two HIV-1 strains. DMJ-II-121 is also a micromolar inhibitor of HIV-1_{YU-2} acting in HIV-1 entry into human immune cells.

Compound **2**, also named as YYA-021, was evaluated against an R5 primary isolate YTA strain with high anti-HIV activity of 8.4 μM by a single round assay and low cytotoxicity with 260 μM in TZM-bl cells (Mizuguchi et al. 2016). Pharmacokinetic analysis showed that YYA-021 has wide tissue distribution and fairly high distribution volumes in rats and rhesus macaques, which may result from hydrophobicity (Mizuguchi et al. 2016). The half-life of YYA-021 in blood of rats and rhesus macaques is 8.4 and 98.4 min, respectively.

Compound **5** with 1,3-benzodioxolyl replaced by the pmethylphenyl of YYA-021 was found to have highly efficient anti-HIV activity with IC_{50} of 6.20 μM and cytotoxicity >200 μM (Mizuguchi et al. 2016). Compound **5** showed higher hydrophilicity and possesses a better profile than compound **2** cause the pharmacokinetics of the intravenous administration of compound **5** in a rhesus macaque indicated that compound **5** has lower tissue distribution than compound **2**. Compound **7** has 4-amino-2, 6-dicyclohexylpiperidine in place of 4-amino-2,2,6,6-tetramethylpiperidine and high antiviral efficiency with IC_{50} of 1.13 μM and CC_{50} of >200 μM (Mizuguchi et al. 2016). Fluorescence-activated cell sorting (FACS) analysis of a CD4-induced (CD4i) Monoclonal antibody (4C11) to the Env-expressing cell surface pretreated with the test compounds was used to study the binding profile by analyzing the mean fluorescence intensity (MFI). The results indicated that pretreatment of compound **5** results in conformational changes in HIV-1 gp120 Env. The opening of gp120 Env raised the binding affinity for 4C11. Pretreatment with compound **5** revealed higher MFI of 4C11 than that with compound **1** (MFI with 5 = 12.3 and MFI with 1 = 8.91; MFI of 4C11 without compound pretreatment = 7.76).

By adding a fluoro group to the meta position of NBD-556, a low-molecular-weight compound, JRC-II-191, was found to inhibit HIV-1 infection by blocking the binding of the HIV-1 glycoprotein gp120 to the CD4 receptor (Lalonde et al.

2011; Madani et al. 2008). Similar to NBD-556, JRC-II-191 also employs protein-ligand interactions in the porch of gp120 Phe 43 cavity (Lalonde et al. 2011; Zhao et al. 2005).

3.2.3 The Acylhydrazone-Containing Small Molecule 18A

18A was found by screening 212,285 compounds from known bioactive collections and commercial libraries using a cell-cell fusion assay. It distinguished distinct conformational states of gp120 in the unliganded Env trimer, each of which was inhibited by CD4-induced destruction of quaternary structures at the trimer apex and the exposure of the gp41 NHR coiled coil (Herschhorn et al. 2014).

By using recombinant HIV-1 pseudovirus with the envelope glycoproteins of different strains, **18A** can effectively inhibit infection of CCR5-tropic (R5) HIV-1 strains from phylogenetic clades A, B, C, and D. However, inhibition of HIV-1 from clade CRF01_AE and HIV-2UC1 was less effective. **18A** can effectively block a wide spectrum of infections from different HIV-1 strains, including primary isolates, with an average IC_{50} of 6.4 μM for all CCR5-using HIV-1 isolates. Among them, IC_{50} values of 66% of these CCR5-using isolates were less than 6 μM. Among the more physiologically relevant target cells for **18A** are primary $CD4^+$ T cells (human PBMC), which present lower levels of CD4 and CCR5 on surface, compared to the Cf2Th cells. Inhibition of HIV-$1_{JR\text{-}FL}$ infection by **18A** was more effective on Cf2Th cells with an IC_{50} of 0.4 μM and complete inhibition at 10 μM (residual infection <5%). The activity was measured against 30 CCR5-tropism HIV-1 isolates of clades A, B, C, D and AE with IC_{50} from 1.5 to 36.8 μM (Herschhorn et al. 2014). However, **18A** showed poor inhibitory activity against HIV-2.

By constructing an Env chimeric of the most sensitivity strains of HIV-$1_{JR\text{-}FL}$ and a comparatively resistant strain of HIV-1_{KB9}, it was also demonstrated that gp120 is the major determinant of sensitivity to **18A** (Herschhorn et al. 2014). One pseudovirus with Env of the JR-FL gp120 and the KB9 gp41 was nearly as sensitive as the JR-FL Env to inhibition by **18A**. Another pseudovirus with Env of the KB9 gp120 and the JR-FL gp41 was found to be about fivefold less effective to **18A** inhibition than the JR-FL Env and lightly susceptible (twofold) than the parental KB9 Env (Herschhorn et al. 2014). But the sensitivity of **18A** to a chimera in which the major variable loops of JR-FL gp120 were grafted onto the KB9 Env inhibition was nearly as HIV-$1_{JR\text{-}FL}$, showing that the major variable regions of gp120 significantly contribute to 18A susceptivity (Herschhorn et al. 2014).

3.3 Small-Molecule HIV Entry Inhibitors Target gp41

3.3.1 ADS-J1 and Its Resistance Profile

Using a virtual screening program, DOCK3.5, researchers screened a database from ComGenex, consisting of the chemical structures of 20,000 organic compounds. Then, 200 top-scoring compounds were selected for in-depth research of their interactions with the amino acid residues in the hydrophobic cavity and neighboring regions by molecular visualization modes (Debnath et al. 1999; Jiang et al. 1999). Finally, the inhibitory activity of 16 commercially available compounds was tested on the inhibition gp41 6-HB formation by a sandwich enzyme-linked immunosorbent assay (ELISA) with a 6-HB-specific monoclonal antibody (MAb), NC-1. Finally, ADS-J1 and ADS-J2, the phenylazo-naphthalene sulfonic acid, were found to inhibit both formation of NC-1 detectable complex and HIV-1 Env-mediated membrane fusion derivative (Debnath et al. 1999; Jiang et al. 1999). Both small molecules have inhibitory activity to the formation of gp41 6-HB core structure at micromolar concentrations (Debnath et al. 1999). In addition, ADS-J1 inhibits semen-derived enhancer of viral infection (SEVI) fibril formation and blocks SEVI-mediated enhancement of HIV-1 infection by

binding to amyloidogenic peptide fragment ($PAP2_{48-286}$) (Armand-Ugon et al. 2005).

Docking analyses showed that the hydrophobic groups (phenyl and naphthalene) of ADS-J1 interact with hydrophobic residues L568, V570, W571 in the gp41 pocket (Debnath et al. 1999). The negatively charged groups (SO_3H) of ADS-J1 are close to K574, a positively charged residue located around the pocket (Debnath et al. 1999). This led us to question the hypothesis that ADS-J1 targets the hydrophobic pocket in the gp41 NHR trimer because resistant mutations induced by ADS-J1 are mostly located in gp120 coding domain, especially in the highly variable V3 loop region (Armand-Ugon et al. 2005; Gonzalez-Ortega et al. 2010). On the other hand, data also showed that HIV-1 mutants resistant to T2635, a peptide derived from the gp41 CHR region with pocket-binding domain (PBD), also have tolerance to ADS-J1 (Yu et al. 2014). Pseudoviruses with Env mutations at positions 64 and 67 in the gp41 pocket region are resistant to ADS-J1 and C34, which is a CHR-peptide with PBD, but relatively susceptible to T20, which is a CHR-peptide without PBD (Yu et al. 2014). These results were identified that ADS-J1 principally targets the pocket region of HIV-1 gp41 NHR trimer.

3.3.2 NB-2, NB-64, A12, GLS-22, and Analogs

By screening a chemical library consisting of 33,040 "drug-like" compounds, two N-substituted pyrroles, *N*-(4-carboxy-3-hydroxy) phenyl-2,5-dimethylpyrrole and *N*-(3-carboxy-4-chloro)phenylpyrrole, designated NB-2 and NB-64, exhibited broad inhibitory activity against both laboratory-adapted and primary HIV-1 strains in distinct genotypes and phenotypes at low micromolar range (Jiang et al. 2004). They have the same parental structure, and both are drug-like property compounds ground on Lipinski's "rule of five," a molecular mass of 500 Da, a calculated CLog *P* value of 5, fewer than five H-bond donors, and fewer than ten H-bond acceptors (Lipinski et al. 2001). As data showed, NB-2 and NB-64 interact with the gp41 hydrophobic pocket, and their COOH groups bind with a positively charged residue (K574) around the hydrophobic pocket to form a salt bridge (Jiang et al. 2004). NB-2 and NB-64 blocked the binding between a D-peptide and the hydrophobic pocket on surface of the HIV-1 gp41 inner trimeric coiled-coil domain, thereby inhibiting HIV-1 Env-mediated membrane fusion.

On the basis structures of NB-2 and NB-64, 42 *N*-carboxyphenylpyrrole derivatives in two categories (A and B series) were synthesized and tested with $\mathbf{A_1}$ at the same time for their HIV-1 inhibitory activity. The compounds $\mathbf{A_2}$–$\mathbf{A_{10}}$ and $\mathbf{A_{11}}$–$\mathbf{A_{20}}$ possess the same backbone structures as those of NB-2 and NB-64, respectively (Teixeira et al. 2008). As determined by ELISA and native-PAGE, $\mathbf{A_1}$–$\mathbf{A_{20}}$ may interact with the gp41 NHR region to disrupt 6-HB core formation, leading to the blockage of gp41-mediated membrane fusion and inhibition of HIV-1 replication in micromolar range. The 2,5-dimethylpyrrole compounds $\mathbf{A_{11}}$–$\mathbf{A_{14}}$, $\mathbf{A_{17}}$, and $\mathbf{A_{19}}$ were significantly more effective than the corresponding pyrrole compounds $\mathbf{A_1}$–$\mathbf{A_4}$, $\mathbf{A_7}$, and $\mathbf{A_9}$, respectively. Theoretical study indicates that two methyl groups on the pyrrole ring and a near perpendicular conformation between benzene and pyrrole rings by steric crowding effect may be beneficial for gp41 binding (Jiang et al. 2004; Teixeira et al. 2008). Further comparisons between $\mathbf{A_{13}}$ (IC_{50} with 11.81 μM) and $\mathbf{A_{16}}$ (IC_{50} with 173.72 μM), $\mathbf{A_2}$ (IC_{50} with 9.66 μM) and $\mathbf{A_8}$ (IC_{50} with 81.67 μM), and $\mathbf{A_3}$ (IC_{50} with 44.81 μM) and $\mathbf{A_6}$ (IC_{50} with 69.25 μM) illustrated that a carboxyl group at the *m*-position of the benzene ring is more effective than the *p*-position for improving anti-HIV activity (Jiang et al. 2004). $\mathbf{A_{14}}$ with a tetrazolyl moiety at the *m*-position of the benzene ring was more potent ($IC_{50} = 7.70$ μM) than $\mathbf{A_{13}}$ because a tetrazolyl moiety is at the *m*-position of the benzene ring (Teixeira et al. 2008).The low IC_{50} value of $\mathbf{A_1}$, $\mathbf{A_2}$, $\mathbf{A_{11}}$, $\mathbf{A_{12}}$, and $\mathbf{A_{18}}$ may suggest that more boundary substituents may greatly increase interaction affinity with the binding site. The docking analyses indicate that the

binding of **A12** and **A14** and the hydrophobic pocket of the gp41 N-trimer showed lower calculation of binding free energies of ΔG, −6.9 and −5.6 kcal/mol, respectively.

A12, *N*-(3-carboxy-4-hydroxy) phenyl-2,5-dimethylpyrrole, is the most active compound among these 42 *N*-carboxyphenylpyrrole derivatives mentioned above. The structure-activity relationship and molecular docking analysis showed that the carboxyl group within **A12** could interact with either Arg579 or Lys574 within gp41 pocket to form salt bridges and two methyl groups on the pyrrole ring, which is conducive to the binding with residues in the gp41 pocket (Teixeira et al. 2008). However, based on docking study using AutoDock 4.0, other researchers found the following three key contact motifs identified within the gp41 binding pocket: (1) a strong salt bridge formed by the flexible and positively charged Lys38 with the negatively charged carboxylic group of **A12**; (2) two H-bonds formed by the hydrophilic Gln41 the carboxylic group of **A12**; and (3) hydrophobic interactions formed by the interactions between the highly hydrophobic pocket, composed of Leu29, Leu32, Thr33, Val34 and Ile37, and the hydrophobic pyrrole ring of **A12** (Wang et al. 2010).

Using GeometryFit, a series of **A12** analogs, designed by replacing the phenolic group of **A12** by a phenyl group, showed better inhibitory efficiency. All five newly designed compounds showed better inhibitory activity than parental **A12**, except GLS-18 in the CPE assay and GLS-12 and 18 in the cell fusion (CF) assay. GLS-21, GLS-22, and GLS-23 targeting two more contact motifs within the gp41 binding pocket all showed better inhibitory activity across all four assays than that of **A12**. GLS-12 and GLS-18 targeting only one more hydrophobic contact motif compared to **A12** mainly showed similar activities, indicating that the second additional contact motif, Arg43, may offer more to the improved binding affinity. All five new ligands had low cytotoxicity with CC_{50} (concentration causing 50% cytotoxicity) of 227–355 μM, and GLS-22, the most potent ligand, had the highest selected index (SI). As the smallest analog of **A12**, GLS-12 was designed by replacing the phenolic group of **A12** by a phenyl group that was fit into the shape of the pocket composed of Trp35, Gln39, Leu40, and Gln41 and that generated favorable hydrophobic interactions with Trp35, Gln39, and Leu40 of gp41 (Wang et al. 2010). The best ligand, GLS-22 with CH=CHCOOH group, showed about six- to sevenfold increased inhibitory activity over that of **A12** in the HIV replication assays, about 12-fold in the HIV CF assay and about 1.4-fold in the gp41 6-HB formation assay (Wang et al. 2010).

3.3.3 NB-206, 12m and Analogs

Fifteen 2-aryl 5-(4-oxo-3-phenethyl-2-thioxothiazolidinylidenemethyl) furans (**11a-o**) were synthesized by a reaction of Knoevenagel condensation (Katritzky et al. 2009). Compared with NB-2 and NB-64, these furan analogs have bigger molecular size with 437–515 Da, which could occupy more space in the deep hydrophobic pocket on the gp41 NHR trimer (Katritzky et al. 2009). All 15 furan compounds had improved anti-HIV-1 activity with IC_{50} ranging from 0.042 to 1.3 μM (Katritzky et al. 2009). The CC_{50} values ranged from 3.29 to 78.29 μM, and the SI ranged from 10 to 915 μM. **11a**, **11b**, and **11d** (NB-206) showed inhibitory activity against HIV-1_{IIIB} and HIV-$1_{94UG103}$ with IC_{50} of $<$100 nM range, about 20-fold more potent than either NB-2 or NB-64 (Katritzky et al. 2009). The activity of four possible isomers was tested for their anti-HIV-1_{IIIB} activity under exclusion of light, as data showed no substantial difference in antiviral activity of the compounds tested under dark and light conditions (Katritzky et al. 2009). This suggests that the origin of their bioactivity is light-activated to give isomers C and D. All 15 compounds inhibited HIV-1 94UG103 replication in a dose-dependent manner with IC_{50} of 0.07–4 μM. The best compounds are **11a**, **11b**, **11d**, and **11l** which have the lowest IC_{50} (70–74 nM). In addition, molecular docking analysis demonstrated that the phenethyl group of compound **11d** filled the space in the deep hydrophobic pocket of gp41 formed by the NHR trimer

that was observed to be unoccupied by NB-64. In addition, the negatively charged COOH group of **11d** interacts with the positively charged area contributed by K574 in the hydrophobic cavity.

The most obvious adverse effect on activity was demonstrated by electron-donating substituents OH and OCH_3, leading to the dropping of anti-HIV-1 activity by ~30-fold. Interestingly, introduction of a nitrogen atom in the ring of unsubstituted **11a** yielded **11l** with IC_{50} of 42 nm, but substituting chlorine or bromine at the R position yielded **11m**, **11n**, or a methyl group was mediated at the R′ position to yield **11o**, and an evident activity decrease was observed. Moving the methyl group of **11b** to the R′ position yielded **11h** with IC_{50} of 43 nM, which was more active than **11b** with IC_{50} of 99 nM. However, similar moves for chlorine and fluorine had tiny effects.

Compound **12m** (NSPD-12m) is an N-substituted pyrrole derivative, which was derived from NB-64 and had strong anti-HIV-1 activity by targeting the gp41 transmembrane subunit of the HIV-1 envelope glycoprotein. According to the data of molecular docking analysis, NSPD-12m can partially occupy the gp41 binding pocket by interacting with several key residues on the surface of the "pocket" to form a "salt bridge" through electrostatic interactions. The positively charged lysine residue (K574) which located in the gp41 pocket region is critical for the binding of NSPD-12m to gp41 because the nonconserved substitution of K574 with alanine (A) resulted in an evident decrease of anti-HIV-1 activity (Qiu et al. 2019). Compared with NB-64 and other derivatives targeting HIV-1 gp41, NSPD-12m occupied more qualifiable chemical space in the hydrophobic pocket of gp41, which may be due to the existence of a rhodanine ring (C-ring) and a phenyl ring (D-ring). The double bond linker between the B-ring and the C-ring enhances the molecular flexibility of NSPD-12m. This flexible structure makes it easier to generate a "salt bridge" between the thioxo group and the side-chain of K574 and introduce the cation-*p* interaction between the phenyl ring and K574 by adjusting the binding conformation of NSPD-12m. This means the flexible linker between the B and C rings might be critical for enhancing the biological activity of NSPD-12m interacting with K574. Additionally, the D-ring can partially occupy a hydrophobic cavity generated by interaction between residues W571, Q577, and R579 located in gp41 NHR and the fluorine combined with R579 to provide more place to bind with the target hydrophobic cavity. After mutating the non-conserved residues W571, K574, Q577, and R579 in the HIV-1_{JR-FL} Env with alanine (Ala, A), they found that there is no apparent loss of inhibitory activity against these three kinds of HIV-1_{JR-FL} Env mutants (W571A, Q577A, and R579A). This result showed that W571A, Q577A, or R579A mutation was not helpful in enhancing the inhibition of 6-HB formation.

3.3.4 Indole-Based Compounds, as Small-Molecule HIV-1 Fusion Inhibitors Targeting gp41, Identified

A key challenge of screening compounds that target the three-stranded coiled coil of gp41 from library is the instability of part-length gp41 and the drug target buried in full-length gp41. Hence, researchers designed a fluorescence resonance energy transfer (FRET) assay to detect inhibitor binding with the gp41 N-peptide coiled coil, which utilized peptide inhibitors derived from the gp41 C-terminal helical region (Gochin et al. 2003, 2006). This FRET assay was designed on the basis of competitive inhibition which involves N-peptide FRET acceptor and C-peptide FRET donor.

The FRET acceptor is a 31-residue N-peptide with magenta fluorescence attaching a metal-ion-binding bidentate ligand, 2,2′-bipyridyl (bpy), which contains a deep hydrophobic pocket stabilized into a trimer by ferrous ion ligation. The 31-residue N-peptide region includes residues 565–584 of gp41 containing a prominent cavity within the grooves of the coiled coil (Chan et al. 1998). As shown several designed peptides before, addition of a metal ion may form a *tris*-bipyridyl metal complex, which can stabilize the coiled-coil structure (Case et al. 1998; Ghadiri

et al. 1992; Gochin et al. 2002, 2003; Lieberman and Sasaki 1991; Lieberman et al. 1994).

The FRET donor is modified with lucifer yellow dye C-peptide probe (C18-Aib-LY being the most efficient) containing the PBD and is designed to identify the specificity of the N-C interaction (Gochin et al. 2006). C18-Aib-LY probe has a fluorescence emission maximum at 540 nm when it was excited at 425 nm. For this donor, it was observed strong correlation between helix formation and metal ion-binding is consistent with hydrophobic collapse of the heptad repeat peptides into a helix bundle and the formation of a highly structured domain using Fe^{II} (Gochin et al. 2006). Fluorescence of recovery relies on Fe^{II}-coiled coil concentration and LY probe peptide affinity.

By FRET, it was confirmed that three new low-molecular-weight gp41 inhibitors less than 500 Da suppressed cell-cell fusion in the low micromolar range by syncytium inhibition assays *in vitro*. Peptidomimetic compounds composing of a 4-benzamidobenzoic acid scaffold with aromatic substituent groups at the 3-position in orientation matched to the i and i + 4 positions of an α helix (Cai and Gochin 2007). Compounds **11 (3,5)** and **11(6,11)** showed positive results, while **11(3,5)** and **11(6,11)** were confirmed to be efficient in viral syncytium formation assays (Cai and Gochin 2007). **11(6,11)** interacted with the gp41 NHR deep pocket, as verified by NMR spectroscopy.

Later, they designed a strategy for compound **1** to increase ligand hydrophobicity and replace the fluorophenyl group of M1 with an indole by Suzuki coupling to maintain solubility (Zhou et al. 2010). Compound **7** inhibits HIV-1 replication with IC_{50} of 1.1 μM and introduces cell-cell fusion inhibition of 2.1 μM (Zhou et al. 2010). According to Autodock 4.0, an indole group on the ligand may simulate the interaction of the bound Trp residues pocket in the 6-HB structure, including the hydrogen bond between the indole NH of Trp 631 and the main carbonyl group of Leu 568 (Caffrey 2001; Zhou et al. 2010). Thus, polar groups were added in compounds **3** and **8** at the 3-position of the indole (Zhou et al. 2010).

A series of compounds with indole rings were designed based on the natural structure of the gp41 6-HB complex in the region of the hydrophobic pocket with IC_{50} of 0.92–92.1 μM. The hydrophobic pocket binding affinity and cell fusion inhibition of these elongated compounds have a strong correlation in the submicromolar to hundreds of micromolar range, but the addition of polar groups at both ends reduced activity (Zhou et al. 2011). Compounds **14g** with a long hydrophobic interface showed the best inhibition activity for cell-cell fusion and HIV-1 replication at submicromolar range (Zhou et al. 2011).

3.3.5 5M038, 5M041 and Analogs Identified by Fluorescence Polarization Assay Using gp41-5 as the Target

Researchers designed a soluble, single-chain protein artificial structure called gp41-5 which can provide a suitable target for a drug screening high-throughput assay (Frey et al. 2006). The gp41-5 has three inner-core segments (residues 35–70), a short connector alternately connected by two outer segments (residues 117–150). The gp41-5 helps dissolve and trimerize the short segment of the gp41 core and allows it to be used at concentrations suitable for NMR. After folding, gp41-5 contains five of the helices existed in the six-helix bundle. This strategy utilizes gp41 post-fusion state, a trimer of hairpins. C38 (residues 117–154) with the sequence of the missing outer helix was labeled with fluorescein at its N-terminus. Fluorescence polarization was monitored if the labeled peptide ($C38^{*}$) bound to gp41-5.

Using a protein-based assay, 34,800 compounds were screened, and four compounds (5M030, 5M038, 5M041, and S2986) can entirely block $C38^{*}$ binding to gp41-5 (Frey et al. 2006). Three compounds have a 2,4-bis (trifluoromethyl) [1,2,4] triazolo [4,3-a] [1,8] naphthyridine ring with diverse substituents in the 9-position. 5M038 and 5M030 had inhibitory activity with IC_{50} values of 5 μM and 9 μM, respectively. 5M041 acted against CF with IC_{50}

of 18 μM. They bound with hydrophobic pocket within the gp41 trimer inner core, which is highly conserved among the HIV-1 clades of group M (Frey et al. 2006). By synthesizing a group of three peptides including 17-residue segments of the gp41 inner core, which was attached to a 29-residue trimerization domain derived from the GCN4 coiled coil, researchers studied the location of binding of 5M038 with 5M038 giving significantly broadened proton resonance in the presence of gp41-5, according to the data of proton NMR experiments. Proton NMR spectral data showed that resonances remained clear in the presence of peptides 1 and 2 (containing residues 34–50 and 41–57) and peptide 3 (containing residues 54–70), the proton lines from 5M038 that broadened and shifted upward in the presence of peptide 3. Peptide 3 of the inner core includes a deep cavity previously exploited and formed by residues Leu57, Trp60, and Lys63 and occupying the post-fusion structure by Trp117, Trp120, and Ile124 from the outer-layer helix (Frey et al. 2006). 5M038 binds this pocket as well. Next, researchers conducted competition assays using peptides missing key residues to probe the further binding site that inserts into the inner-core pocket for these molecules. 5M038 was not showed activity against a shorter peptide containing residues 119–154. In contrast, it can compete against a full-length peptide which contains residues 117–154. The result may provide powerful evidence that 5M038 could occupy the Trp117 pocket.

3.3.6 Alpha-Helical Mimicry and Colorimetric, Affinity-Based Selection Assay

Using split-pool synthesis compatible with recursive deconvolution (Lam et al. 1997), researchers generated a library of 61,275 potential ligands attached to the N-terminus of the biasing peptide. Each of these compounds consists of three building blocks, including cap (C), monomer 1 (M1), and monomer 2 (M2). Each of the C-M1-M2 compounds was then linked to the N-terminus of CHR-peptide C30 which resides in gp41 CHR without the corresponding residues 636–665 of the PDB. Then they evaluate the interaction affinity of a protein with the sequence corresponding to the a-helical inner-core of the gp41 ectodomain by using a colorimetric, affinity-based selection assay. The biotinylated protein target was achieved through a cysteine on the opposite end of the molecule from the targeted site, which was engineered at the N-terminus of the GCN4 moiety. To detect ligand binding, the library of ligand-carrying beads was buit by incubating the biotinylated gp41 inner-core with streptavidin alkaline phosphatase conjugate (SA-AP). Subsequent alkaline phosphatase substrate was incubated with those ligand-carrying beads, which would produce insoluble blue dye on ligand that had occupied the a-helical inner-core of gp41.

Zhou et al. synthesized a combinatorial library of 61,275 potential ligands with all possible combinations of 50 building blocks at the first two positions and 25 different building blocks at the third capping position, which was linked to the N-terminus of an outer-layer peptide lacking the first two α-helical turns (Zhou et al. 2000). The ligand with the highest affinity was selected while remaining attached to its solid synthetic support bead by the ability to capture the labeled soluble form of the gp41 central coiled coils (Zhou et al. 2000). A 445 Da synthetic moiety attached to the N-terminus of a 30-mer outer-layer peptide (Asn-25 to Lys154) could bind to the coiled-coil core of gp41 and block membrane fusion (Zhou et al. 2000). The synthetic moiety includes a terminal cyclopentylpropionic acid, a central E-glutamic acid, and a linking *p*-(*N*-carboxyethyl) aminomethyl benzoic acid. Among these hybrid molecules, C7-Mn34-Mn42-P30 inhibits HIV-1 Env-mediated CF with IC_{50} of 300 nM (Zhou et al. 2000). X-ray crystallographic analyses demonstrated that the C7-Mn34-Mn42 motif in the hybrid molecule could bind in a cavity in the central coiled-coil commonly occupied by three side-chains, Trp-117, Trp-120, and Ile-124, from two turns near the N-terminus of the outer-layer a helix of gp41 (Zhou et al. 2000).

Dale L. Boger et al. analyzed a geometric characterization of 10,245 β-turns in the protein data bank and synthetically designed and synthesized a β-turn mimetic library as a key component of a small-molecule library which targets the major recognition motifs involved in protein–protein interactions. According to theoretical analysis of systematic case studies, the majority of the recognition or binding affinity of protein–protein interactions was produced by the link of a small cluster of key residues near the center of the interface with three main recognition motifs (R-helix, β-turn, and β-strand) (Moreira et al. 2007). Using solution-phase synthesis, this library includes 210 mixtures of 20 compounds for a total of 4200 compounds to mimic all possible permutations of three of the four residues in a naturally β-turn structure. As an effective library template, *trans*-pyrrolidine-3,4-dicarboxamide was used to screen compounds against a series of peptide-activated GPCRs that recognize β-turn structure in their ligands (Moreira et al. 2007). These results showed that appropriately substituted *trans*-pyrrolidine-3,4-dicarboxamides can substitute for the peptide backbone for efficient exhibition of side-chain groups in a β-turn (Moreira et al. 2007).

Using a similar approach, Whitby et al. have established a comprehensive α-helix mimetic library based on the design of a molecular scaffold that mimics the surface of an α-helix, which includes 20 natural amino acids substituted on the three positions of the side-chains of the α-helix, resulting in the creation of an 8000-member library (20 × 20 × 20) in order (Whitby et al. 2011). Using the established FRET-based assay noted above, researchers screened the library to identify the compounds that could bind to the gp41 NHR hydrophobic pocket and show effective inhibitory activity against HIV-1. Three of the α-helix mimetics, H_2N-Trp-[Trp]-Leu-OH, H_2N-Asn-[Trp]-Trp-OH, and H_2N-TyrMe-[Trp]-Trp-OH, could disrupt the assembly of a six-helix bundle with submicromolar Ki value in the FRET-based binding assay and IC_{50} values ranging from 5 to 8 μM in cell-cell fusion assay (Whitby et al. 2011). This intraprotein surface disruption directly results in reduced levels of HIV-1 entry into host cells.

3.3.7 Natural Polyanionic Ingredient

Many natural polyanionic ingredients, or their derivatives, showed anti-HIV activities for preventing or treating HIV infection. Several tea polyphenols, especially those with galloyl moiety, have anti-HIV activity with multiple mechanisms of action. These mechanisms of action include the inhibition of HIV-1 entry into target cells by blocking the formation of the gp41 six-helix bundle, which inhibits HIV-1 envelope glycoprotein-mediated membrane fusion (Table 3.1).

Table 3.1 The inhibition activity of the small-molecule inhibitors to laboratory-adapted strains and primary strains

Inhibitors	IC_{50} (μM)		CC_{50} (μM)	References ©
	Laboratory-adapted strains	Primary strains		
Small-molecule HIV-1 entry inhibitors targeting gp120				
NBD-556	5.28–15.88	19–103	280	Zhao et al. (2005)
NBD-557	4.43–15.93	15–103	223	Zhao et al. (2005)
JRC-II-191	54	–	–	Lalonde et al. (2011)
NBD-10007	4.2–4.6	–	32.8 → 62	LaLonde et al. (2012)
NBD-11009	1.6–3.8	–	58.4–77.8	Curreli et al. (2014)
NBD-11018	1.98–3.5	–	60 → 88	Curreli et al. (2014)
NBD-09027	4.7–9.1	–	23 → 108	Curreli et al. (2015)
NBD-11021	1.5–2.5	0.8–11	28–30	Curreli et al. (2015)
NBD-14009	2.1–2.7	0.32–2.3	25.8–33.6	Curreli et al. (2017)
NBD-14010	0.52–0.59	0.2–0.86	30.5–40.5	Curreli et al. (2017)
NBD-14189	0.089–0.18	–	21.9–22.2	Curreli et al. (2018)

(continued)

Table 3.1 (continued)

Inhibitors	IC_{50} (μM)		CC_{50} (μM)	References ©
	Laboratory-adapted strains	Primary strains		
NBD-14270	0.36	0.11–0.33	92.8	Curreli et al. (2020)
NBD-14235	1.1	0.13–0.74	85	Curreli et al. (2020)
YYA-021	8.40	–	260	Mizuguchi et al. (2016)
Compound **5**	6.20	–	>300	Mizuguchi et al. (2016)
18A	3.6–25	1.5–36.8	44	Herschhorn et al. (2014)
Small-molecule HIV entry inhibitors targeting gp41				
ADS-J1	0.1–4.95	–	292.16	Debnath et al. (1999)
ADS-J2	21.85	–	289.44	Debnath et al. (1999)
NB-2	1.04–11.57	0.89–98	834 → 4000	Jiang et al. (2004)
NB-64	2.21–51.37	4.72–39.68	1521 → 4000	Jiang et al. (2004)
A12	28.19 ± 3.79	–	333.84 ± 22.88	Wang et al. (2010)
GLS-22	4.91 ± 0.69	–	255.28 ± 3.73	Wang et al. (2010)
NB-206	0.017–0.073	–	16.82	Katritzky et al. (2009)
NPSD-12m	0.014–0.99	–	27.85 ± 3.79	Qiu et al. (2019)
11(3,5)	~8	–	–	Cai and Gochin (2007)
11(6,11)	~8	–	–	Cai and Gochin (2007)
Compound **7**	1.1 ± 0.4	–	>100	Zhou et al. (2010)
14g	0.92–2.8		19 → 100	Zhou et al. (2011)
5M038	5	–	–	Frey et al. (2006)
5M030	9	–	–	Frey et al. (2006)
5M041	18	–	–	Frey et al. (2006)
C7-Mn34-Mn42-P30	0.3	–	–	Zhou et al. (2000)
Trilobatin	2.9–27.11	–	94.07–1197.2	Yin et al. (2018)

Phloretin-4′-β-D-glucoside (trilobatin) is a glycosylated dihydrochalcone and a strong natural sweetener, which is extracted from the leaves of the Chinese sweet tea *Lithocarpus polystachyus* Rehd (Fan et al. 2015). It also has antihyperglycemia (Wang et al. 2016), antioxidative (Sun et al. 2015; Wang et al. 2016), and anti-inflammatory properties (Fan et al. 2015). Trilobatin exhibited highly potent inhibitory activities on infection by laboratory-adapted HIV-1Bal (R5) and HIV-1IIIB (X4) strains with IC50 values at 2.91 ± 0.30 and 4.32 ± 0.39 μM, respectively (Yin et al. 2018). In addition, trilobatin has broad-spectrum activity and no significant coreceptor tropism on any tested HIV-1 infectious clones, including HIV-1SF162, HIV-1NL4-3, and HIV-181A, with IC50 of 27.11 ± 5.16, 24.20 ± 4.63, and 24.50 ± 8.62 μM, respectively (Yin et al. 2018). A docking study indicated that trilobatin binds to residues Leu559 and Ile564 by hydrophobic interactions and Leu559 and Gln558 by hydrogen bonds, both of which are on the surface of HIV-1JR-FL gp41 (Yin et al. 2018). The phenol and phloroglucinol groups of trilobatin interact with the hydrophobic residues Leu559 and I564 located in the pocket of gp41 (Yin et al. 2018). Hence, trilobatin can competitively inhibit 6-HB formation to block membrane fusion.

3.4 Future of HIV-1 Small-Molecule Entry/Fusion Inhibitors and Screening Assay

From the early 1990s, discovery of highly potent anti-HIV-1 small-molecule inhibitors had begun. At the same time, the short peptide derived from the HIV-1 gp41 CHR domain opened a new pathway for developing viral fusion/entry inhibitors targeting the viral envelope glycoproteins (Jiang et al. 1993; Wild et al.

1994). As key structures for HIV-1 entry into target cells, gp120 and gp41 are critically important targets of small-molecule inhibitors. Compounds targeting gp120 inhibit HIV-1 entry mainly by blocking the interaction between gp120 and CD4 and changing the conformation of Env to induce ADCC. Compounds targeting gp41 inhibit HIV-1 entry mainly by blocking the formation of 6-HB core to inhibit HIV Env-mediated membrane fusion and virus entry. The main targets of gp41 inhibitors are the hydrophobic pocket in grooves on the surface of the NHR trimer at the fusion-intermediate state of gp41. However, none of these inhibitors has been approved for clinical use. For entry inhibitors targeting gp120 and gp41, designing a lead compound and then enhancing and improving its antiviral activity by changing the chemical group rationally is a dominant direction of inhibitor development. So, the screening of efficient drugs from libraries containing a large number of unknown, but potential, compounds will heavily rely on the establishment of more high-efficiency, sensitive, and time-saving systems. For now, except for a traditional system like ELISA, many more high-throughput screening systems have been built, like peptide FRET, fluorescence polarization assay, and colorimetric, affinity-based selection assay. These assays can support high-throughput screening of a small-molecule inhibitor library. However, rigorous experimental conditions limit the wide application of this new high-throughput assay. Many pharmaceutical companies in Europe and North America, such as Roche, Gilead Sciences, GlaxoSmithKline, Bristol-Myers Squibb, Tibotec (acquired by Johnson & Johnson), Abbott Laboratories, Trimeris (merged with Synageva BioPharma), and BioChem Pharma (merged with Shire Pharmaceuticals), began to concentrate on developing HIV entry/fusion inhibitors targeting the gp41 pocket about a decade ago when enfuvirtide was licensed by the U.S. FDA (Lu et al. 2016). However, most of these programs were discontinued several years later because of the failure to identify druggable leads (Lu et al. 2016). Therefore, more efficient small-molecule inhibitors with remarkable ADMET profiles need to be established in order to withstand the rapid mutation of HIV-1.

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The Genesis and Future Prospects of Small Molecule HIV-1 Attachment Inhibitors

4

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Abstract

Gp120 is a critical viral proteins required for HIV-1 entry and infection. It facilitates HIV-1 binding to target cells, human-to-human transmission, relocation of virus from mucosa to lymph nodes, cell–cell infection and syncytium formation, and the bystander effect that kills uninfected CD4+ T-cells and other human cells. Molecules that bind to gp120 can inhibit its function by stabilizing conformations of the protein, leading to the inability to infect cells, and resulting in non-permissive. Small molecule-mediated stabilization of certain conformations of gp120 may also enhance recognition of HIV-1 infected cells by neutralizing antibodies and make the virus more susceptible to effector functions such as ADCC, which could potentially be part of future cure regimens. Additionally, HIV attachment inhibitors can complex with free gp120 and potentially repress both cytopathic effects from membrane-bound or soluble gp120. Fostemsavir (Rukobia™), a phosphate prodrug of an HIV-1 attachment inhibitor that was recently approved for use in highly treatment experienced (HTE) patients with multidrug resistant HIV-1 is a first-in-class drug with a favorable safety profile that provides an additional treatment option for treatment in this population of patients with a high medical need.

Keywords

HIV-1 · Attachment · Entry · Fusion · Inhibitor

4.1 Introduction

The latest new chemical entity for the treatment of HIV-1 is fostemsavir (Rukobia™) which received FDA approval in the U.S. in July 2020. The discovery program and early development activities were carried out at Bristol-Myers Squibb company and as a result of a business agreement, the successful late stage development work and subsequent marketing of the compound for patients was conducted by ViiV HealthCare Inc.

The Phase 3 BRIGHTE study demonstrated the benefits of fostemsavir for multidrug resistant HIV subjects. Two cohorts were evaluated by the addition of fostemsavir. The randomized cohort

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consisted of subjects who had at least one but no more than two remaining active approved HIV agents. These subjects received 600 mg bid fostemsavir or a placebo, while the non-randomized cohort (subjects with no remaining fully active options) all received 600 mg fostemsavir bid. In the randomized cohort, those who received fostemsavir had a significantly greater decrease in the HIV RNA level than those who received placebo during the first 8 days and this result has been sustained through 48 weeks and longer (Kozal et al. 2020). Efficacy was also observed in the non-randomized cohort.

This drug is the first example of a mechanistically unique class of HIV entry inhibitor called an attachment inhibitor (HIV AI). HIV AIs bind to HIV gp120 and block the initial contact with human $CD4^+$ T-cell, essentially interrupting the HIV life cycle at the earliest stage (Fig. 4.1) (Lin et al. 2003).

Infected $CD4^+$ T-cells express gp120 on their membrane which is packaged into newly formed progeny infectious virus particles after the budding process. These viruses can then infect new $CD4^+$ T-cells. However, cell-bound gp120s are also capable of mediating cell-to-cell transfection (Fig. 4.2), a second route for HIV to deliver its genetic material to uninfected $CD4^+$. HIV AIs can suppress both virus–cell and cell–cell HIV infection.

4.2 Discovery and SAR of Fostemsavir-Related Series (BMS Series)

The original attachment inhibitor lead compound (BMS-‘216) was discovered from a whole cell, antiviral screen of a portion (~100K compounds) of Bristol-Myers Squibb’s chemical inventory (Fig. 4.3). It was the sole hit from the screen and exhibited a novel mechanism of action. BMS-‘216 exhibited an EC_{50} value of 153 nM in a pseudotype assay using envelopes from the CCR5-dependent JRFL strain and CXCR4-dependent LAI strain of HIV-1, and a CC_{50} value of 339 μM in uninfected cells (Wang et al. 2019). A subsequent combinatory effort that included a library of single position changes revealed a F atom at position 4 of the indole (BMS-‘705, EC_{50} 2.6 nM) that enhanced activity by 58-fold (Meanwell et al. 2009a). However, BMS-‘705’s solubility in water, and especially in common excipients, was low, and unsuccessful efforts to identify an oral formulation in the context of other properties precluded advancement. A nitrogen atom was then introduced into the indole ring to improve the solubility and metabolic stability (Wang et al. 2009a). As expected, 4-/5-/6-/7-azaindole analogues of BMS-‘216-Me all displayed better aqueous solubility (>26-fold improvement) and metabolic stability (>2.3-fold improvement) (Wang et al. 2009a).

The addition of a MeO– group in the 7-azadole promptly produced BMS-‘806 (Lin et al. 2003; Wang et al. 2003), which was advanced to first-in-man (FIM) clinical studies. Unfortunately, the targeted exposure was not achieved in the Phase I trial, presumably due to the moderate metabolic stability ($t_{1/2}$ 47 min in HLM) and permeability (Caco-2 Pc 51 nm/s) measured for the compound in preclinical studies. A closely related 6-azaindole molecule, BMS-‘043, was evaluated soon after and proved to be a compound with improved preclinical characteristics ($t_{1/2} > 100$ min in HLM, Caco-2 Pc 178 nm/s) (Wang et al. 2009a). BMS-‘043 was advanced to Phase II clinical trials and the resulting virology data established proof-of-concept (POC) for the new and novel mechanism (Ho et al. 2006; Hanna et al. 2011). Several issues which precluded further advancement of BMS-‘043 were that the targeted viral load drop was observed in only ~68% of patients and that this result was achieved at the relatively high dose requirement of 1.8 g of compound co-administered with a high-fat meal. Further rounds of optimization focused on addressing the abovementioned shortcomings via further modification of position 7 of the 6-azaindole. Installation of amides, aromatic rings, C-linked heteroaryl and ultimately N-linked heteroaryl substituents led to the discovery of BMS-‘529 (temsavir, EC_{50} 0.1 nM) (Wang et al. 2018). Temsavir (Nowicka-Sans et al. 2012) possessed a broader spectrum of anti-HIV activity

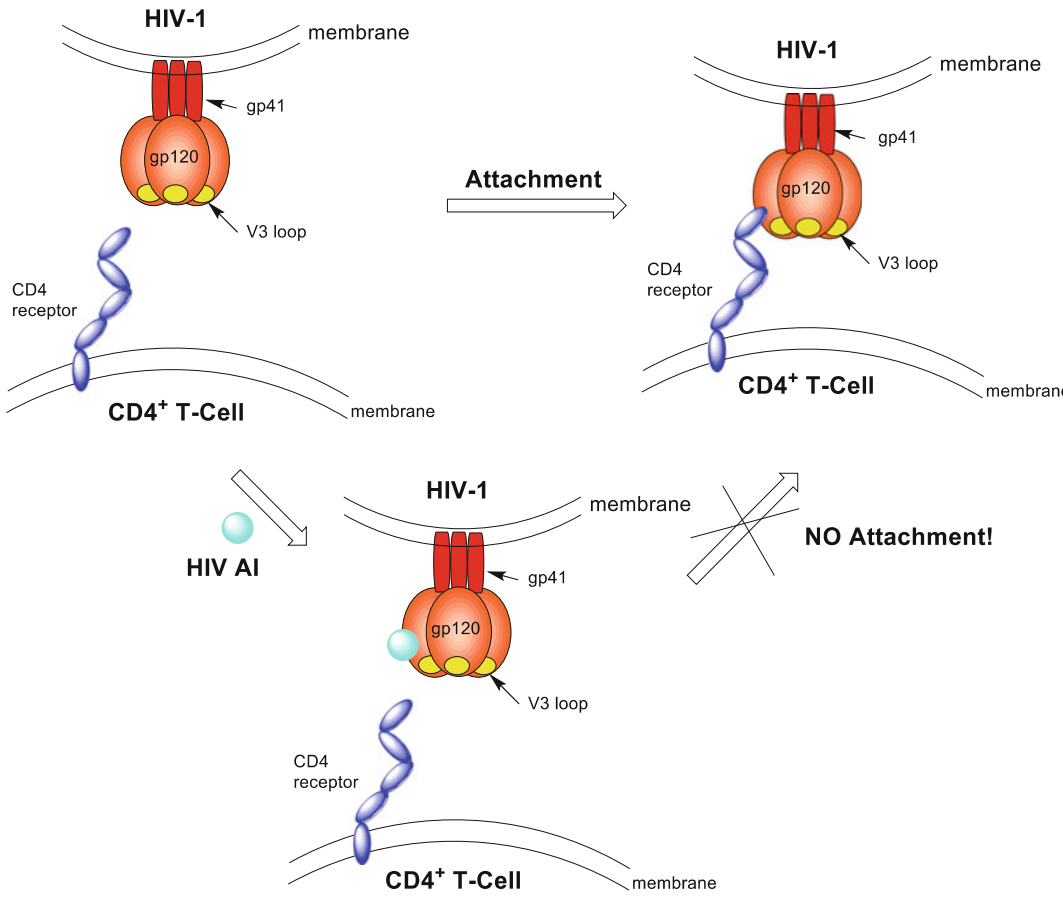


Fig. 4.1 HIV AI interrupts the first step of attachment to CD4

than its predecessors. The preclinical profile achieved all of the necessary safety and physicochemical properties with the exception of aqueous solubility, which appeared to be dictated by the requirements of the target. The less than desired water solubility of 0.022 mg/mL at pH = 7.4 resulted in the lower-than-dose-proportional-increases in exposure in dose-escalation PK studies. The solubility/dissolution limited absorption issue was resolved preclinically by the use of a phosphonooxymethyl prodrug strategy which resulted in the discovery of BMS-‘068 (fostemsavir) (Wang et al. 2018; Nettles et al. 2012). In a subsequent Phase 3 clinical study (the BRIGHTE study), fostemsavir demonstrated a favorable safety and tolerability profile, and showed effective in this difficult to treat HTE population with a high medical need, leading to its approval as a first-in-class HIV AI (Kozal et al. 2020; Markham 2020; Lataillade et al. 2020).

The main focus of the BMS team’s work was the optimization of the physicochemical properties of the (aza)indole benzamide key series described above and shown in Fig. 4.3. In addition, efforts were also spent on surveying and establishing structure activity relationships (SAR) in five regions: (A) (aza)indole core, (B) glyoxamide linker, (C) piperazine spacer, (D) benzamide moiety, and (E) a region which encompasses alterations that concomitantly change both region C and region D that could not be easily described using C or D alone (Fig. 4.4). Additional SAR has also been reported by researchers outside of Bristol-Myers Squibb.

The description of the SAR is based on published data and partially based on that

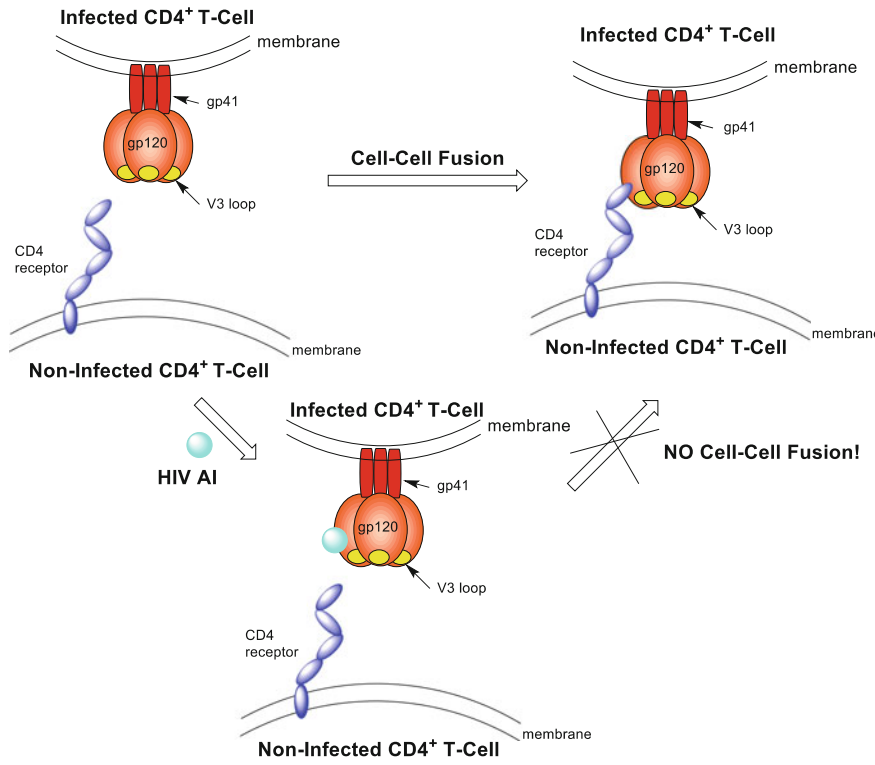


Fig. 4.2 HIV AI's interruption HIV cell-cell fusion

contained in published patent applications and granted patents where for some compounds antiviral activity is reported in categories (e.g., Category A: $EC_{50} < 1$ μM, etc.) and some compounds have specific EC_{50} values disclosed. In Figs. 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, and 4.12, the representative examples were selected to highlight their unique structures or substructures.

In Region A, replacements of the (aza)indole with aromatic or heteroaromatic rings consistently maintained some level of anti-HIV-1 potency. The aromatic(hetero) rings explored were bicyclic, tricyclic, and mono-cyclic (Wang

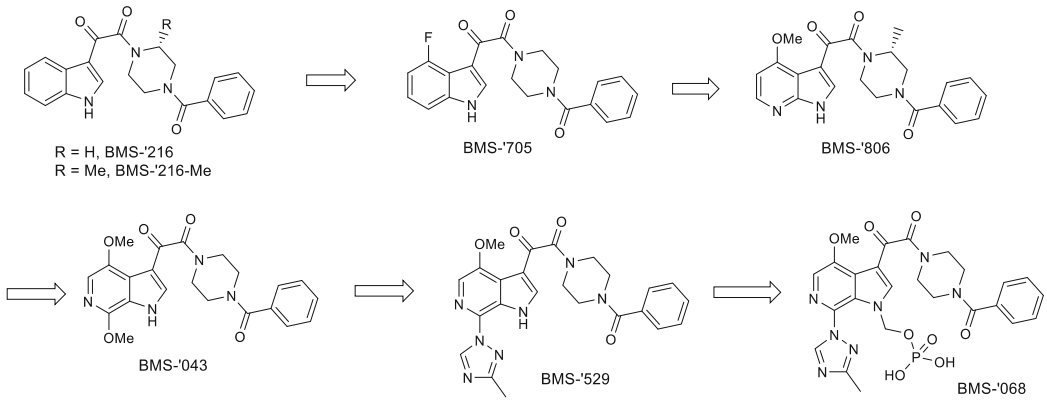


Fig. 4.3 Discovery of fostemsavir

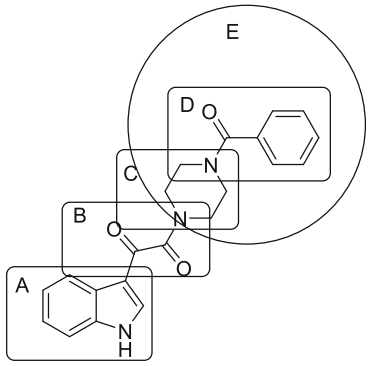


Fig. 4.4 Regions under investigation

et al. 2013, 2019; Lu et al. 2009; Bender et al. 2013). In general, bicyclic heteroaromatic surrogates offered the most interesting results. While monocyclic aromatics(hetero) displayed weak potency in their unsubstituted form, inclusion of a second aromatic(hetero) functionality could rescue the potency to near that of the starting indoles and azaindoles (Wang et al. 2019; Lu et al. 2009). The most impressive (aza)-indole surrogates are shown in Fig. 4.5, as antiviral potencies for (**5a** EC_{50} 2.4 nM, **5b** EC_{50} 0.7 nM, **5c** EC_{50} 4.6 nM) were superior to or competitive with BMS-‘216-Me’s EC_{50} of 4 nM (Fig. 4.3) (Wang et al. 2019). Similar to the (aza)indole series, an aromatic ring at position 6 in **5d** was able to increase activity >100-fold, with the EC_{50} to 0.03 nM (Wang et al. 2019). These novel series remain less studied due to the successful progression of the (aza)indole series.

A bivalent compound **5e** was prepared by dimerizing two molecules of BMS-‘806. In an assay using the MT-2 cell line infected with HIV-1 IIIB virus, compound **5e** exhibited an IC_{50} value of 4.8 nM, half of that of BMS-‘806 (2.3 nM) (Wang et al. 2005). The result initially seemed remarkable in that initial SAR obtained during the optimization of BMS-‘216 seemed to show that the best potency was observed when the C-4 position of the 7-azaindole and indole contained small functionalities and larger groups were deleterious. In the work that identified dimer **5e**, the same expected trends for reduced potency at C-4 with increasing size were observed except for when two BMS-806 molecules were dimerized via a long water-soluble linker. The data suggested that for dimer **5e**, only one of two BMS-‘806 units was involved in gp120 binding, while the second BMS-‘806 unit simply

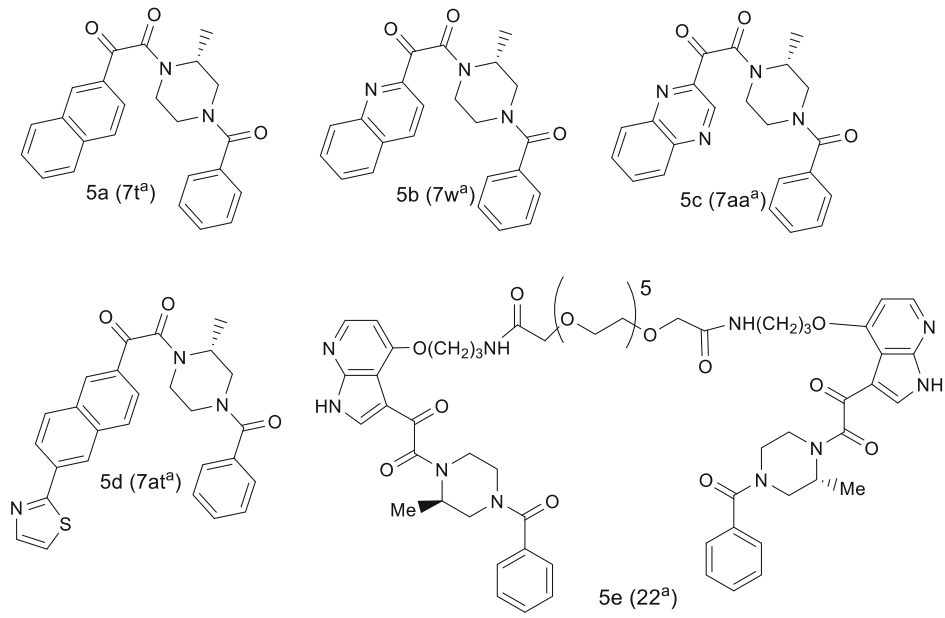


Fig. 4.5 Region A. ([a] The compound number or name in the original literature)

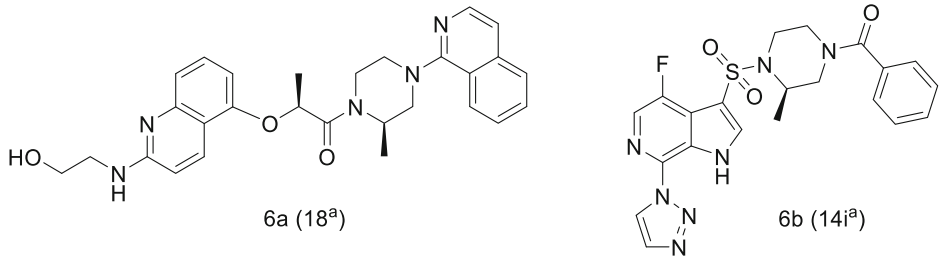


Fig. 4.6 Region B. ([a] The compound number or name in the original literature)

floated around in the extracellular medium (Wang et al. 2005). The tolerance for a bulky extension at C-4 with appropriate linkage was also independently observed in the BMS-‘806 derived conjugates described by others (Sect. 4.4.2).

The modifications of Region B were reported by two independent teams. Replacement of the glyoxamide linkage with a 2-methyl-2-oxo-carboxamide linkage appeared to provide comparable anti-HIV-1 potency to the original glyoxamide series. Example **6a** in Fig. 4.6 exhibited an IC_{50} value of 0.04 nM against HIV-1 fusion in a gp160 induced cell–cell fusion assay that used a Hela P4 cell line and a CHO-15 Tat10 cell line (Fenwick et al. 2005).

Another team made analogs in which the glyoxamide linker was replaced with a sulfonamide. In general, the sulfonamide substitution appeared to exhibit modestly decreased potency vs. the BMS glyoxamide series. The most potent sulfonamide derivative was **6b** (Fig. 4.6), with an IC_{50} of 7 nM in an M33 pseudotype assay, while the benchmark glyoxamide derivative's IC_{50} was <5 nM in the same testing conditions (Lu et al. 2007).

In Region C, replacement of the piperazine linker was explored with a variety of diamines, including fused bicyclic diamine (Wang et al. 2014c), spiral bicyclic diamine (Wang et al. 2014d), and pyrollidine (Kadow et al. 2009) shown in Fig. 4.7. Spiral diamine derivative **7a**

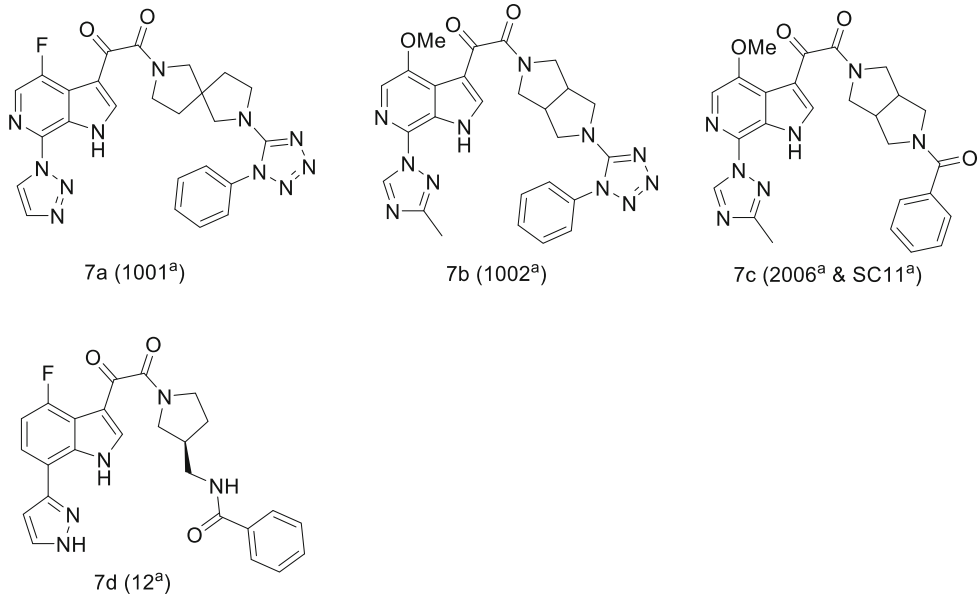


Fig. 4.7 Region C. ([a] The compound number or name in the original literature)

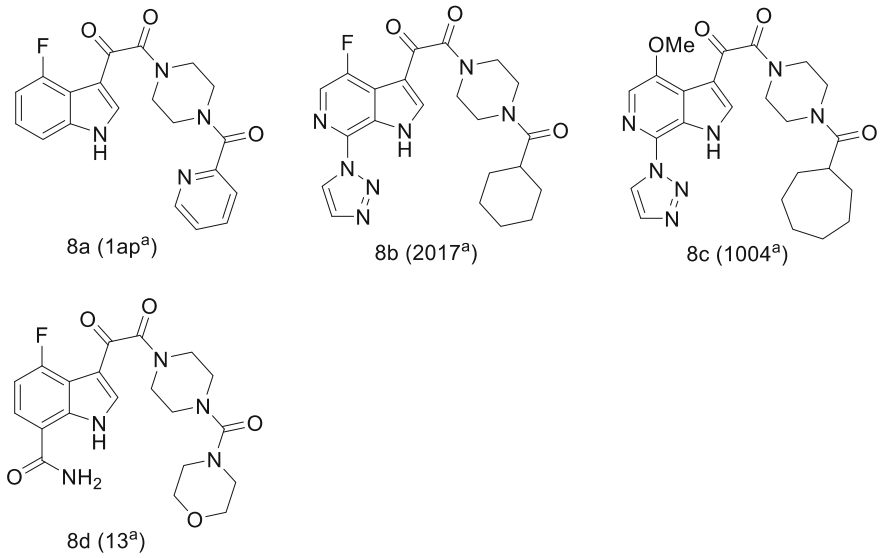


Fig. 4.8 Region D, Approach 1. ([a] The compound number or name in the original literature)

(Wang et al. 2014c), fused diamine derivatives **7b** (Wang et al. 2014d) and **7c** (Wang et al. 2014d) displayed anti-HIV EC_{50} values of 5.65 nM, 1.41 nM and 0.68 nM, respectively, in the pseudo type assay using LAI Env. Additionally, pyrrolidine compound **7d** fell into the activity category A for which the EC_{50} value was less than 1 μM (Kadow et al. 2009).

Interestingly, another independent group also published biological data for **7c**. Against JR-CSF and B41 pseudotyped HIV-1, IC_{50}s of 0.6 nM and 2 nM, respectively, were obtained, while the

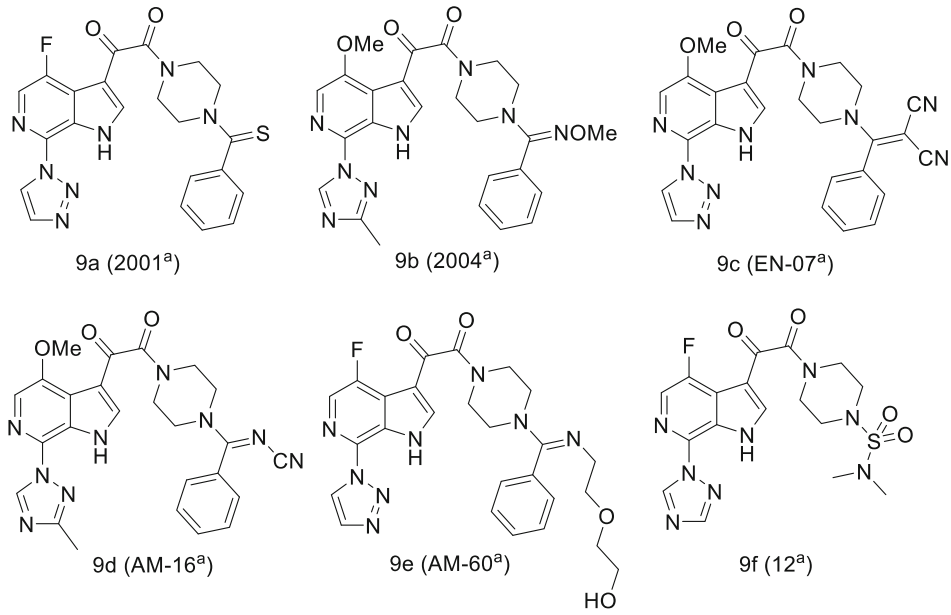


Fig. 4.9 Region D, Approach 2. ([a] The compound number or name in the original literature)

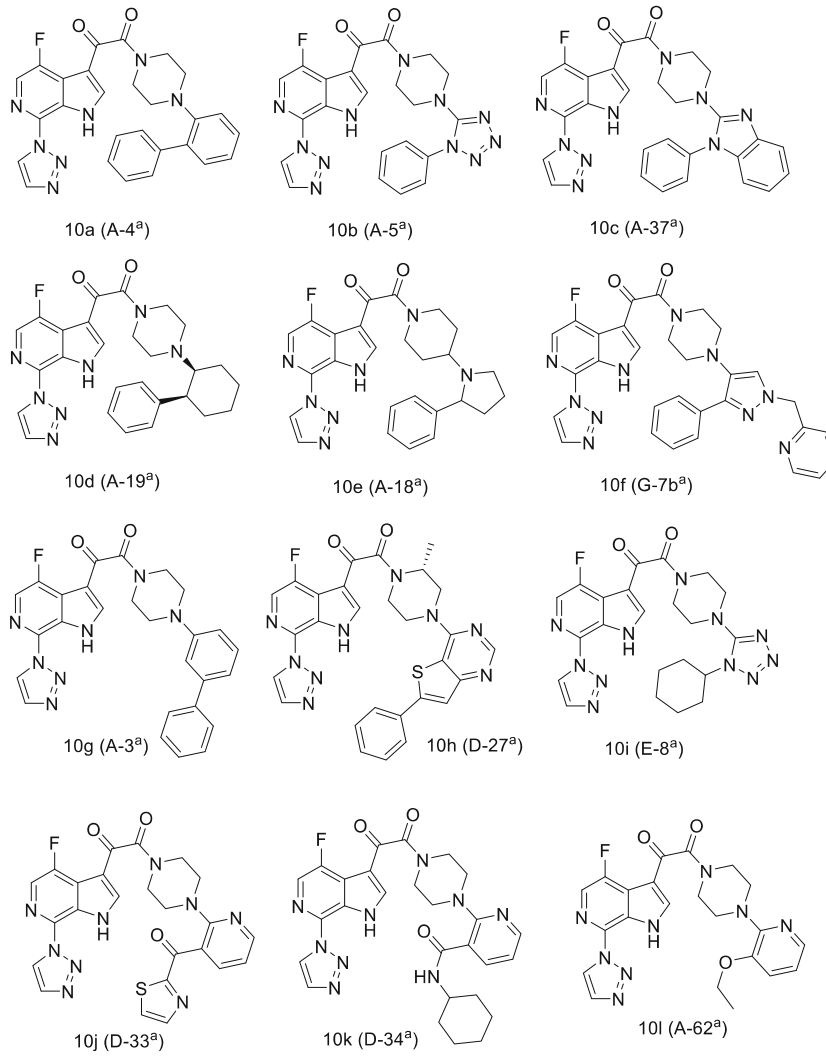


Fig. 4.10 Region D, Approach 3. ([a] The compound number or name in the original literature)

benchmark BMS-'529 showed 0.06 nM and 0.05 nM in the same experiments (Meuser et al. 1940).

Region D was explored extensively. Some of the strategies explored were: (1) replacement of the benzene ring by other aromatic heteroaromatic rings or non-aromatic elements, (2) carbonyl isosteres of the benzamide's carbonyl group, (3) the benzamide's carbonyl group was replaced with an aromatic or heteroaromatic ring, (4) the benzamide's carbonyl group was fused to form a bicyclic ring with either the aryl/heteroaryl ring or alternatively with the piperazine, and (5) the benzamide's amide bond was replaced with an alkene.

In Approach 1, the phenyl ring was replaced either by heteroaromatic rings such as the pyridine contained in analog **8a** (Fig. 4.8) (Meanwell et al. 2009b), or by non-aromatic cyclic rings such as the cyclohexane in **8b** and the cycloheptane in **8c** (Wang et al. 2014a). The resulting compounds furnished comparable potency (EC_{50}: **8a** 4.6 nM, **8b** 0.11 nM, **8c** 0.14 nM) in the initial pseudotype assay compared to their corresponding benzamides BMS-'705 (EC_{50} 2.6 nM) and BMS-'529 (EC_{50} 0.1 nM) (Meanwell et al.

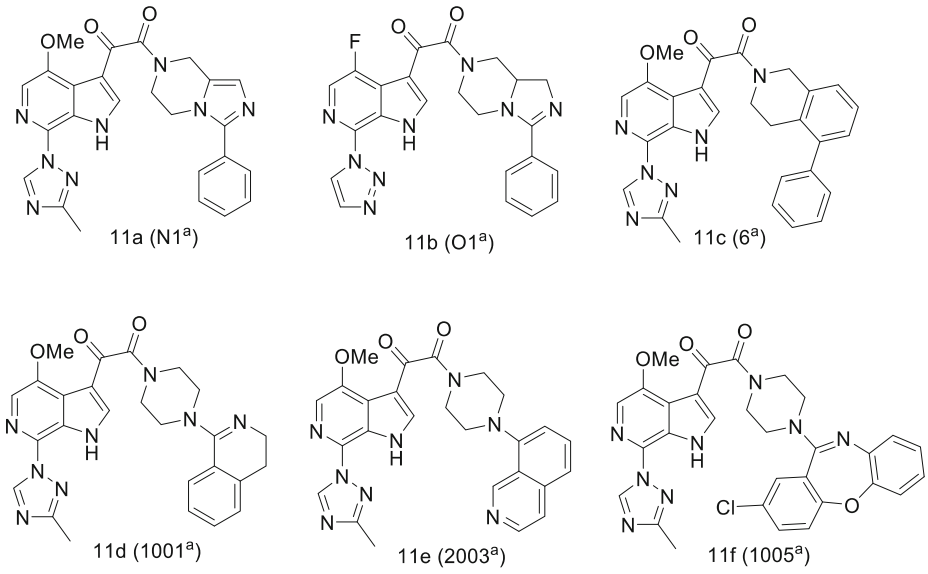


Fig. 4.11 Region D, Approach 4. ([a] The compound number or name in the original literature)

2009b; Wang et al. 2014a). Nitrogen containing saturated heterocyclic rings were also explored which provided ureas. The morpholine urea **8d**'s EC_{50} was reported as Category A ($EC_{50} < 1$ μM) (Regueiro-Ren et al. 2004).

Approach 2 explored Region D via exchange of the benzamide's carbonyl group with its bio-isosteres such as thione (Meanwell et al. 2014), oxime (Meanwell et al. 2014), enamine (Wang et al. 2009b), amidine (Wang et al. 2008a), and sulfone (Regueiro-Ren et al. 2005). As in Fig. 4.9, thione **9a** and oxime **9b** showed superior potency to BMS-'529 (EC_{50}: 0.027 nM and 0.019 nM vs. 0.1 nM) (Meanwell et al. 2014). Furthermore, examples of an enamine **9c** ($EC_{50} < 1$ μM), amidines **9d** ($EC_{50} < 0.5$ μM)

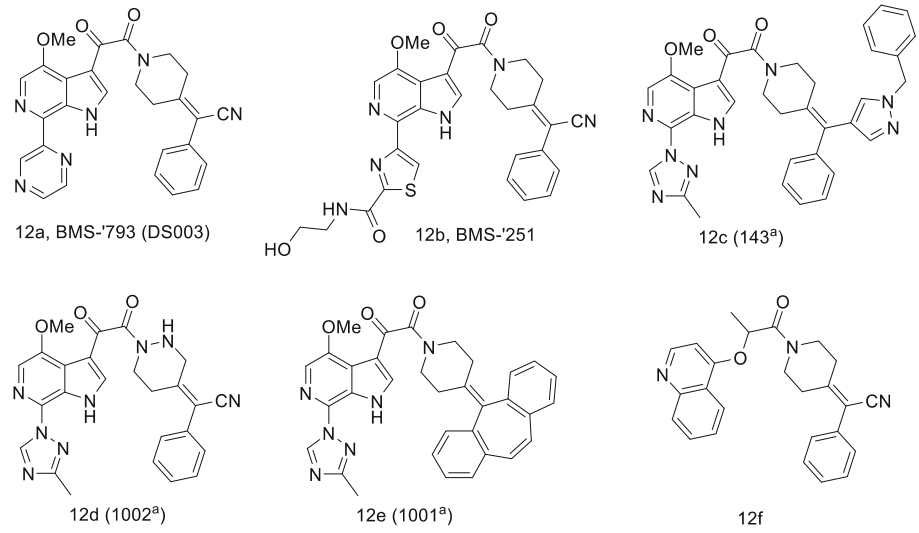


Fig. 4.12 Region D, Approach 5. ([a] The compound number or name in the original literature)

and **9e** (EC50 < 0.5 μM), and sulfone **9f** (EC_{50} < 1 μM) all belonged to the most active Category A. Of note, a lengthy substitution was tolerated on the amidine's nitrogen in **9e**.

Approach 3 in Region D provided potentially useful SAR. As shown in Fig. 4.10, all the examples were in the most active category A (EC_{50} < 0.5 μM). The carbonyl group of the benzamide was able to be changed to a benzene in **10a**, a mono-heterocycle in **10b**, a bicyclic aromatic ring in **10c**, and a saturated cycloalkane in **10d**. In **10e**, the distal nitrogen atom of the piperazine was transposed into the attached cyclopentane resulting in a piperidine ring rather than piperazine as the spacer. In **10f**, an additional large group was attached onto the pyrazole heteroaromatic ring, implying the protein binding interface around the moiety was either flexible or there was some space in this region (Wang et al. 2010).

Retention of potency did not require the phenyl group to be attached exclusively to the 2-position of the ring appended to the piperazine. For example, potency was retained when the phenyl ring was attached to the 3-position of the middle benzene ring, as in **10g** and the 6-position of a diazabenzothiophene, as in **10h** (Wang et al. 2010).

Moreover, further studies showed the carbonyl surrogate at the 2-position could be varied. The terminal benzene was exchanged with a range of functionalities such as a cycloalkane in **10i**, a ketone in **10j**, an amide in **10k**, and a simple ether in **10l** (Wang et al. 2010).

Approach 4 explored what was effectively merging and modifying the benzamide's carbonyl group to form bicyclic rings, either via attachment to the piperazine or with the distal phenyl ring. The majority of the published anti-HIV-1 data for those compounds was expressed as potency categories in the related patents, but these in combination with some published numerical EC_{50}s shed light on the SAR (Wang et al. 2011, 2014b; Swidorski et al. 2016). Forming a new five- or six-membered ring with the piperazine linker in place of the carbonyl group, provided data which clearly showed the preference of aromatic character for retention of potency. This was exemplified by comparison of **11a** (Wang et al. 2011) and **11c** (Swidorski et al. 2016) with **11b** (Wang et al. 2011) in Fig. 4.11. Both fused-imidazole **11a** and fused-benzene **11c** were more potent than BMS-'529 in the initial pseudotype assay (EC_{50}: 0.06 nM and 0.02 nM vs. 0.1 nM). When one of the C=C bonds in the imidazole was saturated, the resulting compound **11b** was significantly weaker, with an EC_{50} of 13 nM (Wang et al. 2011).

A similar trend was observed in **11d** and **11e**, in which the carbonyl element was infused into the distal benzene or pyridine. Both compounds were active, with the aromatic fusion in **11e** having a 10-fold advantage in potency over the non-aromatic fusion in **11d** (EC_{50}: 0.07 nM vs. 0.87 nM) (Wang et al. 2014b).

An extra benzene ring was added to the fused moiety to provide the tricyclic amidine **11f**. Its potency was more than tenfold less than the benchmark BMS-'529, but it was still potent (EC_{50} 1.83 nM). In addition, the bulkiness of **11f**'s tricyclic amidine suggested there was room for structural modification in the region (Wang et al. 2015a).

In Approach 5, the polarized double bond character of the benzamide's amide inspired its transformation into an alkene group, which lacks the amide bond's rotatory freedom and results in a rigid sub-structure. A collection of these piperidine alkene derivatives was described in a patent application (Wang et al. 2008b). Two compounds contained in that application, BMS-'793 (**12a**, DS003) (Schader et al. 2012; Ketas et al. 2007; Frank and Robbiani 2011) and BMS-'251 (**12b**) (Marie Pancera et al. 2017; Lai et al. 2019), have been extensively studied, suggesting the significance of the piperidine alkene chemotype. Both **12a** and **12b** (Fig. 4.12) are discussed in more detail in Sects. 4.4.1 and 4.5. It is noteworthy that **12c** had a bulky benzyl pyrazole on the alkene, along with the distal benzene, which again implied there was sufficient space for optimization around the region (Wang et al. 2008b).

Compound **12d** explored a cyclic hydrazine isostere of the piperidine alkene, which possessed one less carbon atom and one additional nitrogen

atom in the ring. It possessed an EC_{50} of 0.29 nM but was not pursued further (Wang et al. 2015b). Alternatively, two distal phenyl groups on C=C bond were joined to give tricyclic alkene **12e** which exhibited similar potency (EC_{50} 0.28 nM) (Wang et al. 2017a).

An array of piperidine alkene fragments were also incorporated into the 2-methyl-2-oxo-carboxamide chemotype and the example **12f** displayed an $EC_{50} < 1$ μM (Category A) (Wang et al. 2009c).

Region E covers all the variations which span over the piperazine benzamide moiety, but which could not be described by the definitions of Region A–D. The structures in this classification were very diverse, as shown in Fig. 4.13. Compound **13a** was made from the reduction of C=C bond of the corresponding alkene derivative, whilst **13b** was synthesized via the cyclopropanation of its alkene counterpart (Wang et al. 2016). Analogue **13c** was the regioisomer of compound **11b**, with a phenyl ring appended at the 3-position (Wang et al. 2017b). **13a**, **13b** and **13c** all maintained sub-nM potency (EC_{50}: 0.2 nM, 0.75 nM and 0.92 nM) respectively, even though **13c** was 45-fold weaker than **11b**. Compound **13d** contains a homo-piperidine linker rather than a piperazine or piperidine and retained potency with a double-digit pM EC_{50} (0.09 nM) (Wang et al. 2017b).

Another team reported data for the phenyl ketone analog **13e**, which was devoid of the nitrogen of the benzamide. **13e** was tested against JR-CSF, B41, and HxBc2 pseudo typed HIV-1, showing IC_{50}s of 3 nM, 7 nM, and 9 nM, respectively (Tuyishime et al. 2016). The significance of **13e** was that it had multiple freely rotatable C–C bonds and enjoyed much greater entropy than the benzamide analogs. Due to this result, the investigators advocated that there was opportunity for the next-level innovations beyond modifications of the glyoxamide moiety.

4.3 Alternate Chemotypes for Targeting gp120 Unrelated to Keto Amides

Several betulinic acid derivatives with broad subtype coverage were reported to target gp120 (Lai et al. 2008). Structure **14a** (Fig. 4.13) inhibited 25 HIV-1 clinical isolates of clades A, B, and C with an average IC_{50} of 506 nM. **14a** was proposed to bind to the V3 loop of gp120.

Another report described the gp120 antagonist **14b**, which displayed IC_{50} values of 63–170 nM against a panel of 56 HIV-1 pseudoviruses, covering clades A, A/D, A2/D, CRF01_AE, CRF02_AG, CRF13_cpx, B, C, D, and D/A. **14b** appeared to possess pan-subtype coverage (Fig. 4.14) (Curreli et al. 2018, 2020).

The CD4 mimics (CD4mcs), exemplified by JP-III-48 (**14c**) and BNM-III-170 (**14d**), exhibited somewhat complex virology data. They interacted with gp120 trimers with biphasic dose-response curves, showing dose-dependent activation and inactivation of HIV-1 infection. The proposed mechanism of action of these compounds was that gp120 was activated and could bind to receptor when one CD4mc was bound to one gp120 monomer within the trimer, but was inactivated when three CD4mcs occupied three cavities of one Env trimer. In addition to those two scenarios, when two CD4mcs complexed with one Env trimer, the infection of $CD4^+$ $CCR5^+$ T-cells was inhibited, but the infection of $CD4^-$ $CCR5^+$ T-cells was enhanced (Madani et al. 2017). Nevertheless, in the single-round pseudotype assay against YU, JRFL, AD8, and AMLV HIV-1 envelopes, **14c** and **14d**, respectively, exhibited IC_{50} values of 1.3/1.9 μM, 30.6/13.3 μM, 6.7/6.4 μM and >100/>100 μM. Recently, (S)-MCG-IV-210 (**14e**) was uncovered as a new class of CD4mc with a profile distinct from the **14c/14d** series (Ding et al. 2019; Grenier et al. 2020).

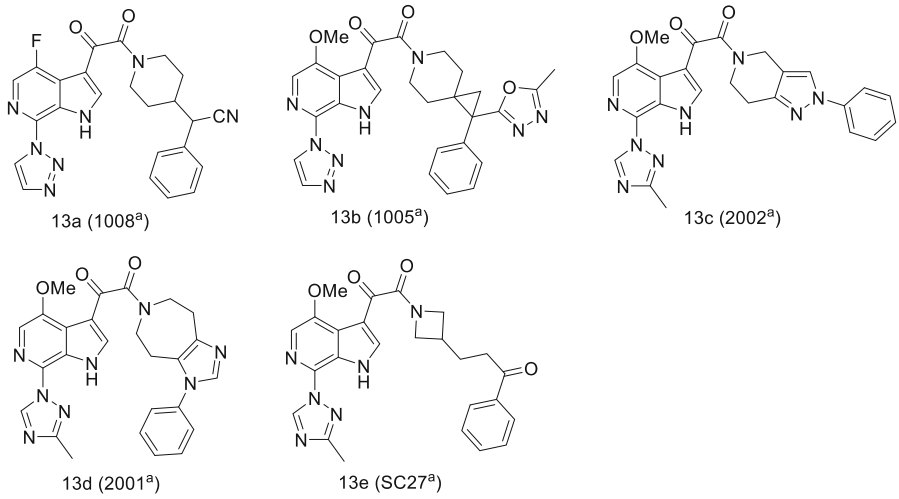
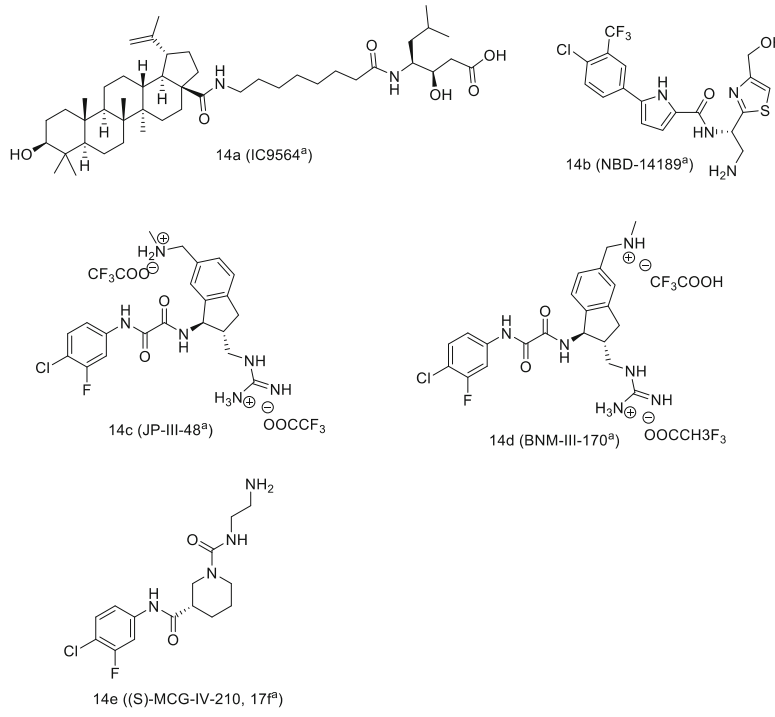


Fig. 4.13 Region E. ([a] The compound number or name in the original literature)

Fig. 4.14 Chemotypes independent from original keto amide series. ([a] The compound number or name in the original literature)



4.4 Application of HIV gp120 Inhibitors

4.4.1 As Microbicides

The best possible course against any viral infection is prevention, fending off the virus's invasion at the earliest possible step. General types of prevention include vaccines, microbicides, or drug treatments (Pre-exposure Prophylaxis, or PrEP). An effective HIV-1 vaccine has not been realized and remains a strategy of great interest. PrEP, uses HIV drugs to prevent HIV infection. Daily oral pill regimens are approved for PrEP and recently, monthly intramuscular injections of cabotegravir have shown superiority over daily pills in two clinical studies, HPTN 083 and HPTN 084 (https://www.hptn.org/news-and-events/press-releases/hptn-083-study-demonstrates-superiority-cabotegravir-prevention-hiv, https://www.hptn.org/news-and-events/press-releases/hptn-084-study-demonstrates-superiority-of-cab-la-to-oral-tdfftc-for). HIV Microbicide is another effective approach to preventing HIV infection. It is known that HIV-1 crosses the human body surface barriers and that uptake occurs by the Langerhans cells in the epithelium and by dendritic cells in the stroma. The interactions between the host cells and virus involve HIV-1 gp120. It was shown HIV AIs' ability to block HIV-1's uptake by skin cells (Nuttall et al. 2007), and, recent *in vitro* models using on an HIV AI from the BMS series, BMS-'793 (**12a**), illustrated the potential of this class of molecules to be used in PrEP. (Herrera et al. 2021).

Initial PrEP studies used BMS-'806, which was evaluated for its ability to protect macaques from vaginal SHIV infection, alone or with C52L (a gp41 inhibitor) and/or CMPD167 (a CCR5 antagonist), which were two other mechanistically distinct types of HIV entry inhibitors. BMS-'806 demonstrated moderate efficacy when used alone, 6 out 8 animals avoided infection when BMS-'806 was dosed at 5.5 mM. The efficacy was dramatically improved in combinations, with 100% protection (three animals) when 5.5 mM of BMS-806 was mixed with CMPD167 (5.0 mM) and C52L (1.5 mM) (Veazey et al. 2005).

Results of studies using BMS-'793 (Schader et al. 2012), showed it to be a much more potent analogue of BMS-'806 as a potential microbicide, either alone or in combination with other types of HIV entry inhibitors (C52L, CMPD167 and CXCR4 ligand AMD3465), against an array of subtypes A-G clinical isolates (25 primary R5, 12 X4 and 7 R5X4 strains). Replication in human donor lymphocytes was inhibited 84% with R5 isolates, 58% with X4 isolates and 14% with R5X4 isolates, by BMS-'793 at a concentration of 1 μM. When BMS-'793 was used in combinations with C52L or CMPD167 or AMD3465, 100% inhibition was consistently achieved (Ketas et al. 2007). Additional studies reported inhibition of dendritic cell-driven HIV-1 infection, the main mechanism for HIV-1 mucosal transmission, using BMS-'793, T1249 (a gp41 inhibitor) and CMPD167. Under all the experimental conditions, BMS-'793 appeared to be the strongest inhibitor. In particular, BMS-'793 was significantly more potent than CMPD167 in preventing infection of DCs (Frank and Robbiani 2011). More recent studies in tissue explant and trans-infection models in combination with a fusion inhibitor confirmed it's potential as an agent for PrEP (Herrera et al. 2021). Currently, BMS-793 is in development by the International Partnership for Microbicide (IPM) as a microbicide candidate in the form of a vaginal tablet. Its Phase I study (IPM 042), a randomized, double-blinded, dose-escalating trial of HIV-negative healthy female participants, to evaluate safety, pharmacokinetics (PK) and pharmacodynamics (PD) was completed.

A molecule of another class of HIV AIs, CD4mc derivative JP-III-48, was evaluated for its ability to prevent HIV-1 vaginal transmission in bone marrow-liver-thymus (BLT) humanized mice. JP-III-48 was capable of inhibiting 100% of HIV-1 infections in experiments where 300 μM of JP-III-48 and JR-CSF virus were applied together. At a higher concentration of 3000 μM, 100% inhibition was achieved when JP-III-48

was administered 30 min prior to the HIV-1 challenge (Princiotto et al. 2018).

The same team also challenged monkeys with a simian-human immunodeficiency virus (SHIV) via the intrarectal route to assess protection by BNM-III-170, another CD4mc. BNM-III-170 had been shown to bind to Env trimer and induced conformational changes which exposed epitopes recognized by non-bNAbs. The immunoprotection in the cohort of gp120 (as immunogen) plus BNM-III-170 was 100% at week 14 and 75% at week 18, while the placebo group of gp120 plus DMSO provided 0% protection at week 9 (Madani et al. 2019).

4.4.2 As Target Recognition Element in Conjugates

The concept of using small molecule warheads to direct antibody recruiting molecules (ARM) to gp160-expressing cells has been explored to assess the potential to treat HIV-1 infection. HIV AIs were tethered to antibody recruiting elements such as a dinitrophenyl (DNP) group. Anti-DNP antibodies, which naturally occur at preexisting concentrations in the blood stream, were directed to gp160-bound viral particles and cells. Compound **15a** was able to disrupt the interaction between HIV gp120 and a $CD4^+$ T-cell, and to kill gp120-expressing cells via complement-dependent cytotoxicity (CDC). At 30 μM, compound **15a** (Fig. 4.15) was capable of killing ~20% of HIV-Env-expressing CHO cells (Parker et al. 2009). Later, the second generation ARM **15b** eliminated ~30% and ~70% of HIV-1 Env (JRFL)-expressing HEK293T cells at a concentration of 30 μM and 60 μM, respectively (Parker et al. 2014). It appears a more robust immune response will be needed to make this approach viable.

An alternative approach to recruiting antibodies used covalently linked HIV AIs (e.g., BMS-'806 and BMS-'043) and mAb 38C2 Fabs to generate multi-valent gp120 binders. Exploration of linking strategies determined that a 7-position linker strategy such as that used in the BMS-'043 derived programmed antibody **15c** performed best, with an IC_{50} value of 128.6 nM in a single-round neutralization assay using U87.CD4.CCR5 cells and the HIV-1 JRFL strain. The research demonstrated the ability of HIV AIs to be used to create bivalent binders. The authors stated that further assessment of **15c** was warranted since the approach was also designed to use the small molecule for localization but have the conjugate provide improved properties such as pharmacokinetics and half-life since it was anticipated those properties would be defined by the antibody. Furthermore, Fc engineering of the antibodies could optimize the conjugates ability to potentially elicit antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) or complement dependent cytotoxicity (CDC), to enhance specific destruction of infected cells (Sato et al. 2013).

Another approach to selectively kill HIV infected cells was to link cell killing or cytotoxic agents to HIV AIs. HIV AIs were used to direct the toxic agent to gp160-expressing cells, which was expected to provide selective killing of HIV infected cells (Spiegel and Parker 2017). One example **15d** demonstrated a consistent ~10% gp160-selective toxicity in an assay using a single addition of drug, while multiple additions increased gp160-selective toxicity to ~35%. Given the excellent safety of current HIV regimens, considerable progress will need to be made if cell killing agent delivery approaches are to become viable.

4.4.3 As Immuno-Protectants or Immuno-Stimulators

It is known that not all HIV-1 viral particles are infectious. The infectious viral particles (VPs) carry out their replication by invading human $CD4^+$ T-cells and producing off-spring. The defective, non-infectious viral like particles (VLPs) that constitute the majority of gp120 particles also interact with uninfected $CD4^+$ T-cells, $CD8^+$ T-cell and NK cells. The cytotoxicity of gp120 in these interactions manifests itself in multiple ways. For example, binding of VLPs to some cells can induce apoptosis or

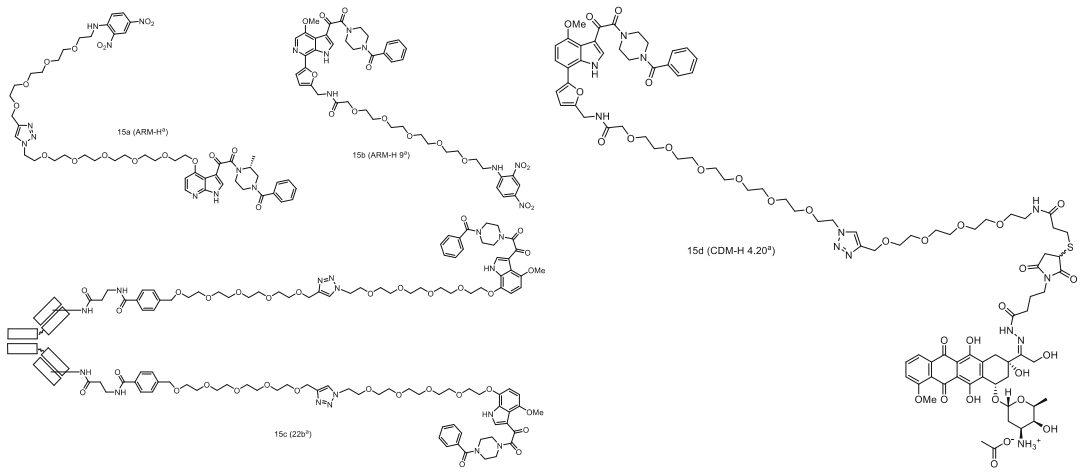


Fig. 4.15 HIV AI conjugates. ([a] The compound number or name in the original literature)

initiate ADCC effects against these immune cells. This killing of what appear to be bystander, non-HIV infected $CD4^+$ T-cells contributes to the diminished CD4 counts found in HIV-1 patients.

Early work showed VLPs induced the loss of $CD4^+$ T-cells. VLPs with Env initiated $CD4^+$ T-cell apoptosis in a dose-dependent manner, while VLPs without Env had no effect on apoptosis. Because HIV AIs bind to gp120, regardless of whether it is on VPs or VLPs, AIs have the capacity to inhibit bystander killing of uninfected $CD4^+$ T-cells. In one study, the potency against the NL_{4-3} strain and the anti-apoptotic potency against VLPs packaged with NL_{4-3} Env of HIV were evaluated with entry inhibitors BMS-'806, BMS-'043, soluble CD4 (sCD4), AMD-3100 (a competitive inhibitor of gp120 binding to the co-receptor CXCR4) and T-20 (a peptide anti-HIV fusion inhibitor) (Alexander et al. 2009). BMS-'806 and BMS'-043 demonstrated similar EC_{50} values in both the antiviral (2.9 nM and 17.4 nM) and anti-apoptotic (5.8 nM and 11.7 nM) assays. In comparison, sCD4 had an eightfold difference (12.4 nM vs. 98.1 nM) and T-20 showed a 24-fold divergence (22.4 nM vs. 548 nM), whilst AMD-3100 displayed equal potencies (10.8 nM vs. 8.8 nM). The conclusions were that HIV AIs are potent inhibitors of T-cell deletion, and secondly that the IV AI's antiviral and anti-apoptotic effects may share a common mechanism, presumably by preventing binding of the gp120 protein to its cell surface CD4 receptor.

Immunologically, gp120 can also deregulate an immune response, as for example by blocking T-Cell activation and contributing to viral escape. One of the main mechanisms of HIV's immune evasion is to acquire mutations in gp120 that lead to heterogenic and interconverting conformations not easily recognized by antibodies. It has also been postulated that mutations in gp120 that reduce susceptibility to HIV attachment inhibitors may stabilize epitopes and enhance antibody neutralization. This hypothesis was explored in relatively early work on AIs and showed mutations selected by HIV AI treatments could increase susceptibility of certain HIV variants to broadly neutralizing antibodies (Zhou et al. 2010). Three escape mutants of LAI virus selected by an early HIV AI contained one of the following mutations: F423Y, an amino acid located in gp120-CD4 binding pocket, and I595F or K655E, both of which are found in gp41 ectodomain. As anticipated, BMS-'043 displayed a loss in potency against the three mutants with EC_{50} values of 10.9 nM (F423Y), 19.0 nM (I595F), and 6.06 nM (K655E), compared to 0.98 nM for the wild-type virus. The potency of two bNAbs antibodies, 2F5 and 4E10, were increased by >9-fold against these mutant viruses. The EC_{50} values for inhibition by 2F5 were 1.88 μg/mL,

0.06 μg/mL, 0.22 μg/mL and 0.02 μg/mL against the wild-type, mutant F423Y, mutant I595F and mutant K655E, respectively. The EC_{50} values for inhibition by 4E10 were 9.29 μg/mL, 0.72 μg/mL, 0.27 μg/mL and 0.22 μg/mL against the wild-type, F423Y, I595F and K655E, respectively. As controls, HIV entry inhibitors AMD-3100 and Enfuvirtide (T-20) did not show any differential inhibition against the variant viruses.

Recent studies by another team investigated the effects of combining temsavir and anti-HIV-1 antibodies (1:1 molar ratio) against a HIV ADA pseudotyped virus (Zhang et al. 2019). Synergy was observed between temsavir and 5 out of 7 CD4 binding site (CD4bs)-targeting bNAbs. The CD4bs-targeting bNAbs combinations were able to maintain IC_{50} values under 0.45 nM. Moreover, all the bNAbs, including those which targeted gp120's CD4 binding site, gp120's V1V2 segment, gp120's V3 loop, and gp41-gp120 interface, were potent vs. virus containing temsavir's key escape mutants, such as M426L, S375M, M426L/M434I, M426L/M475I and S375M/M434I. For example, the S375M/M434I mutations decreased temsavir's IC_{50} from 0.14 nM to 29.58 nM. All eleven bNAbs demonstrated IC_{50} values against the S375M/M434I variant with values of 0.001–0.23 nM.

Mechanistically, HIV AIs are now believed to stabilize the closed conformation of gp120, which prevents access to gp120 conformations permissive for binding to the CD4 receptor. The closed conformation is also thought to be the most efficient conformation for induction and binding of bNAbs. The open conformation of gp120 trimer, which is permissible for binding to CD4 receptor on cells, also is more susceptibility to the binding of non-neutralizing Abs. The binding of non-neutralizing Abs could compete with bNAb binding, effectively inhibiting neutralization. Recent studies show how temsavir constrains the conformation change of the gp120 trimer when presented either in a soluble form or a membrane-bound mode. The stabilized Env trimers remained constrained in the pre-CD4 binding closed form and the immunogenicity of select Env immunogens were improved (Alam et al. 2020).

In contrast, the CD4mc inhibitors bound to gp120 induce the Env to convert to the open form and uncover conserved regions for non-neutralizing Abs to bind. These non-neutralizing antibodies trigger ADCC-mediated killing of HIV-1 infected cells (Richard et al. 2015, 2017). For instance, JP-III-48 (**14c**) increased the percentage of ADCC-mediated killing of wild-type HIV-1 infected primary $CD4^+$ T-cells from ~2 to 8% (Richard et al. 2015). In a FACS-based ADCC assay of killing infected p24+ cells, at 50 mM concentration, BNM-III-170 (**14d**) and (S)-MCG-IV-210 (**14e**) enhanced the ADCC effect from 5% to 14% and 10%, respectively (Ding et al. 2019; Grenier et al. 2020). The key questions to be addressed in this area of research is whether enhanced levels of ADCC-mediated cell killing can be attained and will they be sufficient to provide a physiologically relevant impact.

4.4.4 As Agents Against Cytopathic and Non-cytopathic Effects of gp120

The cytopathic effects of gp120 are not specific towards $CD4^+$ T-cell and other immune cells, as the effects are more widespread (Levy 2007). Neuronal cells, tubular epithelial cells, cardiac myocytes and endothelial cells, among others, can be damaged in the presence of bystander effects elicited by gp120 binding to CD4 present on many different cell types.

There are also numerous deleterious effects reported within cells that express gp160. Among the effects attributed to its expression are the release of calcium on mucosal surfaces, compromising integrity and enhancing monocyte migration across the blood-brain barrier, induction of pro-inflammatory cytokine release and blocking the endogenous GHRH receptor. Pathophysiological effects attributed to HIV include dementia, nephropathy, cardiomyopathy, enteropathy encephalitis, pain, and wasting, all of which may be observed in HIV-1 infected

patients and may be exacerbated by the expression of gp160 (Levy 2007).

The ability of HIV AIs to bind to accessible gp160 expressed on envelope or surfaces provides considerable potential for drugs with this mechanism to improve the quality of life. For instance, the protection of neuronal cell apoptosis induced by HIV VLP was reported. In the study, two neuronal cell lines, SH-SY5Y and BE (2)-M17, were $CD4^-$, $CCR5^+$ and $CXCR4^+$, which suggested that virus could not infect these cells due to lack of CD4. BMS-‘806, BMS-‘043, sCD4, TAK779 (a maraviroc-like molecule that binds to CCR5) and T-20, all HIV entry inhibitors, were assessed against the impact of HIV-1 VLP’s cell killing in the SH-SY5Y and BE(2)-M17 cells. EC_{50}s of BMS-‘806 and BMS-‘043 were 14.7 nM and 11.3 nM against SH-SY5Y killing and 17.5 nM and 10.6 nM against BE(2)-M17 killing, respectively. sCD4 had a similar protecting capacity, whilst TAK779 and T-20 showed no effect. It was concluded that HIV gp120 was capable of interacting with and eliminating neuronal cells in a CD4-independent manner. The mechanism is believed to be distinct from that involved in the viral entry process. Thus, it was postulated that HIV AIs may protect neuronal cells from gp120’s cytopathic effects (Zhang et al. 2010). The actual benefits of fostemsavir in reducing the effects caused by VLPs or noninfective HIV particles, if clinically significant, should become more evident with expanded clinical experience and use.

4.5 Future Directions

Fostemsavir is the culmination of considerable preclinical optimization and sustained development efforts. An extended-release formulation of this prodrug enabled the twice daily dosing schedule (BID), which was ultimately pursued to maximize the chances of success in development for HTE patients, despite the fact that early clinical trials demonstrated the potential feasibility of daily (QD) dosing. Critical for its continued development has been the excellent safety profile displayed by fostemsavir and the optimization of the process chemistry and manufacturing processes. Substantive efforts were made to identify a HIV attachment inhibitor with even better properties but at this point in time, none have been disclosed to be in active development (Brown et al. 2013).

A hypothetical next generation AI could offer expanded utility to patients if ever pursued and realized. Once daily (QD) oral dosing or long-acting injectable (LAI) molecules could offer decreased frequency of dosing. Increasing the intrinsic $t_{1/2}$ or potency beyond that of temsavir would help achieve these goals as might additional application of slow release technologies.

In addition, the selected dose of AIs is dependent on covering the minimum effective concentration of as broad a range of HIV-1 virus clades and subtypes as feasible. Since gp120 envelope possesses sequence variability in a number of regions, pre-existing heterogeneity has been reported to result in a wide range of susceptability (Soulie et al. 2013; Charpentier et al. 2012; Fofana et al. 2015; Alessandri-Gradt et al. 2018; Lepore et al. 2020; Bouba et al. 2020). However, in a recently published study using a Phenosense assay, 1337 individual envelopes encompassing 20 different HIV-1 subtypes were examined for their susceptibility to temsavir (Gartland et al. 2021). While there was variability in susceptibility observed within all subtypes, against the majority of tested viruses, temsavir was highly potent, with most viruses exhibiting IC_{50}s < 10 nM. An exception was CRF01_AE viruses, where all five isolates examined exhibited IC_{50}s > 100 nM. The spectrum and potency of temsavir is broad but because it was originally optimized vs. the HIV-1 B subtype, it does not cover every subtype such as, noted above, genotype CRF01_AE. Gp120 is heavily and nonuniformly glycosylated and is highly flexible and conformationally mobile. It is possible that a recently emerging better understanding of the exact mode of binding to gp120 and its ligands will help in further refining potential future generations of gp120 binders. For example, X-ray co-crystal structures of a number of AI analogs including gp120 + BMS-‘806, gp120 + temsavir and gp120 + BMS-‘251 have

recently been reported, which may result in new insights for rational design improvements (Marie Pancera et al. 2017; Lai et al. 2019). Moreover, one of the analogs, BMS-‘251 and temsavir were screened against 208 HIV-1 strains in a cellular assay, with BMS-251’s potency enhanced versus some less susceptible HIV-1 viruses from clades A, CRF01_AE, CRF02_AG, B, CRF07_BC, C, and G (Lai et al. 2019). The IC_{50} geometric means were >40 nM for temsavir and <2 nM for BMS-‘251. Specifically for the CRF01_AE subtype, BMS-‘251’s IC_{50}s against four isolates were 730 nM, 89 nM, 471 nM and 32 nM, in comparison with temsavir’s >20,000 nM, 6210 nM, > 20,000 nM and 5840 nM, respectively (Lai et al. 2019). The study demonstrates that improving the pan-subtype coverage is achievable *in vitro* if it could be incorporated into a molecule with an excellent drug-like profile. HIV AIs with an expanded pan-subtype coverage would potentially enable a better microbicide for HIV prevention in the absence of vaccines but realistically only if the synthesis was much shorter and less complex than that of fostemsavir. If a long-acting injectable formulation of an AI were discovered, it is possible it could find use in PrEP regimens.

Considering the recent approval of fostemsavir, there has not been time yet for definitive studies looking to see if fostemsavir will have at clinically meaningful positive additive or synergistic effects on the actions of therapeutic antibodies or bNAbs. Positive data in this arena may suggest a potential role in regimens moving closer to realizing a functional cure. The potential for using AIs for targeting and eradicating HIV infected cells has been demonstrated so if further optimization of the conjugation strategies for delivery of immunological or other specific mediators of killing those cells are realized, they could also potentially be useful in future cure regimens (Sloan et al. 2015).

4.6 Conclusions

The discovery of agents that target the HIV viral envelope gp120 has been fruitful and there is still potential for further treatment advances. The recent approval of fostemsavir (RUKOBIA™) has provided an important new option in the treatment of HTE HIV patients and clinical experience and expanded usage should further inform about potential other benefits in the future. In addition to AIs, CD4 mimics, agents that target other regions of gp120, and conjugates of these small molecules designed to elicit specific killing of HIV selected cells all offer the potential for utility in regimen’s that deplete reservoir or move closer to realizing HIV cure.

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Endogenous Peptide Inhibitors of HIV Entry

5

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Abstract

The discovery of the G-protein coupled-receptor (GPCR) CXCR4 as a major coreceptor of HIV-1 entry about three decades ago explained why the chemokine SDF-1/CXCL12 inhibits specific viral strains. The knowledge that RANTES, MIP-1α, and MIP-1β specifically inhibit other primary HIV-1 strains allowed the rapid discovery of CCR5 as second major viral coreceptor and explained why individuals with deletions in CCR5 are protected against sexual HIV-1 transmission. Here, we provide an update on endogenous ligands of GPCRs that act as endogenous inhibitors of HIV-1, HIV-2, and simian immunodeficiency virus (SIV) entry. In addition, we summarize the development of optimized derivatives of endogenous GPCR ligands and their perspectives as antiviral agents and beyond. Finally, we provide examples for other endogenous peptides that may contribute to our innate immune defense against HIV-1 and other viral pathogens and offer prospects for preventive or therapeutic development.

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5.1 Introduction

Our innate immune response represents the first line of defense against viral pathogens. Detection of foreign viral invaders by immune sensors induces the interferon system and consequently increases expression of numerous antiviral factors as well as activation of immune-associated proteases. Antimicrobial peptides (AMPs) are an important component of the innate immune response (Ahmed et al. 2019; Sørensen et al. 2008). While many intracellular antiviral factors restrict viral replication in cells that are already infected, AMPs usually act outside of the cell and may destroy viral particles directly and/or protect uninfected cells against viral entry. Many human AMPs, such as defensins and cathelicidin LL-37, are positively charged and helical and best known for their broad antibacterial activity (Diamond et al. 2009; Wang et al. 2014). It has become clear, however, that AMPs are structurally more versatile than initially thought and also display antiviral and immunomodulatory activities (Vilas Boas et al. 2019; Pahar et al. 2020).

In the case of HIV-1 and related nonhuman primate lentiviruses, endogenous inhibitors may not only play a role in controlling viral replication

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but were also instrumental in elucidating the viral entry process. Since depletion of CD4+ T cells is a hallmark of HIV-1 infection *in vivo*, CD4 was identified as primary receptor soon after the discovery of this virus as causative agent of AIDS (Sattentau and Weiss 1988). While it also rapidly became clear that additional factors are required for viral entry, their identification took more than a decade (Alkhatib and Berger 2007). The breakthrough came with the discovery of CXCR4 as entry cofactor of HIV-1 strains causing strong cytopathic effects in immortalized T cell lines. CXCL12 (initially named SDF-1), the chemokine ligand of CXCR4, inhibited infection of some HIV-1 strains (Ahmed et al. 2019). Many other HIV-1 strains, however, were inhibited by CCL5 (initially named RANTES) and to a lesser extent by CCL3 (MIP-1α) and CCL4 (MIP-1β), which were all already known to interact with CCR5 (Lusso 2006). This knowledge allowed the rapid discovery of CCR5 as major coreceptor of primary HIV-1 strains (reviewed in: Alkhatib and Berger 2007). Subsequent studies showed that CCR5 plays the key role in virus transmission and during chronic infection, while CXCR4-tropic HIV-1 strains emerge in about half of all AIDS patients and are associated with rapid disease progression in the absence of combined antiretroviral therapy (cART) (Connor et al. 1997; Schuitemaker et al. 2011).

Since the initial discovery of CCR5 and CXCR4 as major coreceptors of HIV-1, several other GPCRs have been reported to mediate HIV-1 entry albeit with lower efficiency (Pollakis and Paxton 2012; Wetzel et al. 2018). It has also been established that HIV-2 and related simian immunodeficiency viruses (SIVs) are more promiscuous in coreceptor usage. A variety of chemokines and peptidic GPCR ligands have been reported to specifically inhibit HIV-2 and SIV entry (Fig. 5.1). Most but not all of them also regulate the physiological signaling function of the respective GPCRs and may thus play important roles in the trafficking and function of immune cells. In this review, we summarize key findings on endogenous peptide inhibitors of HIV-1 and related primate lentiviruses, with a focus on entry inhibitors. We also discuss their mechanism of action, the optimization of endogenous agents, and their potential prospects for preventive or therapeutic development and application.

5.2 Antiviral Host Defense Peptides

5.2.1 Defensins

Peptides exhibiting antimicrobial, antifungal, or antiviral activity are referred to as Host Defense Peptides (HDPs). These are commonly constitutively expressed in mucous tissue and induced early during immune activation as a first line of defense against human pathogens (Schröder and Harder 1999). Defensins form a diverse group of 30–40 amino acid long peptides sharing similar charge, morphology and antimicrobial properties (Shafee et al. 2016). While the α-defensin precursors DEFAs (Defensin, alpha) proteins, cluster on chromosome 8, β-defensin are encoded on a variety of genes and grouped based on their structure and function. All defensins are characterized by three intramolecular cysteine-disulfide bonds, β-sheet structure and their cationic and amphipathic properties (Bulet et al. 2004). The human α-defensins 1–3, and human β-defensin-2 and 3 inhibit replication of CCR5- and CXCR4-tropic strains of HIV-1, including several primary isolates in the low μM range (Hu et al. 2019; Quiñones-Mateu et al. 2003; Wu et al. 2005). Structural integrity of the three intramolecular cysteine-disulphide bonds, as well as side chain hydrophobicity, were shown to be critical for antiviral activity. Human α-defensins 1–3 and 5 are lectin-like and bind the glycosylation sites of the HIV-1 gp120, thereby inhibiting virion attachment. However, the antiviral activity of human α-defensin 5 remains controversial (Ding et al. 2013; Furci et al. 2012). α-defensin concentrations in human saliva vary between 1 and 10 μg/ml, being in the range of antimicrobial and antiviral active concentration (Gardner et al. 2009).

Of three known classes of defensins (α-, β-, and θ-defensin) only α- and β-defensins are

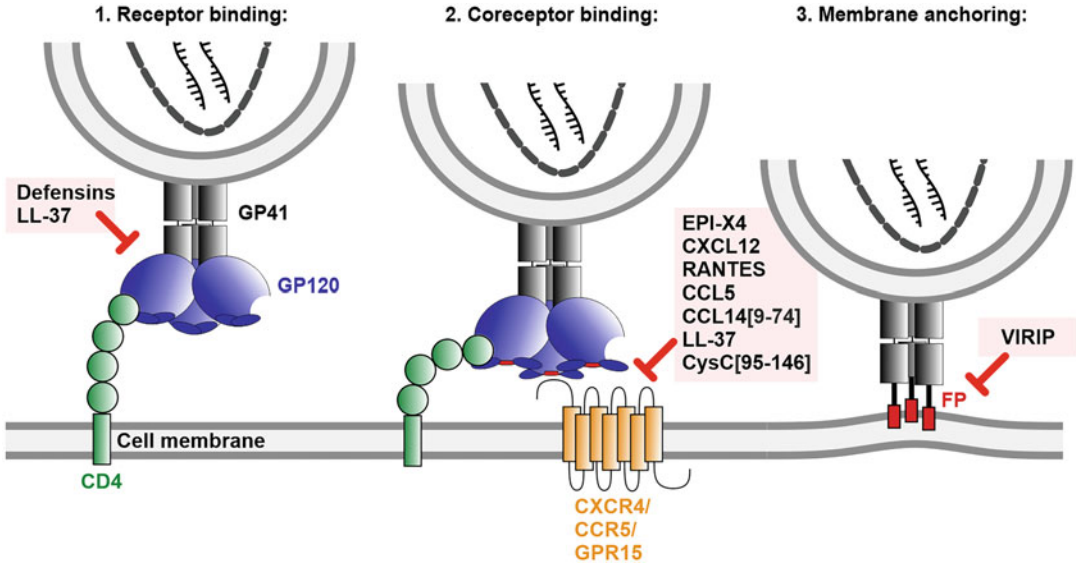

Fig. 5.1 Schematic presentation of HIV and SIV entry and endogenous (poly)peptides inhibiting this process. CD4 binding of the viral glycoprotein (e.g., HIV-1 GP120) and subsequent interactions with GPCRs result in the liberation and membrane insertion of the GP41 fusion peptide (FP). Subsequent six-helix bundle formation triggers the fusion of viral and cellular membranes and ultimately leads to the release of the viral capsid into the cytosol of the host cell. Virus infection can be blocked at several stages by the indicated endogenous antiviral chemokines and peptides

transcribed in humans. Human θ-defensin is a pseudogene harboring a premature stop codon (Daher et al. 1986; Zhao et al. 2013). θ-defensins, which are expressed in monkeys, or synthesized based on the human pseudogene sequence, are named retrocyclins (Cole et al. 2002; Nguyen et al. 2003). Unlike α- and β-defensin, retrocyclins are circular peptides but contain the disulphide bonds that are characteristic for defensins. Retrocyclins were found to bind and restrict the HIV-1 glycoproteins gp120, gp41 and its cellular receptor CD4 due to its lectin-like ability to bind N-and O-linked carbohydrates, while having little to no effect on cell fusion of HIV-2 and SIV (Münk et al. 2003; Owen et al. 2004; Wang et al. 2003). Additionally, retrocyclins may bind the HIV-1 heptad repeats, thereby inhibiting 6-helix bundle formation of gp120 and virus-cell fusion (Cole et al. 2006; Gallo et al. 2006). The retrocyclin congener RC-101 was confirmed safe during *in vivo* application in pigtailed macaques. RC-101 was applied in the cervix and vagina of the primates as a quick-dissolving film, where it remained for several days and kept its activity against HIV-1 and SIV (Cole et al. 2010). Recent studies characterize the role of defensins in HIV response in human macrophage systems and connect the antiviral effect of defensins to GPCR mediated signaling pathways (Bharucha et al. 2021). It remains to be determined, whether defensins play a role in the innate defense against HIV-1 *in vivo*. However, their broad antiviral and antimicrobial effects make defensins interesting for future therapeutic applications (Park et al. 2018).

5.2.2 LL-37

Most HDPs are expressed as precursor proteins and proteolytic processing leads to the release of the bioactive peptides. A well-studied member of the cathelicidin protein family is hCAP-18. hCAP-18 can be processed in 16 different fragments some of which show antimicrobial and antiviral activity (Agerberth et al. 1995;

Steinstraesser et al. 2005). The latter, in particular, has been described for LL-37, a 37-aa cationic peptide generated by proteinase 3 mediated cleavage of the C-terminal end of hCAP18. LL-37 has an α-helical structure and forms aggregates in solution (Shahmiri et al. 2016; Wang et al. 2014). Its amphipathic nature allows LL-37 to insert into lipid bilayers. Thus, unlike other HDPs, LL-37 is protected from proteolytic degradation (Oren et al. 1999). Further optimization of LL-37 led to the identification of FK-17 as minimal active antiviral peptide (Wang et al. 2008). The antibacterial activity of LL-37 is mediated by permeabilization of bacterial membranes. Cholesterol, which is absent in bacterial cell membranes, protects mammalian cells from LL-37 mediated pore formation (Brender et al. 2012; Sancho-Vaello et al. 2020). The antiviral mechanism of LL-37 is under debate. An inhibitory effect of LL-37 on the HIV-1 protease has been observed (Wong et al. 2011) but effects on membrane integrity of enveloped viruses have also been proposed (Wang et al. 2014). Current studies focus on the antimicrobial activity of LL-37 and its application in multivalent systems (Lakshmaiah Narayana et al. 2021; Mori et al. 2021). Notably, LL-37 was also found to directly interact with the HIV-1 cofactor CXCR4 (Pan et al. 2018; Podaza et al. 2020), but it is currently unclear whether LL-37 inhibits CXCR4-tropic HIV-1 infection.

5.3 Endogenous Ligands Targeting CXCR4-Mediated HIV Infection

C-X-C chemokine receptor type 4 (CXCR4) is a typical G-protein coupled receptor composed of seven transmembrane domains (Wu et al. 2010). It plays important roles in immunity, tissue regeneration and hematopoietic stem cell homeostasis (Pozzobon et al. 2016). CXCR4 dysfunction is associated with several malignancies, such as inflammatory diseases and cancer, making CXCR4 an important drug target (Pozzobon et al. 2016). Besides this, CXCR4 is also a major coreceptor for HIV-1 entry (Moore et al. 2004) (Fig. 5.1). HIV-1 infection is typically initiated by unspecific attachment of the virion to a host cell by its external gp120 envelope glycoprotein (Env). Subsequent binding to the primary CD4 receptor induces structural rearrangements in gp120 resulting in the interaction with viral coreceptors, mainly CXCR4 and CCR5, triggering conformational changes in gp41 that finally drive membrane fusion (reviewed in Chen 2019). CCR5-tropic HIV-1 variants dominate during acute and chronic infection. During or after AIDS progression a coreceptor switch or expansion is observed in some patients. CXCR4-tropic viruses are associated with a more rapid deterioration of the immune system leading to faster disease progression (Mosier 2008) in the absence of cART. The gp120 V3 loop determines coreceptor interaction and only a few amino acid changes are required to switch from CCR5 to CXCR4 coreceptor usage (De Jong et al. 1992). So far, it is largely unknown, which factors trigger the coreceptor switch (Connell et al. 2020; Regoes and Bonhoeffer 2005).

5.3.1 CXCL12

C-X-C motif chemokine 12 (CXCL12, formerly named SDF-1) is so far the only classical chemokine known to interact with CXCR4 and to block CXCR4-tropic HIV-1 infection (Bleul et al. 1996; Janssens et al. 2018). It is constitutively expressed in many tissues, especially the bone marrow and lymph nodes, where it acts as chemoattractant for lymphocytes (Nagasawa 2007). CXCL12 is encoded by a single gene and several splice variants are described with molecular weights between 8 and 14 kDa (Gleichmann et al. 2000; Yu et al. 2006). CXCL12 shares the common structure of chemokines: a disordered N-terminus followed by a globular core domain. Binding of CXCL12 to CXCR4 is initiated by interactions with the receptor N-terminus and then the binding pocket, which is shaped by the receptor transmembrane domains and extracellular loops (Wu et al. 2010; Xu et al. 2013). This so called "two-site"-binding model has also been implicated for gp120 interaction with CXCR4,

which interacts with the N-terminus of CXCR4 and subsequently with the second and third extracellular loops of the receptor (Kalinina et al. 2013; Lin et al. 2003; Wu et al. 2010). The basic V3 loop may also penetrate the positively charged receptor binding pocket (Wu et al. 2010). Considering the similar binding modes, it is plausible that CXCL12 inhibits CXCR4-tropic HIV-1 by blocking access of the viral gp120 to the GPCR. However, CXCL12 also induces CXCR4 downmodulation and this effect correlates with the antiviral activity of CXCL12 isoforms, suggesting that receptor downmodulation contributes to its antiviral activity (Altenburg et al. 2010; Amara et al. 1997). CXCL12α and CXCL12β are the two most abundant isoforms in humans, both inhibiting CXCR4-tropic HIV-1 with IC_{50} values in the nanomolar range (Altenburg et al. 2007, 2010). CXCL12γ has been shown to have even more potent anti-HIV-1 activity due to increased affinity to CXCR4 and more efficient receptor internalization. However, this isoform is mainly expressed in the adult human heart and hardly detected in other tissues (Yu et al. 2006).

It is tempting to speculate that CXCL12 might be involved in HIV-1 transmission and pathogenesis. HIV-1 is mainly sexually transmitted and in most cases single, so called "transmitted/founder" (T/F) viruses establish infection (Parrish et al. 2013; Joseph et al. 2015). T/F viruses are almost exclusively CCR5-tropic, indicating a selective strong transmission barrier for CXCR4-tropic viruses (Grivel et al. 2010). The reason(s) for specific restriction of CXCR4-utilizing viruses are currently unclear. One plausible explanation is the presence of inhibitory CXCR4 ligands at sites of initial virus infection. CXCL12 is constitutively produced by epithelial vaginal cells and might contribute to selective inhibition of CXCR4-tropic viruses (Francis et al. 2016). However, CXCL12 levels in vaginal fluids vary and are frequently low (17.4–2071.5 pg/ml) (Francis et al. 2016), arguing against a major role of CXCL12 in preventing sexual transmission of CXCR4-tropic HIV-1 strains.

In infected individuals, CXCL12 plasma levels reach up to 10 ng/ml (Ikegawa et al. 2001). Concentrations may even be higher in tissues that are relevant for HIV-1 pathogenesis, such as lymph nodes and the gut (González et al. 2010; Müller et al. 2001). In addition, genetic CXCL12-polymorphisms affect disease progression (Modi et al. 2005; van Rij et al. 1998; Winkler 1998) suggesting that CXCL12 may restrict CXCR4-tropic HIV-1 *in vivo*. However, other studies did not confirm a role of CXCL12 in viral pathogenesis (Brambilla et al. 2000; Ioannidis 2001; Mehlotra et al. 2015; Petersen et al. 2005; Watanabe et al. 2003; Wei et al. 2018). A more recent study identified CXCR4-tropic HIV-1 variants that are resistant to inhibition by CXCL12 (Armani-Tourret et al. 2021). These variants emerged in late stage AIDS patients with low CD4 T cell counts and may show an enhanced ability to infect naive CD4 T cells surrounded by CXCL12 (Armani-Tourret et al. 2021). Altogether, the relevance of CXCL12 in viral transmission, propagation, and pathogenesis is far from clear and warrants further studies.

The identification of CXCL12 as potent inhibitor of CXCR4-tropic HIV-1 infection stimulated research to develop CXCL12-based antiviral agents for therapeutic approaches. N-terminal truncations and sequence modifications allowed to design CXCL12 analogs lacking agonistic and thus pro-inflammatory activity (Crump 1997; Heveker et al. 1998). Findings showing that not only the CXCL12 N-terminus but also residues in the loop region (Crump 1997) and the C-terminal α-helix (Luo et al. 1999a, b) contribute to receptor interaction led to the design of more sophisticated CXCL12-derivatives (Tudan et al. 2002). The lead compound, CTCE0021 is composed of CXCL12(5–14) linked to CXCL12(55–67) by a four-glycine linker mimicking the distance between the N- and C-terminal regions of CXCL12. In the optimized CTCE0214 derivative, the α-helical structure was stabilized by lactamization leading to enhanced receptor binding and the peptide was cyclized to improve plasma stability (Zhong et al. 2004). Furthermore, modifications and dimerization of the N-terminus allowed to convert derivatives into CXCR4 antagonists (Faber et al. 2007; Loetscher et al.

1998). To our best knowledge, none of these CXCL12-derived peptides has been evaluated as therapeutic agents against CXCR4-tropic HIV-1 in advanced clinical trials. Among other reasons, lack of oral bioavailability of CXCL12-derived peptides may have accounted for the termination of further development as antiviral drugs. However, some CXCL12 derivatives showed potent anti-inflammatory properties or mobilized stem cells *in vivo* and may be further developed for other CXCR4-linked diseases (Fan et al. 2012; Zhong et al. 2004).

5.3.2 EPI-X4

A second CXCR4 ligand with antiviral activity is EPI-X4. This peptide was identified in a fraction of a peptide library derived from human hemofiltrate that selectively inhibited CXCR4-tropic HIV-1 infection (Zirafi et al. 2015). The active compound turned out to be an 1832 Da and 16 amino acid long peptide derived from serum albumin (position 408–423), which was termed EPI-X4 (**E**ndogenous **P**eptide **I**nhibitor of C**X**CR**4**). EPI-X4 is generated from human serum albumin under acidic conditions by aspartic proteases (e.g., Cathepsin D and E) (Buske et al. 2015; Gilg et al. 2021, Mohr et al. 2015; Zirafi et al. 2016). The peptide is evolutionary conserved and interacts with the CXCR4 binding pocket thereby antagonizing CXCL12-induced signaling and cell migration (Sokkar et al. 2021; Zirafi et al. 2015). In addition, EPI-X4 suppresses basal CXCR4 signaling, and thus also acts as inverse agonist of the receptor (Zirafi et al. 2015). Notably, EPI-X4 only interacts with CXCR4 but no other GPCRs including CXCR7. Thus, this peptide is a highly selective inhibitor of CXCR4 function. However, its physiological role remains to be clarified.

EPI-X4 not only antagonizes CXCR4 but also prevents CXCR4-tropic HIV-1 infection in cell culture with an IC_{50} value of ~10–20 μg/ml, while having no effect on CCR5-tropic HIV-1 infection (Harms et al. 2020a, b; Zirafi et al. 2015). EPI-X4 interacts with the CXCR4 binding pocket via its seven N-terminal amino acids, presumably blocking access of the viral glycoprotein to the coreceptor (Sokkar et al. 2021). The CXCR4 antagonizing peptide is not detectable at relevant concentrations in plasma or serum of healthy individuals or HIV-1 patients (Mohr et al. 2015; Zirafi et al. 2015), suggesting that EPI-X4 does not play a major role in controlling CXCR4-tropic HIV-1 infection *in vivo* (Mohr et al. 2015; Zirafi et al. 2015). However, it is currently not known whether EPI-X4 may also be locally produced in lymphoid tissues, the major sites of viral replication, and affect CXCR4-tropic HIV-1. Notably, high amounts of EPI-X4 sufficient to block CXCR4-tropic HIV-1 can be easily generated upon acidification of plasma, which activates proteolytic digestion of the abundant precursor albumin (Mohr et al. 2015; Müller et al. 2016; Zirafi et al. 2015). Acidic pH values are also characteristic for vaginal fluid (Boskey et al. 1999). Thus, it is conceivable that EPI-X4 might be locally generated from albumin-rich semen in the acidic environment of the vaginal tract, where the peptide might selectively restrict CXCR4-tropic HIV-1 upon sexual intercourse.

EPI-X4 is a promising candidate for further development as CXCR4 antagonist to treat CXCR4-tropic HIV-1 infection or other CXCR4-linked diseases (Buske et al. 2015; Zirafi et al. 2015). EPI-X4 is not cytotoxic, can be easily modified, acts as antagonist and inverse agonist of CXCR4, and was shown to reduce airway inflammation in a mouse asthma model without having side effects (Zirafi et al. 2015). Based on computational modeling and empiric approaches, EPI-X4 derivatives with increased plasma stability and reduced size (<1000 Da) were developed, that antagonize CXCR4 and inhibit CXCR4-tropic HIV-1 infection in the low nanomolar range (Harms et al. 2020a, b; Sokkar et al. 2021; Zirafi et al. 2015). The optimized EPI-X4 derivatives WSC02 and JM#21 were successfully tested in preclinical mouse models of Waldenström's macroglobulinemia (a CXCR4-linked disease with constitutive overactivation of the receptor) and acute myeloid leukemia (Kaiser et al. 2021), as well as atopic dermatitis, and allergic asthma (Harms et al. 2020a, b). Currently, they are evaluated as antiviral agents against

CXCR4-tropic HIV-1 in humanized mice. The small size of some improved EPI-X4 with molecular weights below 1000 Da might also pave the way for future oral administration (Sokkar et al. 2021).

5.3.3 Other CXCR4 Ligands

CXCL12 was long-thought to be the only chemokine ligand of CXCR4. In 2007, however, the lymphokine macrophage migration inhibitory factor (MIF) was reported as novel interaction partner for CXCR4 and CD74 (Bernhagen et al. 2007). However, in contrast to CXCL12, addition of MIF to HIV-1 infected cell cultures promoted viral replication independently of coreceptor usage (Regis et al. 2010), demonstrating that MIF does not inhibit CXCR4-tropic HIV-1 strains.

Ubiquitin is a small regulatory protein found in most human tissues (Mayor and Peng 2012). Extracellular ubiquitin functions as an immune modulator with anti-inflammatory properties (Majetschak 2011) and was shown to bind and agonize CXCR4 (Saini et al. 2010a, b). Similar to CXCL12, ubiquitin evoked signal transduction via CXCR4, and CXCR4-expressing cells migrated along a ubiquitin gradient (Saini et al. 2010a, b). However, ubiquitin did not inhibit CXCR4-tropic HIV-1 at concentrations up to 10 μM (Saini et al. 2011). Since extracellular ubiquitin levels usually do not exceed concentrations of 10 nM (Majetschak 2011), it does most likely not contribute to the control of HIV-1 *in vivo*.

As discussed in Sect. 5.2.2, LL-37 is an antimicrobial peptide which may also inhibit viral infections (Pahar et al. 2020)**.** It has been reported that LL-37 affects CXCR4 distribution on the cell surface and its incorporation into lipid rafts (Wu et al. 2012). Interestingly, LL-37 induces CXCR4 signaling and internalization via interaction with an alternative binding site on the receptor, revealing it as a novel agonist for CXCR4 (Pan et al. 2018; Podaza et al. 2020). However, whether LL-37 binding to CXCR4 inhibits CXCR4-tropic HIV-1 infection is unclear.

Another host defense factor interacting with CXCR4 is the human β-defensin-3 (hBD-3) (Feng et al. 2006, 2013). hBD-3 acts as CXCR4 antagonist and inhibits CXCL12-induced receptor signaling and chemotaxis toward CXCL12. It has been reported that hBD-3 also reduces the infection by CXCR4-tropic HIV-1 *in vitro*, albeit only at high concentrations (20 μg/ml) that may not be reached in the human body (Feng et al. 2013; Sun et al. 2005).

More recently, the chemokine CXCL17 was described as novel ligand of CXCR4 (White et al. 2019, 2021). CXCL17 is expressed by mucosal tissues where it is presumably involved in innate immune response and angiogenesis (Burkhardt et al. 2012). CXCL17 inhibits CXCR4-mediated signaling and ligand binding via a glycosaminoglycan-containing accessory protein. If CXCL17 also has an impact on CXCR4-tropic HIV-1 and contributes to HIV-1 pathogenesis remains to be determined.

5.4 Chemokine Ligands of CCR5 Inhibit CCR5-Tropic HIV-1

C–C chemokine receptor type 5 (CCR5) is a GPCR possessing the typical seven transmembrane helical structure of all GPCRs. It is expressed on cells of the immune system including helper and effector T lymphocytes and antigen-resenting cells. CCR5 and its chemokine ligands are involved in immune regulation and inflammatory processes and have been associated with the pathogenesis of several inflammatory diseases (Vangelista and Vento 2018). CCR5-targeting strategies gained significant interest and the small molecule CCR5 antagonist Maraviroc has been approved by the FDA for HIV-1 treatment (Lieberman-Blum et al. 2008).

CCR5 interacts with several chemokines and most of them have been reported to inhibit infection by CCR5-tropic HIV-1. The first chemokines that were described to block HIV-1 infection were the CD8 T cell derived inflammatory proteins CCL3 (MIP-1β, i.e., macrophage inflammatory protein 1 beta), CCL4 (MIP-1α), the respective isoforms CCL3L1 and CCL4L1, and CCL5

(RANTES, i.e., regulated on activation, normal T expressed and secreted) (McBrien et al. 2018). It was later discovered that also CCL2, CCL7, CCL8, CCL11, CCL13, CCL14, and CCL16 are ligands for CCR5, of which CCL7, and the latter two were the only ones not reported to reduce CCR5-tropic HIV-1 infection (Blain et al. 2007; Blanpain et al. 1999; Detheux et al. 2000). Among all CCR5 chemokine ligands, CCL3, CCL4, truncated versions of CCL14 (see below) and in particular CCL5 most efficiently inhibit CCR5-tropic HIV-1 (Blanpain et al. 1999; Detheux et al. 2000; Münch et al. 2002). As discussed above for CXCR4/CXCL12, inhibition of CCR5-tropic HIV-1 by chemokines involves two distinct mechanisms: (1) downmodulation of the CCR5 receptor from the cell surface, and (2) sterically hindrance of CCR5 interaction with the viral glycoprotein gp120 (Alkhatib et al. 1997; Blanpain et al. 1999; Cocchi et al. 1995; Oberlin et al. 1996). Although native CCR5-chemokines are described to bind to G-protein coupled CCR5 with high affinity, they appear to have low affinity to the uncoupled receptor. In contrast, CCR5-tropic HIV-1 interacts with its coreceptor independently of coupled G-protein. This difference might limit antiviral activity of native chemokines and explain why CCR5-tropic HIV-1 persist despite high chemokine production at inflammatory sites (Brelot and Chakrabarti 2018).

Another CCR5 ligand, CCL14, is converted into an active chemokine by proteolytic processing (Detheux et al. 2000). Full-length CCL14 is a weak ligand for CCR1 and lacks potent chemotactic activity (Tsou et al. 1998). It is a 74 amino acid protein that shares ~46% sequence identity with CCL3 and CCL4 (Detheux et al. 2000). A truncated form of CCL14, termed CCL14[9–74], was isolated from human hemofiltrate and shown to be a potent CCR1 and CCR5 agonist that blocks CCR5-tropic HIV-1 (Detheux et al. 2000). CCL14[9–74] targets the second extracellular loop (ECL-2) of CCR5, induces CCR5 internalization, and inhibits CCR5-tropic HIV-1 strains in primary T cells and macrophages (Münch et al. 2002). CCL14[9–74] promotes calcium flux and migration of T lymphocytes, eosinophils, and monocytes (Münch et al. 2002).

β-chemokine mRNA expression is markedly upregulated in HIV-1 infected patients (Trumpfheller et al. 1998) and genetic variations in CCR5 ligands or copy numbers might be linked to progression to AIDS (Brelot and Chakrabarti 2018). Also, it has been suggested that decreased CCL5-sensitivity of CCR5-tropic primary HIV-1 isolates correlates with CD4+ T cell decline and disease progression in infected patients (Karlsson et al. 2004; Kwa et al. 2003). However, findings are debated and it is presently unclear, if the direct inhibition of CCR5-tropic HIV-1 by respective chemokines influences the course of HIV-1 infection (Brelot and Chakrabarti 2018).

Based on the anti-HIV-1 activity of natural chemokine ligands, several groups developed analogues of CCL5 to treat or prevent HIV-1 infection. To avoid receptor mediated signaling that could lead to adverse effects, initial studies focused on the design of CCR5 antagonists. It has been shown that the N-terminus of CCL5 is critical for receptor interaction and signaling (Choi et al. 2012). One of the first CCL5-derived therapeutic peptides was CCL5(9–68). Deletion of the first eight N-terminal amino acids abrogated the signaling function but also reduced binding efficiency to CCR5 (Arenzana-Seisdedos et al. 1996). A second strategy for eliminating agonistic functions of CCL5 was the extension of the N-terminus by a methionine residue. Since [Met]CCL5 showed no agonistic activity, CCL5-derivatives were generated with N-terminal modifications mimicking the hydrophobic nature of methionine (Gaertner et al. 2008; Kawamura et al. 2004; Lederman 2004; Mack et al. 1998; Wilken et al. 1999). One of those derivatives was [5P12]CCL5, which revealed no calcium signaling activity and did not induce receptor internalization (Gaertner

et al. 2008; Nedellec et al. 2011). [5P12]CCL5 protected rhesus macaques against vaginal challenge with simian/human immunodeficiency virus (SHIV) (Veazey et al. 2009) and successfully surpassed pharmacokinetic studies in a sheep model following vaginal administration (McBride et al. 2017). In addition, [5P12]CCL5, demonstrated unusually high thermal and biological stability and could be produced at low-cost (Cerini et al. 2016, 2017; McBride et al. 2017). Therefore, the construct is currently further developed as vaginal and rectal microbicide for prevention of sexual HIV-1 transmission (McBride et al. 2017, 2019a, b).

Other studies focused on new CCL5-derived therapeutic molecules, including other N-terminally modified variants (Hartley et al. 2003; Nardese et al. 2001), polymer-conjugated derivatives (Shao et al. 2005), or other derivatives of the endogenous sequence (Nishiyama et al. 1999; Secchi et al. 2012; Vangelista et al. 2006; Vyroubalova et al. 2006). One of those peptides [5P7]CCL5 has been used to determine a crystal structure of CCR5 in complex with the modified chemokine analogue showing occupation of all gp120 binding sites within the receptor (Zheng et al. 2017).

One concern of using chemokine-based therapeutic agents is their potential pro-inflammatory activity, which could lead to detrimental chronic activation of the immune system (Baggiolini 2001). In addition, immune activation might lead to increased susceptibility of activated T cells to HIV-1 infection (Kinter et al. 1998). Another point to consider is the broad specificity for different chemokine receptors for most of the CCR5 ligands. CCL5 additionally interacts with CCR1, CCR3, and GPR75, what might lead to unwanted therapy-related effects. Due to the lack of CCR5 signaling, high stability and tolerability in preclinical studies, [5P12]CCL5 might be a good candidate for further development as microbicide for the prevention of HIV-1 infection. However, despite 25 years of research, none of the chemokine derivatives successfully passed through phase II/III trials or were approved for the therapy of HIV-1/AIDS.

5.5 CysC Fragments Inhibit GPR15-Mediated HIV-2 and SIV Infection

The basic entry mechanism of HIV-1, HIV-2, and SIVs are highly similar but the latter show broader coreceptor usage. As described above, HIV-1 almost exclusively utilizes CCR5 and/or CXCR4. In comparison, HIV-2 frequently also uses the CXC-chemokine receptor 6 (CXCR6) and G protein-coupled receptor 15 (GPR15) in addition to CCR5 and CXCR4 for viral entry (Gilbert et al. 2003; Mörner et al. 1999; Popper et al. 1999; Reeves et al. 1999).

GPR15 has been proposed to be a key player in mucosal immunity as it is supposedly involved in the homing and trafficking of T cells to the inflamed mucosa of the gut (Fischer et al. 2016; Nguyen et al. 2015; Suply et al. 2017). It is a GPCR with a molecular weight of ~40.8 kDa expressed by e.g. lymphocytes, endothelial cells and synovial macrophages (Cartwright et al. 2014; Clayton et al. 2001; Pan et al. 2017). In addition to its physiological functions, GPR15 is an entry cofactor for HIV-2 and SIVs (Kiene et al. 2012; Unutmaz et al. 1998). In a recent study, C-terminal fragments of the abundant plasma protein Cystatin C, e.g. CysC95-146 (5914 Da), were identified as specific, neutral ligands of GPR15 that prevent lentiviral infections via this GPCR (Hayn et al. 2021). Cystatin C is small, basic protein expressed by all nucleated cells in the human body at constant rates (Grubb et al. 1985; Onopiuk et al. 2015; Zi and Xu 2018). In healthy individuals, Cystatin C plasma levels are ~0.1 μM, however, they can reach up to 0.5–0.7 μM under conditions of uremia and inflammation (Abrahamson et al. 1986). Notably, the plasma levels of CysC are increased in HIV infected individuals and decrease with the initiation of cART (Longenecker et al. 2015).

Antiviral Cystatin C fragments can be generated by proteolytic digestion of CysC with the proteases Cathepsin D, chymase, and Napsin A (Hayn et al. 2021). *In vivo*, these proteases are either secreted by specialized granules or lysosomal exocytosis during immune responses and

activated under acidic conditions (Okajima 2013; Rodríguez et al. 1997; Yamamoto et al. 2012). The generation of these GPR15-specific peptides shows parallels to the generation of the CXCR4 antagonist EPI-X4 (Sect. 5.3.2) from serum albumin by cathepsin D and E under acidic conditions (Zirafi et al. 2015), as well as the CCR5 agonist (CCL14[9–74]) from CCL14 (Detheux et al. 2000b; Münch et al. 2002). It is therefore tempting to speculate that these GPCR-targeting peptides are locally generated and cooperate to inhibit GPR15-, CXCR4-, and CCR5-mediated lentiviral infection. SIVs are most likely infecting primate species for millions of years (Compton et al. 2013; Gifford et al. 2008) and the presence of such endogenous peptides might have been a driving force for promiscuous coreceptor usage.

In 2017, Suply et al. reported the discovery of a chemokine ligand of GPR15, a peptide they termed GPR15L (Suply et al. 2017). GPR15L is a polypeptide consisting of 57 amino acids which is expressed in the colon, stomach, tonsils, skin, and the cervix in humans (Suply et al. 2017). GPR15L affects downstream pathways upon binding to GPR15 (Ocón et al. 2017; Suply et al. 2017) but fails to inhibit GPR15-dependent infection by lentiviral pathogens (Hayn et al. 2021). This was unexpected since the agonistic chemokine ligands of CCR5 and CXCR4, e.g. CCL5 and CXCL12, inhibit CCR5- or CXCR4-tropic HIV infection, respectively (Bleul et al. 1996; Mosier et al. 1999; Oberlin et al. 1996). Conversely, antivirally active, C-terminal CysC peptides do not induce GPR15 signaling, making them neutral ligands of this GPCR (Hayn et al. 2021). These findings show that endogenous peptide ligands may prevent a detrimental activity of a GPCR (e.g., virus entry) without compromising its physiological signaling function.

5.6 VIRIP Blocks Fusion Peptide Insertion into the Cell Membrane

Many viral glycoproteins utilize hydrophobic fusion peptides as membrane anchors (Albertini et al. 2012; Söllner 2004; White et al. 2009). Upon exposure, those 20–30 amino acid, nonpolar domains perturbate the proximal layer of its target cell membrane (Agirre et al. 2000; McMahon and Gallop 2005). FPs are enriched in hydrophobic and aromatic amino acids. In almost all FP sequences repetitive patterns of two to four hydrophobic amino acids connected by glycine are found (Epand 2003). Fusion peptides mediate an essential step during host cell entry, which makes them useful targets for therapeutic approaches (Badani et al. 2014; Fumakia et al. 2016; Vigant et al. 2015).

VIRIP (VIRus-Inhibitory Peptide) is a 20 amino acid fragment of α1-AT (α1-antitrypsin). It blocks HIV-1 entry by binding to the gp41 FP, preventing the insertion of the FP into the cellular membrane and consequently the viral anchoring and fusion process. The antiviral peptide was identified by screening of a human hemofiltrate library and found to inhibit a wide variety of HIV-1 strains (Münch et al. 2007). Full-length α1-AT can reach plasma concentrations of up to 250 μM during infection or inflammation (Brantly et al. 1988). VIRIP is produced by the proteolytic digest of α1-AT by matrix metalloproteases and enriched in the plasma of HIV-1 patients during acute viremia (Kramer et al. 2010). Notably, a HIV-1-infected patient with severe α1-AT deficiency showed very rapid progression to AIDS (Potthoff et al. 2007) suggesting that α1-AT or VIRIP may contribute in suppressing HIV-1 replication.

The unique mode of action and high barrier to resistance made VIRIP an interesting candidate for further development. A structure activity study resulted in the generation of optimized VIRIP derivatives, such as VIR-576, with IC_{50} values of 10–50 nM, which is about two orders of magnitude more potent than endogenous VIRIP (Münch et al. 2007). The increased antiretroviral efficacy is due to additional hydrophobic residues enhancing its interaction with the gp41 FP and a cysteine bridge stabilizing the active conformation (Münch et al. 2007; Venken et al. 2011) VIR-576 was safe and efficient in a phase I/II trial and reduced the mean plasma viral loads by 1.23 log10 copies per ml without causing severe

adverse effects (Forssmann et al. 2010). The genetic barrier for HIV-1 to overcome VIRIP mediated restriction is very high. During long-term passage of HIV-1 for more than 1 year with increasing concentrations of VIR-353 on MT-4 cells, resistance was achieved but associated with strongly reduced viral fitness (Gonzalez et al. 2011; Müller et al. 2018). Despite high efficacy *in vitro*, treatment with VIR-576 required infusion of large amounts of the peptide *in vivo*. Proteolytic degradation, tissue distribution, and/or absorption to the extracellular matrix or serum components may reduce the efficacy of VIR-576 in patients (Forssmann et al. 2010). However, this study provided evidence that endogenous peptides can be optimized and suppress viral loads and established the HIV-1 FP as therapeutic target. Studies to generate VIRIP derivates with further increased antiviral activity and improved pharmacokinetic properties are ongoing.

5.7 Conclusions and Perspectives

HIV-1 entry into target cells is a complex multi-step process involving attachment, coreceptor binding, anchoring, and fusion. Essentially each step can be inhibited by (poly)peptides naturally existing in the human body. Defensins, retrocyclin and VIRIP directly target the virion and may prevent CD4 binding and viral fusion with the host cell. In comparison, chemokines and other GPCR ligands (EPI-X4, CysC fragments) inhibit entry through occupation and/or downregulation of the viral coreceptors. These endogenous antiviral factors may play important roles in the innate immune response against HIV-1. However, their contribution in controlling viral transmission and replication remains to be clarified. Notably, some of these peptides are generated by proteolytic processing of abundant precursor proteins with non-related function and inhibit HIV-1 entry by novel and unexpected mechanisms: Degradation of the protease inhibitor Cystatin C results in fragments that specifically bind GPR15 without agonizing or antagonizing the receptor but preventing GPR15-mediated infection. VIRIP is released from the acute phase protein α1 antitrypsin and blocks HIV-1 fusion by preventing the insertion of the viral fusion peptide into the cellular membrane. EPI-X4, a highly specific CXCR4 antagonist, is produced by processing of human serum albumin, the most abundant protein in the circulation and extravascular space. The common principle underlying the generation of these peptides is a low pH trigger, which activates acidic proteases that release the effector peptide. These endogenous viral entry inhibitors are particularly promising for development as drugs for HIV-1/AIDS or GPCR-linked diseases, because they are already evolutionarily optimized to perform their respective function(s) in humans. Furthermore, improved derivatives thereof should be better tolerated and are less immunogenic than those based on foreign antigens. Moreover, EPI-X4 and VIRIP are relatively small peptides and hence easy to modify by means of peptide synthesis and/or chemical modifications allowing to develop analogs with improved bioavailability and pharmacokinetic properties.

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Peptide-Based Dual HIV and Coronavirus Entry Inhibitors

6

Huan Wang and Chao Wang

Abstract

The continued HIV/AIDS epidemic worldwide and the battle against emerging infectious diseases caused by coronaviruses underscore the need for the development of an ever-expanding repertoire of antiviral drugs. Entry inhibitors are of particular interest because of their potential to be used as therapeutic or prophylactic treatments for blocking viral invasion. HIV and coronaviruses utilize class I fusion proteins to facilitate their entry and membrane fusion. Discovery of a common hexameric coiled-coil fusion complex resulting from the packing of three C-terminal heptad repeat region from the fusion-mediating subunit of viral fusion proteins against trimeric coiled-coil made up by their N-terminal heptad repeat prompted the search for peptides mimicking the heptad repeat regions that could potentially inhibit viral entry. This has led to the development of effective peptides that are specific to the virus that is developed for. In this review, we focus on peptide-based entry dual inhibitors that block fusion process not only of HIV but also coronaviruses through interrupting their fusogenic six-helical bundle core and which hopefully will help to gain insight into the α-helical secondary structure- and coiled-coil superstructure-based strategies to design entry inhibitors with broad-spectrum antiviral activity against enveloped viruses with class I fusion proteins.

Keywords

HIV · Coronavirus · Fusion inhibitor · Lipopeptide · Coiled-coil

6.1 Introduction

Human immunodeficiency virus (HIV), a retrovirus that causes acquired immunodeficiency syndrome (AIDS), remains a worldwide health treat. According to UNAIDS (United Nations on HIV/AIDS) in its most recent update in 2020, there are 38.0 million people living with HIV in 2019 with 690,000 deaths from AIDS-related illnesses and 1.7 million people newly infected with HIV. An effective vaccine is the best hope for prevention. However, its foreseeable future is still unclear (Gray et al. 2021). Current antiretroviral therapy that consists of more than 30 approved drugs or regimens has transformed HIV infection into a chronic disease (Ma et al. 2021). Owing to the early establishment of anatomic reservoirs for HIV latency, there is still no cure for the HIV infection (Pitman et al. 2018). Moreover, HIV readily mutates to develop drug resistance during the lifelong therapy (Koay et al.

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2021). These facts call for the development of new antiviral agents to fight HIV infection. In addition to the HIV pandemic, emerging infectious diseases caused by coronaviruses pose another devastating threat to global health and economy. Coronaviruses (CoVs) comprise four genera: α, β, γ, and δ (Pillaiyar et al. 2020). Up to now, seven strains of coronaviruses have been identified that are pathogenic to humans, in which four low-pathogenicity human coronaviruses (HCoVs), including HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1, induce mild respiratory tract diseases (Wang et al. 2020) and three highly pathogenic human-infecting β-coronaviruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and new coronavirus SARS-CoV-2 that responsible for the coronavirus disease 2019 (COVID-19), cause severe lower respiratory tract infections (Drosten et al. 2003; Zaki et al. 2012; Zhu et al. 2020a). SARS-CoV infection resulted in 8098 SARS cases and 774 SARS-related deaths around the world (Skowronski et al. 2005). As of November 2019, 2494 laboratory-confirmed cases of infection with MERS-CoV, including 858 deaths, had been reported to the World Health Organization. While efforts for the development of antivirals against SARS-CoV and MERS-CoV are still in process, SARS-CoV-2 rapidly spread all over the world so that by May 23, 2021, more than 166 million people have been confirmed to be infected with SARS-CoV-2 and more than 3.4 million deaths due to COVID-19 have been recorded worldwide. The development of broad-spectrum pan-HCoV inhibitors that are active against a wide range of HCoVs may become the ultimate treatment strategy for addressing the current urgency and those coronaviruses outbreaks that are likely to emerge in the future (Wang et al. 2021b). This review summarizes case studies and highlights the correlation of therapeutic intervention strategies between HIV and HCoVs, focusing especially on peptide-based dual HIV/HCoVs fusion inhibitors. This leads to a better understanding regarding the chemically manipulating viral entry step in the HIV/HCoVs infection cycle by shared mechanisms, therefore expending the current repertoire of both anti-HIV agents and anti-HCoV therapeutics.

6.2 Common Hexameric Coiled-Coils in Viral Envelope Proteins as Conserved Drug Target Sites

Membrane fusion plays a crucial role in the entry of enveloped viruses, occurring in either the endosomal/clathrin-dependent route or nonendosomal/clathrin-independent pathway (Dimitrov 2004). Enveloped viruses such as HIV and HCoVs present envelope (Env) or spike (S) glycoproteins, known as class I fusion proteins, on their viral membrane surface (Harrison 2008). These viral fusion proteins are synthesized as precursors that are later cleaved by host proteases into a receptor-binding subunit (gp120 in HIV and S1 in coronaviruses) that is responsible for the interaction with cellular receptor(s) and a fusion-mediating subunit (gp41 in HIV and S2 in coronaviruses) that plays key roles in mediating membrane fusion (Sanders and Moore 2021). HIV infection is initiated by the binding of gp120 to the cell surface receptor CD4 and a coreceptor (CCR5 or CXCR4) that triggered a series of conformational changes in gp41 subunit for viral entry into host cells (De Feo and Weiss 2012). Coronavirus S1 proteins are believed to be functionally equivalent to the surface subunit gp120 of HIV Env. The key functional host receptors utilized by HCoVs includes angiotensin-converting enzyme 2 (ACE2), dipeptidyl peptidase 4 (DPP4), aminopeptidase N, and *O*-acetylated sialic acid (Li et al. 2003; Raj et al. 2013; Yeager et al. 1992; Huang et al. 2015). Although HIV and HCoVs dock onto the host cell membrane by engaging different viral receptors and entry into host cells via different pathways, their fusion-mediating subunits go through a similar conformational change resulting in formation of a conserved six-helical bundle (6-HB) core structure to bring viral and cellular membranes close (Fig. 6.1a). In 1997, three groups independently

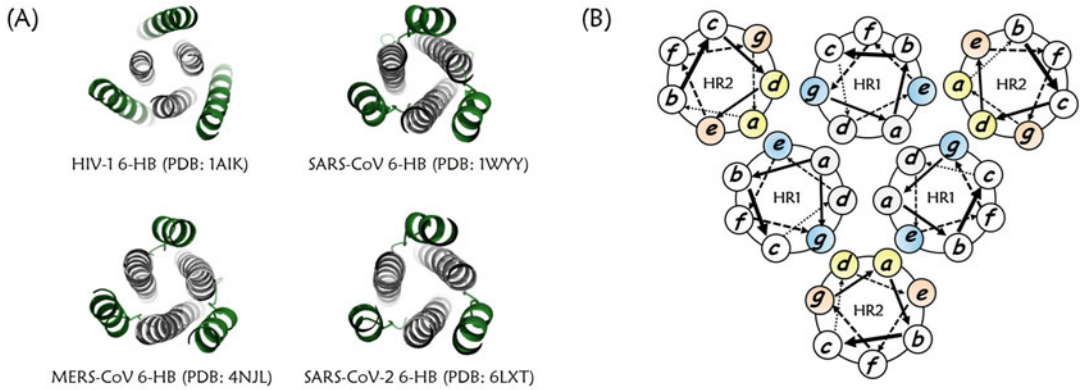

Fig. 6.1 Research and development of peptide-based entry inhibitors targeting viral fusion proteins. (**a**) Top view of the HIV-1 6-HB (PDB: 1AIK) and crystal structures of coronavirus fusion core. The PDB codes are 1WYY for SARS-CoV, 4NJL for MERS-CoV, and 6LXT for SARS-CoV-2. Colored identically, the HR1 trimers and HR2 helices within these 6-HB fusion core structures are shown as gray and forest, respectively. (**b**) Formation of 6-HB between HR1 and HR2. In the 6-HB formation process, the residues at the *a* position and *d* position (shown in gray) in a HR1 helix can interact with the *d* position and *a* position (shown in gray) of other HR1 helices, respectively, to form a homotrimeric assembly. The residues at the *a* and *d* positions (shown in yellow) in a HR2 helices interact with residues located at the *e* and *g* positions (shown in blue) in the HR1 to form 6-HB

solved the crystal structure of 6-HB fusogenic core of HIV type 1 (HIV-1) gp41 by using peptide pairs derived from the N-terminal heptad repeat (NHR or HR1) and C-terminal heptad repeat (CHR or HR2) (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). In these peptide models of HIV-1 gp41, three NHR-helices associate to form the central trimeric coiled-coil through the interaction between residues located at the "a" positions in the α-helical wheels of one helix and those at the "d" positions in another helix. The central trimeric NHR coiled-coil creates three conserved hydrophobic grooves. Three peptides from the CHR region pack obliquely in an anti-parallel configuration into the three exposed hydrophobic grooves on the surface of internal N-trimer, in which the residues at the "e" and "g" positions in the α-helical wheels of the N-helices interact with "a" and "d" positions in the CHR helices during the process of 6-HB nucleation (Fig. 6.1b). In a similar manner to HIV-1 gp41, the S2 domain of SARS-CoV S protein can fold into a 6-HB structure, consisting of three inner HR1 helices in association with three HR2 helices in an antiparallel orientation (Ingallinella et al. 2004; Deng et al. 2006; Bosch et al. 2004). In 2014, Lu et al. characterized the 6-HB fusion core structure of MERS-CoV spike protein S2 subunit by X-ray crystallography (Lu et al. 2014). The crystal structure of HR1/HR2 of MERS-CoV shows a canonical 6-HB that adopts a similar fold as that of SARS-CoV.

After the outbreak of COVID-19, Jiang's group immediately solved the X-ray crystal structure of SARS-CoV-2 6-HB structure (Xia et al. 2020a). Together with the crystal structural information of 6-HBs of HCoV-229E and HCoV-NL63 (Zheng et al. 2006; Yan et al. 2018), these findings suggested that the 6-HB is a conserved and critical architecture for the fusion and entry of coronaviruses. In addition, 6-HB has become an important target for the design of peptide fusion inhibitors against viral infection.

6.3 Virus-Specific Peptide-Based Fusion Inhibitors That Antagonize 6-HB Formation

In the early 1990s, Jiang et al. discovered that a synthetic peptide derived from the CHR domain

(C-peptide) of HIV-1, designated SJ-2176, as highly potent HIV-1 fusion inhibitor (Jiang et al. 1993) (Table 6.1). Later, Wild's group identified another synthetic peptide T20 (designated DP178 in the original publication), corresponding to the sequence from the gp41 CHR domain and overlapping more than 2/3 of the SJ-2176 sequence, which inhibited HIV-1 replication at nanomolar levels (Wild et al. 1994). T20 (brand name: Fuzeon; generic name: enfuvirtide) was licensed by the US FDA in 2003 for salvage therapy of patients who are infected with multidrug-resistant HIV (Lalezari et al. 2003). A noteworthy feature of the contact between the N- and C-terminal heptad repeat regions is a hydrophobic pocket on the NHR surface, which receives the side chains of three conserved hydrophobic residues (Trp-628, Trp-631, and Ile-635) within the pocket-binding domain (PBD) of the CHR region (Chan et al. 1997). Early studies have shown that T20 lacks the PBD, thereby being unable to interact with the NHR-derived peptides to form a stable 6-HB (Liu et al. 2005, 2007). However, Zhang et al. have recently reported the crystal structure of T20 with N39, a target mimic peptide derived from the NHR domain of gp41 (Zhang et al. 2019). The complex of T20 and N39 formed a typical 6-HB structure, in which T20 inhibitor helices packed into each of the grooves created in the interior N39 trimeric coiled-coil. Subsequently, Jiang's group demonstrated that T20 inhibited native gp41 6-HB formation at the early stage of the HIV-1 entry process (Xu et al. 2019). All these results suggest that T20 acts as a dominant-negative inhibitor to antagonize the gp41 transition into the stable 6-HB structure by specifically targeting the gp41 NHR domain, therefore, inhibiting the function of HIV-1 gp41 to prevent viral entry into host cells.

In fact, the development of CHR-peptide-based HIV-1 fusion inhibitors arises from the early discovery of NHR-derived peptides (N-peptides) such as DP107 (Wild et al. 1992). Conceptually, N-peptides could bind to the gp41 CHR region and form heterogeneous 6-HB to block fusogenic gp41 core formation. Unfortunately, liner N-peptides are much less potent in inhibiting HIV-1 Env-mediated virus-cell membrane fusion than C-peptides. The major reason is that N-peptides have the tendency to aggregate because of their strong hydrophobicity and cannot form stable trimeric coiled-coil conformation under physiological conditions (Dwyer et al. 2008). To address this issue, therefore, researchers have long sought chemical interventions to reinforce the bioactive tertiary structure of gp41 NHR-based peptides and thereby restoring binding affinity toward their protein targets. One of the most established methods involves introducing an exogenous trimeric motif into the NHR-derived peptides to facilitate their folding into stable and soluble trimers. Kim's group introduced an exogenous trimeric motif, such as GCN4-pI_QI' (IQ) or IZm (IZ), into the peptide derived from the HIV-1 gp41 NHR region so that three copies of this peptide form a stable and soluble trimer with the assistance of the trimeric motif. The resultant chimeric N-peptide fusion inhibitors, e.g., IQN17, IZN17, and an intermolecular disulfide bond-stabilized $(CCIZN17)_3$, have much improved inhibitory activity than their parent liner peptide N17 (Eckert and Kim 2001; Bianchi et al. 2005). It has also been shown that a series of recombinant protein-based N-peptide fusion inhibitors, such as N36Fd, N28Fd, and ccN28Fd, which consists of three copies of peptides derived from the HIV-1 gp41 NHR and a trimeric motif, foldon (Fd), forms a conformation of highly stable trimeric helices and inhibits HIV-1 infection in nanomolar range (Chen et al. 2010; Tong et al. 2013). Inspired by the tractability of coiled-coils and ultrastability provided by isopeptide bond crosslinks found in certain bacterial surface proteins, our group has developed an attractive and efficient isopeptide bridge-tethering strategy to produce de novo designed α-helical coiled-coil trimers with exceptional resistance to thermal and chemical unfolding and proteolysis, as structure directing auxiliaries to guide the trimerization of HIV-1 gp41 N-terminal heptad repeat peptides (Wang et al. 2015). We found that these ultra-stable trimerization motifs directed the self-assembly of NHR peptides into well-characterized trimers, which had highly potent

Table 6.1 Amino acid sequences of peptide inhibitors

Peptide	Amino acid sequence (from N- to C-terminus)
SJ-2176	EWDREINNYTSLIHSLIEESQNQQEKNEQEGGC
DP178	YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF
C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL
PBD-4HRu	WMEWDREAEELAKKAEELAKKAEELAKKAEELAKK
PBD-m4HR	WMEWDREIEELIKKSEELIKKIEEQIKKQEESIKK
DP107	NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
IZN17	IKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL
$(CCIZN17)_3$	$(CCGGIKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL)_3$
N28Fd	SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILGYIPEAPRDGQAYVRKDGEWVLLSTFL
ccN28Fd	$(CCGGIEAQQHLLQLTVWGIKQLQARILAVERYGYIPEAPRDGQAYVRKDGEWVLLSTFL)_3$
$(3HRN23)_3$	$(WRIQQIEQ\underline{K}IHHI\underline{E}QRIQQIEQRIEAQQHLLQLTVWGIKQLQARIL)_3$
$(N36M)_3$	$(SGIVQ\underline{K}INNI\underline{E}RAIEAQQHLLQLTVWGIKQLQARIL)_3$
HR2-8	ELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIK
HR2	ISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL
GST-removed HR2	DVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYI
HR2-18	IQKEIDRLNEVAKNLNESLIDLQELGK
PBD-m4HR	WMEWDREIEELIKKSEELIKKIEEQIKKQEESIKK
HR1-1	NGIGVTQNVLYENQKQIANQFNKAISQIQESLTTTSTA
CP-1	GINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYE
HR2P	SLTQINTTLLDLTYEMLSLQQVVKALNESYIDLKEL
P1	LTQINTTLLDLTYEMLSLQQVVKALNESYIDLKEL
229E-HR2P	VVEQYNQTILNLTSEISTLENKSAELNYTVQKLQTLIDNINSTLVDLKWL
229E-HR1P	AASFNKAMTNIVDAFTGVNDAITQTSQALQTVATALNKIQDVVNQQGNSLNHLTSQ
2019-nCoV-HR2P	DISGINASVVNIQKEIDRLNEVAKNLNESLIDLEQL
EK1	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL
EK1V1	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKELK(Chol)
IPB02	ISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELK(Chol)
EK1C4	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL-GSGSG-PEG4-C(Chol)
EK1C2A	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL-PEG4-C(Chol)
IIQ	IEEIQKKIEEIQKKIEEIQKKIEEIQKKIEEIQKK-βAla-K(C16)
N3G	$(IEEIVK\underline{K}IHHI\underline{E}RAIEAQQKLLQLTVWGIKQLQARIL)_3$

antiviral activities in the low nanomolar range, reaching the clinically used C-peptide-based HIV-1 fusion inhibitor T20, and exhibited exceptional resistance to proteolysis. Although successful formation of soluble and stable uniform trimers in physiological solutions was achieved via introducing an exogenous trimeric motif into the NHR-derived peptides, these modifications suffer from extra-large auxiliary protein domains, which may attenuate the binding of the NHR trimer to their native ligand. Therefore, we report an effective strategy to recapitulate NHR trimeric coiled-coils in HIV-1 gp41, containing no any exogenous trimerization motif (Lai et al. 2016). We have used an NHR-peptide with 36 amino acid residues, N36, as original peptide for modification. First, a number of residues of N36 peptide were substituted under the guidance of established rules-of-thumb that relate protein sequence to structure in coiled-coil domains. Then, three weak inter-helical ionic bonds at the *g*-*e*′ positions were replaced with isopeptide bridges using our developed isopeptide bond-tethering methodology. As a result, $(N36M)_3$ is highly effective in blocking HIV-1 Env-mediated membrane fusion and inhibiting infection by both laboratory-adapted and primary HIV-1 strains, as well as T20-resistant HIV-1 strains. The data from biophysical studies, including native-PAGE, sedimentation velocity analysis, and circular dichroism spectroscopy, suggested that $(N36M)_3$ was able to interact with the gp41 CHR region to form stable unproductive 6-HB complex, thereby, blocking HIV-1 entry into host cells.

Discovery of anti-HIV-1 peptides analogous to either one of the two gp41 heptad repeat regions triggered tremendous efforts to explore peptide fusion inhibitors of HCoV targeting the six-helix coiled-coil bundle (Table 6.1). A peptide called HR2-8 (aa 1126–1193) that is derived from HR2 region of the SARS-CoV S protein inhibited SARS-CoV infection of Vero cells in a concentration-dependent manner with an EC_{50} value of 17 μM (Bosch et al. 2004). Peptides derived from SARS-CoV HR2 region, such as HR2 (aa 1151–1185) and GST-removed HR2 (aa 1145–1192), possess potent inhibitory activity against SARS-CoV pseudotyped virus entry with EC_{50} values of 0.34 μM and 2.15 μM, respectively (Chu et al. 2008). Yuan et al. obtained two peptides, including SARS-CoV HR2-derived peptide HR2-18 (aa 1161–1187) and SARS-CoV HR1-derived peptide HR1-1 (aa 889–926), with promising inhibitory activity against SARS-CoV (Yuan et al. 2004). Jiang's research group designed another peptide known as CP-1 (aa 1153–1189) that had inhibitory activity on SARS-CoV-induced cytopathic effect in Vero E6 cells with an IC_{50} value of about 19 μM (Liu et al. 2004). In addition to peptide analogues as SARS-CoV fusion inhibitors, numerous fusion inhibitory peptides derived from the S2 subunit of spike proteins of other coronaviruses have been identified, such as HR2-based MERS-CoV fusion inhibitors HR2P (aa 1251–1286) and P1 peptide (Lu et al. 2014; Gao et al. 2013), HCoV-229E-specific peptide fusion inhibitors 229E-HR2P and 229E-HR1P (Xia et al. 2018), and peptide spanning the HR2 domain of SARS-CoV-2 named 2019-nCoV-HR2P (Xia et al. 2020b). However, most of these fusion inhibitors showed little, or no, cross-inhibitory activity against heterologous HCoVs. For instance, MERS-CoV-HR2P had no effect on SARS-CoV infection, and SARS-CoV-specific CP-1 peptide failed to cross-inhibit MERS-CoV infection. Interestingly, three peptides derived from the HR2 region in spike protein of bat coronavirus HKU4 were found to be able to bind to the MERS-CoV HR1 region and have anti-MERS-CoV activity (Xia et al. 2019a). Furthermore, Xia et al. found that a peptide, OC43-HR2P, which derived from the HCoV-OC43 spike protein HR2 region, can inhibit cell-cell fusion mediated by the S proteins of a broad range of HCoVs, including MERS-CoV, SARS-CoV, SARS-CoV-2, HCoV-229E, HCoV-NL63, and HCoV-OC43. Following further modification, they successfully designed EK1 peptide that effectively inhibits infection of five live HCoVs, including MERS-CoV, SARS-CoV-2, HCoV-229E, HCoV-NL63, and HCoV-OC43. Crystal structure data show that EK1 as a pan-HCoV fusion inhibitor can interact with HR1s from different HCoVs to form 6-HB structures (Xia et al. 2019b). The

introduction of a cholesterol component to EK1 dramatically enhances its pan-HCoV inhibitory activity from the micromolar to low nanomolar range. The lipopeptide EK1C4, in which a cholesterol group was attached to the C-terminus of EK1 through a glycine/serine-based 5-amino acid linker "GSGSG" and a tetraethylene glycol derivative, exhibits highly potent activities in inhibiting a panel of live HCoVs (Xia et al. 2020a). On the basis of EK1 sequence, He's group engineered another cholesterol conjugated peptide designated EK1V1. This conjugation strategy is to add cholesterol onto a lysine that was additionally appended outside of the EK1 sequence in the absence of linkers. They also identified another SARS-CoV-2 HR2 sequence-based cholesteroylated peptide IPB02 that can bind avidly to the target mimic peptides from SARS-CoV and SARS-CoV-2 and inhibit viral entry of SARS-CoV and SARS-CoV-2 into 293T/ACE2 cells with IC_{50} values of 0.25 μM and 0.08 μM, respectively (Zhu et al. 2020b).

6.4 Pan-Coronavirus Fusion Inhibitors Possess Cross-Inhibitory Activity Against HIV Infection

Recently, He's group reported that pan-coronavirus fusion inhibitors could inhibit HIV-1 infection (Yu et al. 2021). In a HIV-1_{NL4-3} Env-mediated cell-cell fusion assay, both pan-coronavirus fusion inhibitors EK1 and EK1V1 showed fusion inhibitory activity against HIV-1_{NL4-3} with IC_{50} values of 6.63 μM and 0.02 μM, respectively (Table 6.1). The pseudovirus assay suggested that the antiviral activity of EK1 and EK1V1 against HIV-1_{NL4-3} infection with IC_{50} values of 8.89 μM and 0.04 μM, respectively. Nevertheless, EK1 peptide showed no cross-inhibitory activity against infection of pseudoviruses bearing the primary HIV-1 Env. In contrast, EK1V1 inhibited divergent HIV-1 pseudoviruses with a mean IC_{50} value of 0.9 μM. Additionally, the anti-coronavirus peptide fusion inhibitor EK1V1 exhibited similar activity with HIV-1 specific fusion inhibitor T20 in inhibiting HIV-2 and simian immunodeficiency virus (SIV). Subsequently, He's group further demonstrated that the inhibitory activity of lipopeptide EK1C4 is similar or slightly more potent than EK1V1 against HIV-1 isolates, including NL4-3, JRFL, and SF162, in terms of Env-based cell fusion and pseudoviruses. Lipopeptides IPB02 and its derivatives also exhibited potent inhibitory activity against HIV_{NL4-3} Env-mediated cell-cell fusion and pseudovirus infection. In vitro cytotoxicity assay determined that EK1- and IPB02-based lipopeptides are not cytotoxic at a concentration of 10 or 25 μM on HEK293T and TZM-bl cells. Importantly, EK1V1 and IPB02-based pan-HCoV fusion inhibitors possessed potent activities in inhibiting HIV-1 variants resistant to HIV-specific fusion inhibitors such as enfuvirtide and short C-peptides targeting the gp41 pocket. These results illustrate that pan-coronavirus fusion inhibitors are important leads as novel generation of HIV entry inhibitors. Furthermore, the cross-inhibition of pan-HCoV fusion inhibitors on HIV-1, HIV-2, and SIV has provided potential arsenals to combat viral strains resistant to the specific anti-HIV drugs.

Very recently, Jiang's group further tested whether lipopeptide-based pan-HCoV fusion inhibitors are active against HIV-1 infection (Lan et al. 2021). Evaluation of pan-HCoV fusion inhibitor EK1 and its cholesterol-tagged and palmitic acid (C16)-conjugated derivatives by a HIV-1 Env-mediated cell-cell fusion assay indicated that cholesterol conjugates effectively inhibited fusion between the HIV-1_{IIIB} chronically infected H9 cells and TZM-bl target cells with IC_{50} values ranging from 65 to 862 nM. In comparison with cholesterol-tagged pan-HCoV fusion inhibitors, C16-modified EK1 peptide named EK1P1A has less potency in vitro with an IC_{50} value of 1932 nM and their parental peptides without cholesterol or lipid introduction showed weak or no inhibitory activity with the concentration up to 5 μM. Consistent with the cell–cell fusion assay results, cholesterol-tagged inhibitors could significantly inhibit HIV-1_{Bal} pseudovirus entry with IC_{50} values ranging from 1.7 to 8.3 nM, which was 44-fold more potent

than fatty acid-conjugated peptide and 600-fold more potent than a peptide without lipid conjugation designated EK1C0. Among these lipopeptide-based pan-HCoV fusion inhibitors, EK1C2A, a peptide that was created by adding cholesterol moiety to the C-terminus of EK1 through a flexible polyethylene glycol 4 (PEG4)-derived linker, displayed the most potent inhibitory activity against HIV-1 Env-mediated cell-cell fusion and HIV-1 pseudovirus infection. In addition, EK1C2A could efficiently inhibit various HIV-1 isolates, including HIV-1 laboratory-adapted strains, HIV-1 clinical isolates, and T20- or T-2635-resistant strains, with nanomolar activity.

6.5 HIV-1 Fusion Inhibitors Possess Cross-Inhibitory Activity Against Coronaviruses Infection

In light of the urgency of emerging highly pathogenic human CoVs, repositioning of already approved drugs represents a viable drug discovery approach with safety profile and side effects data in hand (Serafin et al. 2020). Unfortunately, most HIV-1 fusion inhibitors were not effective against human CoVs. For example, in the emergence of SARS pandemic, Yamamoto et al. found that T20 could not inhibit SARS-CoV infection (Yamamoto et al. 2004). After the MERS-CoV outbreak, Lu et al. reported HIV-1-specific T20 peptide exhibited no significant inhibition of MERS-CoV infection at the concentration up to 5 μM (Lu et al. 2014). Recently, Yu et al. tested the potential inhibitory activity of a panel of peptide HIV-1 fusion inhibitors, including T20, albuvirtide, T1249, T2635, sifuvirtide, and 2P23, on SARS-CoV-2 replication and found that these HIV-1 entry inhibitors did not affect the replication of SARS-CoV-2 at a concentration as high as 25 or 50 μM (Yu et al. 2021).

Interestingly, our group found that an isopeptide bridge-tethered gp41 NHR trimeric coiled-coil named N3G as a potent HIV-1 fusion inhibitor showed pan-CoV fusion inhibitory activity against multiple HCoVs (Wang et al. 2021a). N37 peptide, which consists of sequence spanning HIV-1_{HXB2} residues 546–581 with an additional Ile residue at its N-terminus, was used as parent molecule to engineer the gp41 NHR trimer. After trimerization of N37 with a variety of powerful approaches, e.g., placing Ile residues at *a* and *d* positions and introducing intra- or inter-helical electrostatic interactions between Glu and Lys, the isopeptide bridge-tethered coiled-coil trimer was constructed via a site-directed acyl transfer reaction (Table 6.1). N3G showed inhibitory activity against HIV-1 Env-mediated cell-cell fusion with an IC_{50} value of 6.58 nM. As determined by single-cycle infection assays, N3G inhibited infection by two pseudotyped HIV-1 strains, JRFL and HIV_25710-2, with anti-HIV-1 activity similar to that of T20. N3G was also shown to effectively inhibiting infection of HIV-1 laboratory-adapted HIV-1 strains at nanomolar levels. Moreover, N3G inhibited MERS-CoV S protein mediated cell-cell fusion and infection by pseudotyped MERS-CoV with IC_{50} of 0.34 μM and 0.25 μM, respectively. In comparison, HR2P-M2, a specific MERS-CoV peptide fusion inhibitor, inhibited MERS-CoV pseudovirus infection with an IC_{50} value 0.45 μM. Compared with HR2P-M2, N3G exhibited a potent and long-lasting ex vivo anti-MERS-CoV activity. The pseudovirus assay also suggested that the antiviral activity of N3G against SARS-CoV and SARS-CoV-2 infection with IC_{50} values of 5.68 μM and 3.45 μM, respectively, in Caco-2 cells and a CC_{50} value of >20 μM. Finally, N3G demonstrated concentration-dependent inhibition of HCoV-OC43 infection with an IC_{50} value of 1.26 μM and was not cytotoxic at 40 μM. From structure-activity relationship and biophysical-based studies on N3G peptide, we found that the broad-spectrum antiviral activities against HIV-1 and β-coronaviruses were both dependent on its trimeric coiled-coil structure and the original gp41 NHR trimer mimetic sequences. These data suggested that HIV-1 gp41 NHR-derived N3G highlight a promising strategy for inhibitor development combating numerous β-coronaviruses.

6.6 Design of Dual HIV/HCoV Entry Inhibitors Based on Artificial Peptide Sequences

The anti-HIV-1 peptides T20 and it analog C34 share a common 4–3 heptad repeat (HR) sequence, through which both peptides interact with viral gp41 NHR region to form heterologous 6-HB and block the fusion-active core of gp41 formation. A PBD motif in C34 is considered critical for the interaction between C34 and a target mimic NHR peptide named N36. T20 lacks the PBD but contains a tryptophan-rich motif (TRM) that plays essential roles for its inhibitory activity. Our group found that anti-HIV-1 C-peptides could be divided into a structural domain that is composed of 4–3 HR sequence and is responsible for maintaining an appropriate length and conformation of C-peptides and a functional domain, e.g., PBD and TRM, which specifically interact with corresponding target sites (Qi et al. 2008). On the basis of this design strategy, we have developed an artificial peptide designated PBD-4HRu that contains four copies of a non-native 4–3 HR sequence (AEELAKK) as a structural domain with an additional PBD motif located at the N-terminus of the HR sequence as a functional domain. As expected, PBD-4HRu exhibited inhibitory activities on gp41 6-HB formation, HIV-1 Env-mediated cell-cell fusion, and HIV-1_{IIIB} replication. Introducing mutations at the NHR-binding sites in PBD-4HRu according to the helical wheel model, we found that the inhibitory activity of PBD-m4HR was dramatically improved (Shi et al. 2008, 2012). PBD-m4HR peptide displayed stable α-helical conformation and exhibited high potency against HIV-1 Env-mediated cell-cell fusion and both T20-sensitive and resistant HIV-1 strains, possibly by binding gp41 NHR region to block its fusogenic hexameric structure formation. These results suggested that a high variability in the primary sequence of CHR motif within the HIV-1 6-HB is allowed as long as the driving force for coiled-coil assembly, i.e., amphiphilic characteristics of the individual helices, is maintained. Accordingly, we identified a de novo designed α-helical lipopeptide named IIQ (Wang et al. 2018). Its sequence contains three parts: five copies of a 7-mer sequence IEEIQKK, a β-alanine linker, and a palmitic acid (Table 6.1). IIQ adopted stable α-helical structure and could interact with multiple HR1 target surrogates to form 6-HB structures. IIQ exhibited relatively broad-spectrum antiviral activity against viruses including MERS-CoV, influenza A viruses, and HIV-1. For inhibition of MERS-CoV S protein mediated cell-cell fusion and HIV-1 Env-mediated cell-cell fusion, IIQ showed IC_{50} values of 0.11 μM and 3.63 μM, respectively. Moreover, the activities displayed by IIQ was far below its cytotoxicity threshold ($CC_{50} > 100$ μM on Huh-7 and TZM-bl cells that were used for the fusion assay).

6.7 Conclusion and Outlook

In the last three decades, significant progress has been made in the development of peptide-based HIV entry inhibitors targeting viral envelope gp41. The commercialization of the first peptide HIV-1 entry inhibitor, enfuvirtide, has stimulated the research and development of numbers of peptide-based entry inhibitors targeting fusion proteins of HCoVs. HCoVs used a six-helix bundle similar to that of HIV-1 to mediate its entry into host cells. It has been established that the extended pre-hairpin intermediates that span the virus and cell membrane before the 6-HB assembly can be optimal targets for therapeutically active peptides design. HIV fusion inhibitors derived from the heptad repeats in the viral fusion protein are able to inhibit HIV virus–target cells fusion via interaction with their counterpart regions. In the case of HCoVs, most of the HR2-mimicking peptides have been shown to be effective against homologous HCoVs fusion process. Strikingly, EK1 and its cholesterylated peptides as pan-CoV fusion inhibitors show promise for further development to combat both current and future pandemics/epidemics of CoV diseases. Despite the similarity between 6-HB cores of HIV and HCoV, these fusion-inhibiting

peptides usually lack cross-inhibitory activity. Two perspectives exist and define HR1–HR2 interactions in the trimer-of-hairpins core structures of viral fusion proteins. First, each of the fusion proteins contain a unique HR sequence in the HR2 region to specifically interact with the HR sequence in the HR1-trimer of the same virus, rather than that of other viruses. The specific interaction between HR2 and HR1-trimer involves matched residues at their binding faces. This could be the reason why most HIV peptide fusion inhibitors cannot act as a potent HCoV fusion inhibitor, and vice versa. From a second perspective, the packing of three HR2 helices against three HR1 helices inner core is essentially the result of a coiled-coil assembly (Apostolovic et al. 2010). Compared with antiviral peptides, such as HIV-1 fusion inhibitors T20 and T1249, that have a random coil conformation in solution, the binding between α-helical peptide fusion inhibitors and their target exacts less energetic penalty due to the reduced changes in entropy of binding. N3G as a mimetic of the HIV-1 gp41 NHR trimer is a stable supramolecular assembly of α-helical peptides. One explanation for the observed cross-inhibitory activity of anti-HIV-1 peptide N3G against β-HCoVs is that the relatively low entropy penalty accompanying heterologous 6-HB formation resulted from the preorganization of the trimeric coiled-coil conformation favoring its accessibility to the HR2s of HIV-1 and some β-HCoVs. Both the HR2-based pan-HCoV fusion inhibitor EK1 and a potent HIV-1 fusion inhibitor T2635 adopt α-helical conformation. However, EK1 had no inhibitory activity against HIV-1 and T2635 had no inhibitory potency against SARS-CoV-2. In contrast, the α-helical cholesterol conjugated EK1 peptides effectively inhibited HIV-1 isolates. Consistently, our study found that a de novo designed α-helical lipopeptide exhibited broad-spectrum antiviral activity against infection by MERS-CoV and HIV-1. The cholesterol and fatty acid conjugation strategies could increase effective inhibitor concentration at membrane sites of viral entry, thus enhancing the probability for interaction between HR2-based peptides and viral HR1 target (Pessi 2015). In addition, lipid conjugation could further amelioration of peptide α-helicity (Chong et al. 2016). According to such theories, HR2-based lipopeptides as dual HIV and HCoV entry inhibitors could because of the pre-positioning inhibitors on the cell surface and the increased α-helical content that translates into a reduction in conformational entropy. It is well evidenced that mutations in the buried binding sites of artificially designed α-helical C-peptide fusion inhibitors to precisely match the subtle interacting network between interfaces of the inhibitor and gp41 NHR helices resulted in their stronger binding affinity, thereby increasing the biological activity of the α-helical peptides. Therefore, in combination with primary structure optimization through introducing target-specific interaction, the α-helical secondary structure- and coiled-coil superstructure-based strategies to inhibitor design would identify dual HIV and HCoVs entry inhibitors with higher potency and lower toxicity. These broad-spectrum antiviral agents to control both the HIV and HCoVs fusion with target cells lead to a better understanding regarding the process of viral entry and the knowledge of the nature of coiled-coil interaction between two heptad repeat regions in class I viral fusion proteins, hence, opening a new avenue for design and development of novel fusion inhibitors to combat fatal infectious disease caused by HIV and the present and future coronavirus pandemics.

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Coronavirus Entry Inhibitors

7

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Abstract

Coronaviruses (CoVs) are enveloped RNA viruses that widely exist in the environment. Several CoVs possess a strong ability to infect humans, termed as human coronavirus (HCoVs). Among seven known HCoVs, SARS-CoV-2, SARS-CoV, and MERS-CoV belong to highly pathogenic HCoVs, which can cause severe clinical symptoms and even death. Especially, the current COVID-19 pandemic severely threatens human survival and health, which emphasizes the importance of developing effective CoV vaccines and anti-CoV agents to protect humans from HCoV infections. Coronavirus entry inhibitors can block various processes in viral entry, such as receptor binding, proteolytic activation of spike protein, or virus-cell membrane fusion. Coronavirus entry inhibitors, alone or in combination with other drugs, play important roles in the treatment of coronavirus diseases. Thus, we summarize and discuss the development of coronavirus entry inhibitors in this chapter.

Keywords

Coronavirus · Entry inhibitor · Receptor binding · Membrane fusion · Proteolytic activation

7.1 Introduction

Coronaviruses (CoVs) are a group of enveloped RNA viruses, belonging to the family Coronaviridae, subfamily Coronavirinae, consisting of four genera: Alphacoronavirus (αCoV), Betacoronavirus (βCoV), Gammacoronavirus (γCoV), and Deltacoronavirus (δCoV) (Zumla et al. 2016). Coronaviruses widely exist in the environment and can infect various animals. Some coronaviruses possess the potential to cross-species to infect humans by intermediate hosts (Cui et al. 2019). Coronaviruses can be divided into human CoVs (e.g., HCoV-229E) and animal CoVs (e.g., mouse hepatitis virus) according to their abilities to infect humans or other animals.

However, few specific drugs are currently available for the clinical treatment of coronavirus diseases (Chen et al. 2020). Many drugs used clinically are nonspecific antiviral drugs, and their coronavirus inhibitory activity is relatively limited. It is necessary for us to find more effective anti-coronavirus drugs to protect humans against current and possibly coming coronavirus infections.

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Coronavirus entry inhibitors, including a broad range of neutralizing antibodies, recombinant proteins/peptides, and small molecules, inhibit coronaviruses at the entry stage and block viral particles from entering target cells. Coronavirus entry inhibitors play important roles in stopping coronavirus infections, which are worthy of more attention and further studies. In this chapter, we introduce the basic characteristics of human coronaviruses, including epidemiology, structure, and life cycle. More importantly, we summarize and discuss the development of coronavirus entry inhibitors targeting the process of receptor binding, proteolytic activation, and membrane fusion.

7.2 Epidemiology and Pathogenesis of Human Coronavirus (HCoVs)

To date, seven coronaviruses are known to infect human beings: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (βCoV), Middle East respiratory syndrome coronavirus (MERS-CoV) (βCoV), SARS-CoV (βCoV), HCoV-229E (αCoV), HCoV-OC43 (βCoV), HCoV-NL63 (αCoV), and HCoV-HKU1 (βCoV). Among them, SARS-CoV-2, SARS-CoV, and MERS-CoV usually cause severe disease, accounting for their classification as highly pathogenic HCoVs. Other HCoVs usually lead to mild illnesses after infecting humans, and these are classified as low-pathogenic HCoVs (Jiang and Du 2020; Ma et al. 2020).

SARS-CoV first emerged at the end of 2002 in Guangdong Province, China, and then quickly spread to more than 30 countries and regions around the world, causing 8273 infections and 775 deaths (fatality rate of 9%) (Chen et al. 2020). The natural hosts and intermediate hosts of SARS-CoV are considered as bats and palm civets, respectively (Cui et al. 2019). The common symptoms of SARS-CoV-infected patients are fever, myalgia, headache, and chills, followed by coughing, dyspnea, and respiratory distress. SARS-CoV has a strong transmission capacity, which led to several super-spreading events in Guangzhou, Hong Kong, or other places (Sung et al. 2009) and caused serious panic around the world.

In 2012, MERS-CoV was first identified in the Middle East and then spread to countries outside of the Middle East (de Wit et al. 2016). The natural hosts of MERS-CoV are considered as bats, and its intermediate hosts are dromedary camels (Cui et al. 2019). MERS-CoV infection can cause severe respiratory symptoms, accompanied by the failure of multiple organs, such as the liver and kidney. In 2015, an outbreak of MERS-CoV happened in South Korea, followed by super-spreading events that caused alarm in South Korea and neighboring countries (Hui 2016). MERS-CoV still exists in Saudi Arabia and causes viral infections and even death (Zhang et al. 2021). As of April 2021, MERS-CoV had infected more than 2574 individuals and caused 886 deaths with a case fatality rate of 35%, which is much higher than that of SARS-CoV.

SARS-CoV-2 (previously named 2019-nCoV) first emerged in 2019 (Zhou et al. 2020). Diseases caused by SARS-CoV-2 were termed as coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO). The natural host and intermediate host of SARS-CoV-2 are still unknown, but the most probable natural hosts are bats (Hu et al. 2021). SARS-CoV-2 poses a huge threat to the health and life of people all over the world since its transmission rate is higher than that of SARS-CoV and MERS-CoV. In the early COVID-19 outbreak, WHO announced this pandemic as a public health emergency of international concern (PHEIC).

By March 2022, SARS-CoV-2 has spread to more than 200 countries and regions, infected more than 4.4 billion individuals, and caused six million deaths (https://covid19.who.int). During its worldwide spread, several SARS-CoV-2 variants have also emerged, arousing huge concern (Davies et al. 2021). The symptoms of COVID-19 vary from mild (fever, fatigue) to

critical (acute respiratory distress syndrome, multi-organ failure) (Hu et al. 2021). In addition, some patients infected with SARS-CoV-2 were asymptomatic, nevertheless, serving as sources of infection (Wiersinga et al. 2020).

Other HCoVs, including HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1, are distributed widely in the world (Su et al. 2016). These low-pathogenic human coronaviruses usually cause mild illnesses after infecting humans, but they can also cause serious clinical signs in immunocompromised people or patients with underlying comorbidities (Pinana et al. 2021; Chen et al. 2020).

7.3 Structure of Human Coronavirus Virion

HCoV genomes are non-segmented, positive-sense, and single-stranded RNA with lengths ranging from 26 to 32 kb (Fung and Liu 2019). Viral proteins encoded by genomes of coronavirus can be divided into structural, accessory and nonstructural proteins. Structural proteins, including spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N), are important for virion assembly and mediating viral infection (Zumla et al. 2016).

HCoV S protein is a class I fusion protein (Rey and Lok 2018) responsible for receptor binding and mediating virus-cell membrane fusion. S protein consists of subunits S1 and S2. The S1 subunit includes signal peptide (SP), N-terminal domain (NTD), and receptor binding domain (RBD). RBD in the S1 subunit can interact with the cellular receptor to mediate the entry of virus particles into target cells. Both NTD and RBD are important and vulnerable targets for neutralizing antibodies. The S2 subunit includes fusion peptide (FP), heptad repeat 1 (HR1) and 2 (HR2) domains, transmembrane domain (TM), and cytoplasm domain (CP) (Chen et al. 2020). The stable six-helical bundle (6-HB) structure formed by the interaction of HR1 and HR2 can shorten the distance between the viral and cellular membrane to promote cell-virus fusion (Du et al. 2009). Thus, disrupting membrane fusion by inhibiting the 6-HB formation is an important strategy to develop novel coronavirus entry inhibitors.

M protein is an important component of the virion. Studies have shown that M protein can interact with viral N protein, E protein, and S protein to mediate virus assembly and budding (Schoeman and Fielding 2019). N protein plays an important role in virus packaging and the organization of the viral genome. N protein can interact with viral genomic RNA to form nucleocapsid complexes and also interact with membrane proteins to maintain the shape and structure of viral particles (McBride et al. 2014). E protein is divergent in different coronaviruses. E protein mainly participates in the assembly and release of the virus together with M protein. In addition to their involvement in virion assembly, M, N, and E proteins of some HCoVs can suppress host immune response to facilitate viral replication (Zumla et al. 2016). Different from other HCoVs, genomes of HCoV-HKU1 and HCoV-OC43 encode hemagglutinin-esterase (HE) protein (structural protein), which is expressed on the surface of viral particles and acts as a receptor-destroying enzyme (Lang et al. 2020).

Accessory proteins are encoded by accessory genes interspersed between genes encoding structural proteins (V'Kovski et al. 2021). Accessory proteins are varied in different CoVs, but they are typically involved in the process of virus-host interaction and have immunomodulatory effects (V'Kovski et al. 2021; Liu et al. 2014).

Nonstructural proteins (nsps) produced by HCoV genome (nsp1–nsp16) play vital roles in the process of replication and transcription of the viral genome (Yadav et al. 2021). Some nsps have also become important targets for the development of antiviral drugs based on their important roles in coronavirus replication and transcription, such as the SARS-CoV-2 - RNA-dependent RNA polymerase (nsp12) is the primary target for remdesivir (Zhu et al. 2020a). In addition, some nsps, the specific functions of

which are not yet clear, are worthy of further studies.

7.4 Life Cycle of Human Coronaviruses

HCoVs share a similar life cycle, mainly including viral attachment and entry, uncoating, replication transcription complex (RTC) formation, genome replication and transcription, translation of structural proteins, virion assembly, budding, and exocytosis (Fung and Liu 2019; Arya et al. 2021).

Receptor binding is the first step of the viral entry process. HCoV particles utilize their RBD in S1 protein to recognize and bind cellular receptors, such as angiotensin-converting enzyme 2 (ACE2) for SARS-CoV-2 and SARS-CoV, dipeptidyl peptidase 4 (DPP4) for MERS-CoV, aminopeptidase N (APN) for HCoV-229E. Then, the S2 subunit undergoes a series of conformational changes. The FP is inserted into the cell membrane, and then HR1 and HR2 (heptad repeat 2) interact to form a stable 6-HB structure to shorten the distance between the virus and cell membrane, mediating membrane fusion and promoting virus entry into the cytoplasm.

After viral entry, genomic RNA is released into the cytoplasm, in which two large open reading frames, ORF1a and ORF1b, encode polyproteins pp1a and pp1ab, respectively. These polyproteins are proteolytically cleaved into nsp1-16 by viral papain-like protease (PLpro) and main protease (Mpro, also termed 3CLpro), followed by the formation of the replication and transcription complexes (Arya et al. 2021). Next, viral RNA synthesis is finished in double-membrane vesicles (DMVs) derived from the endoplasmic reticulum (ER), and then structural proteins are translated and translocated into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) for virion assembly (V'Kovski et al. 2021). Finally, the mature virus particles are released in the manner of budding, as shown in Fig. 7.1.

7.5 Entry Inhibitors Targeting Receptor Binding Domain

7.5.1 Neutralizing Antibodies Targeting RBD

RBD, which is responsible for receptor binding, serves as a vulnerable target for entry inhibitors. Indeed, many monoclonal antibodies targeting RBD have been developed. Forms of neutralizing antibodies used in anti-coronavirus studies are manifold, including whole monoclonal antibody (mAb), antigen-binding fragment (Fab), single-chain variable region fragment (scFv), or single-domain antibody (sdAb) (Jiang et al. 2020). These antibodies have respective advantages in size, cost, and therapeutic efficacy. While many studies on neutralizing antibodies against HCoVs were reported after the outbreaks of human CoVs, we can only introduce some representatives here.

7.5.1.1 SARS-CoV Neutralizing Antibodies

SARS-CoV neutralizing antibodies targeting S protein were usually isolated from SARS patients or S protein-immunized animals (Du et al. 2009). Several antibodies were reported to inhibit SARS-CoV infection, such as 80R, CR3014, and CR3022 (ter Meulen et al. 2006; Sui et al. 2004). CR3014 and CR3022 are two human mAbs able to potently neutralize SARS-CoV infection, and CR3022 can potently neutralize CR3014 escape virus infection (ter Meulen et al. 2006). CR3022 was reported to cross-react with SARS-CoV-2 RBD, but it could not neutralize wild-type SARS-CoV-2 infection (Zhang et al. 2020).

7.5.1.2 MERS-CoV Neutralizing Antibodies

Many neutralizing antibodies targeting MERS-CoV RBD were developed after the outbreak of MERS, such as mouse mAbs isolated from immunized mice (4C2 and Mersmab1) (Li et al. 2015), human mAbs isolated from humanized mice or human donors (MERS-27, REGN3048, REGN3051, and m336) (Jiang et al. 2014; Ying

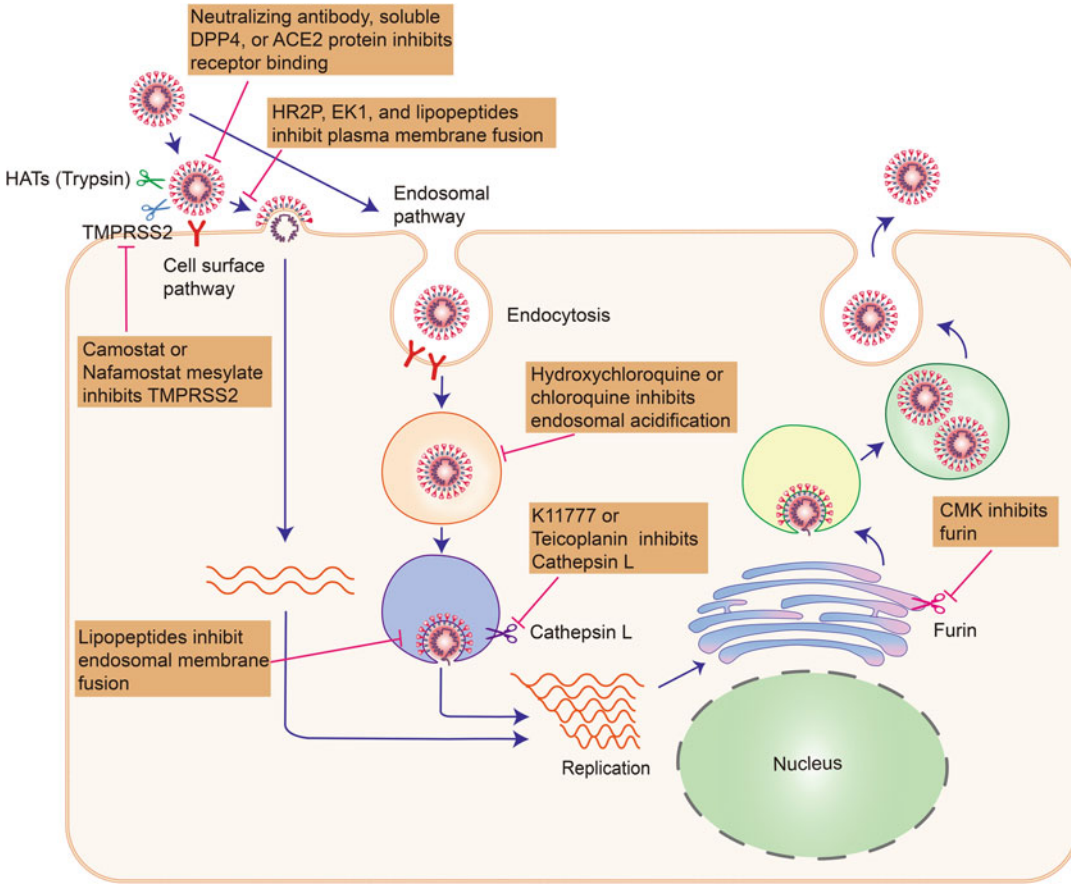

Fig. 7.1 The life cycle of human coronaviruses and therapeutic targets of entry inhibitors. HCoVs enter the target cells by the cell-surface and endosomal pathways. After viral entry, ORF1a and ORF1b are translated into polyproteins pp1a and pp1ab and proteolytically cleaved into nsp1-16, followed by the formation of replication and transcription complexes. Next, structural proteins are translated, and virion particles assemble in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). Finally, the mature virus particles are released through budding. Entry inhibitors inhibit several processes during entry to stop viral infections. Neutralizing antibodies, soluble ACE2 and DPP4 proteins inhibit receptor binding to stop viral entry; CMK, Camostat, and E64D inhibit proteolytic activation of spike protein to stop viral entry; HR2P and EK1 mainly inhibit plasma membrane fusion, while lipopeptides (e.g., EK1C4) also inhibit endosomal membrane fusion

et al. 2015). These neutralizing antibodies block the interaction of MERS-CoV RBD and DPP4 to stop receptor binding and viral entry. Additionally, antibodies targeting receptor DPP4 (2F9, YS110) can also block receptor binding and prevent MERS-CoV entry (Ohnuma et al. 2013).

The binding site of 4C2 and MERS27 to MERS-CoV RBD is very similar. The binding affinity of humanized 4C2 antibody to RBD is high with Kd of 217 nM, and it has sufficient inhibitory activity against MERS-CoV pseudovirus and live virus infection (IC50 is 1.8 μg/ml and 6.25 μg/ml, respectively) (Li et al. 2015). MERS-27 also has potent inhibitory activity against MERS-CoV live virus infection (IC50 is 13.33 nM) (Jiang et al. 2014). REGN3048 and REGN3051 are two fully human noncompetitive monoclonal antibodies targeting MERS-CoV RBD, which were isolated from immunized VelocImmune mice. Both antibodies show potent MERS-CoV inhibitory activity in vivo and can reduce viral titers in hDPP4 mice (Pascal et al.

2015). REGN3048 and REGN3051 can cobind to RBD of MERS-CoV S protein, and co-administration of these mAbs shows more potent MERS-CoV inhibitory activity. The phase I study shows that this antibody cocktail has good safety (Sivapalasingam et al. 2021).

7.5.1.3 SARS-CoV-2 Neutralizing Antibodies

After the outbreak of COVID-19, mAbs targeting SARS-CoV-2 RBD were urgently developed, and some were under clinical studies or approved for emergency use (Taylor et al. 2021). For example, LY-CoV555 (Bamlanivimab), CT-P59, and REGN-COV2 (REGN10933 plus REGN10987) have all entered clinical trials (Tuccori et al. 2020).

LY-CoV555 was isolated from a convalescent COVID-19 patient. LY-CoV555 showed potent SARS-CoV-2 inhibitory activity in vitro and reduced SARS-CoV-2 viral titers in the upper and lower respiratory tract of rhesus macaques (Jones et al. 2021). Clinical trials suggested that bamlanivimab administration can reduce hospitalization and mortality among COVID-19 patients (Chen et al. 2021; Bariola et al. 2021; Verderese et al. 2021). Additionally, a related study demonstrated that bamlanivimab injection in healthy individuals can effectively prevent SARS-CoV-2 infection (Cohen et al. 2021). Bamlanivimab monotherapy was the first mAb to receive an Emergency Use Authorization (EUA) from FDA. However, bamlanivimab showed poor inhibitory activity against SARS-CoV-2 variants and its EUA was revoked. A combination of bamlanivimab and etesevimab also received the EUA, this antibody cocktail has showed greater clinical efficacy than bamlanivimab monotherapy (Gottlieb et al. 2021; Dougan et al. 2021).

REGN10933 and REGN10987 are fully human mAbs derived from VelocImmune mice and humans. Both REGN10933 and REGN10987 can potently neutralize the SARS-CoV-2 live virus with IC50 values at the low pM level (Hansen et al. 2020). REGN-COV2 (a combination of REGN10933 and REGN10987) shows improved SARS-CoV-2 neutralizing activity (Baum et al. 2020). Structural analysis revealed that REGN10933 and REGN10987 can simultaneously bind with the different regions of SARS-CoV-2 RBD, suggesting that a combination of REGN10933 and REGN10987 is a promising approach to improve antiviral efficacy. Related studies demonstrated that REGN-COV2 can reduce hospitalization and viral load in COVID-19 patients (Verderese et al. 2021; Weinreich et al. 2021). Similarly, an antibody cocktail therapy (BRII-196 plus BRII-198) developed by Chinese scientists also showed clinical efficacy and was approved for emergency use in China.

Some antibodies show compromised neutralizing activity against newly emerging SARS-CoV-2 variants (Wang et al. 2021c; Planas et al. 2021). For example, B.1.351 (South Africa), B.1.617 (India), and P.1 (Brazil) variants are fully resistant to bamlanivimab neutralization (Hoffmann et al. 2021a; Planas et al. 2021), and the B.1.351 (South Africa) variant is partially resistant to REGN10933 neutralization (Corti et al. 2021). However, REGN-COV2 can normally neutralize entry of B.1.351, P.1, and B.1.617 variants (Hoffmann et al. 2021a, b), hinting at the efficacy of antibody cocktails in avoiding resistance of some SARS-CoV-2 variants. It should be noted that SARS-CoV-2 variants able to escape neutralization of antibody cocktails may still emerge with the epidemic of COVID-19. Therefore, it is important to find more broadly neutralizing antibodies or develop more antibody cocktails to overcome the resistance of SARS-CoV-2 variants.

7.5.2 Recombinant Proteins and Peptide Analogues

Recombinant proteins and peptide analogues can competitively bind to viral RBD to block the process of receptor binding, which is widely investigated in the studies of anti-SARS-CoV-2, -MERS-CoV, and -SARS-CoV (Chan et al. 2021; Hofmann et al. 2004; Inn et al. 2018). Human recombinant soluble ACE2 protein (hrsACE2) was reported to inhibit SARS-CoV and SARS-

CoV-2 infection in vitro (Monteil et al. 2020; Hofmann et al. 2004). Similarly, soluble DPP4 protein can also inhibit MERS-CoV in vitro (Inn et al. 2018). However, since the sequence and structure of natural hrsACE2 is basically the same as that of human ACE2 receptor expressed on the cell surfaces, the in vitro activity of natural hrsACE2 protein to inhibit SARS-CoV-2 entry is limited. HrsACE2 protein has, however, been used to treat a severe COVID-19 patient (Zoufaly et al. 2020), and it did improve the clinical outcome of this patient, but its clinical efficacy in large-scale clinical trials remains to be reported.

Because of the limited SARS-CoV-2 inhibitory activity and short half-life of natural hrsACE2 protein, various improved protein entry inhibitors based on ACE2 protein were reported (Tada et al. 2020). We select some representative ACE2-based protein inhibitors and describe them as follows. The ACE2 microbody is an ACE2 dimer formed by ACE2 ectodomain fused with Fc domain 3, which was reported to effectively inhibit SARS-CoV-2 infection with tenfold elevation compared with the antiviral activity of soluble ACE2 (Tada et al. 2020). In addition, this ACE2 microbody potently inhibited SARS-CoV and some PsV infections of SL-CoVs. Guo et al. (2021) designed the trimeric ACE2 protein, which can potently inhibit SARS-CoV-2 live and PsV infections with IC50 values at the low-nanomolar level. This trimeric ACE2 protein can also broadly inhibit SARS-CoV-2 variants and some SARS-like viruses.

However, these ACE2 monomers, dimers, and trimers all have huge molecular mass and size, which may decrease tissue penetration and increase production costs if they are applied in clinical use. Based on the interface of SARS-CoV-2 RBD and human ACE2 protein, Cao et al. developed a mini-protein inhibitor, termed LCB1, which can potently bind to SARS-CoV-2 RBD and inhibit WT SARS-CoV-2 infection in vitro with an IC50 value at the picomolar level (Cao et al. 2020). Next, an improved mini-protein inhibitor, termed LCB1v1.3, was developed on the basis of LCB1. LCB1v1.3 can potently inhibit WT SARS-CoV-2 and B.1.1.7 variant infections and shows excellent protective efficacy in hACE2 mice (Case et al. 2021).

7.5.3 Other Entry Inhibitors Targeting Receptor Binding Domain

Receptor molecules are also important targets for inhibiting the receptor binding process. Dalbavancin, a lipoglycopeptide antibiotic, was previously reported to inhibit SARS-CoV and MERS-CoV PsV infections (Zhou et al. 2016). Wang et al. recently reported that dalbavancin can bind ACE2 protein to inhibit SARS-CoV-2 infection in vitro, and dalbavancin shows potent SARS-CoV-2 inhibitory activity in vivo and significantly reduces viral titers in the rhesus macaque model (Wang et al. 2021b). Adenosine deaminase (ADA), which can bind DPP4 protein, also showed MERS-CoV inhibitory activity (Raj et al. 2014).

Apart from main receptor molecules, some attachment factors, or auxiliary receptors, also mediate the process of viral attachment and facilitate the entry of HCoVs, such as neuropilin-1 for SARS-CoV-2 (Daly et al. 2020) and CD209L (L-SIGN) for SARS-CoV (Jeffers et al. 2004). Entry inhibitors targeting these attachment factors and/or these interactions are worth follow-up studies.

7.6 Entry Inhibitors Targeting N-terminal Domain

In addition, entry inhibitors targeting the NTD, mainly neutralizing antibodies, were developed, such as antibodies 4A8 and S2X333 targeting SARS-CoV-2 NTD (Chi et al. 2020; McCallum et al. 2021). While 4A8 and S2X333 can also potently neutralize SARS-CoV-2 infections, neither antibody could interfere with the interaction between SARS-CoV-2 RBD and ACE2. Instead, they neutralize SARS-CoV-2 infection through other mechanisms. Although SARS-CoV-2 variants having mutations in NTD could escape

NTD-targeting mAbs-mediated neutralization (Wang et al. 2021c; Corti et al. 2021), rational combinations of NTD-targeting mAbs and RBD-targeting mAbs may overcome the resistance of these SARS-CoV-2 variants.

MERS-CoV NTD-based vaccines can induce potent anti-MERS-CoV immunity and antigen-specific neutralizing antibodies (Jiaming et al. 2017), suggesting that MERS-CoV NTD is also an attractive target for developing MERS-CoV neutralizing antibodies. Many neutralizing antibodies targeting MERS-CoV NTD were reported, such as CDC2-A2, 7D10, and G2 (Zhou et al. 2019; Wang et al. 2018, 2019c). 7D10 was an NTD-specific antibody isolated from MERS-CoV spike protein-immunized mice, showing potent MERS-CoV PsV and live virus neutralizing activities. Related studies showed that 7D10 not only inhibits receptor binding, but also inhibits subsequent conformational changes of spike protein to inhibit MERS-CoV entry (Zhou et al. 2019).

7.7 Entry Inhibitors Targeting Proteolytic Activation of Spike Protein

Many host proteases can cleave S protein, which is important for the entry of HCoVs. Two cleavage sites are found in HCoV spike protein: S1/S2 cleavage site and S2′ cleavage site (Tortorici and Veesler 2019). Cleavage sites also influence the infectivity of HCoVs (Xia et al. 2020a; Johnson et al. 2021). Before the entry process of some HCoVs, S protein is cleaved into S1 and S2 subunits by proprotein convertases (PPCs) (Shang et al. 2020), such as furin. Transmembrane protease serine 2 (TMPRSS2) and Cathepsin B/L cleave S protein during the process of HCoV entry.

TMPRSS2 usually cleaves S protein in the cell-surface fusion pathway (plasma membrane pathway) (Hoffmann et al. 2020a), while Cathepsin B/L usually cleaves S protein in the endosomal pathway. Because these proteases can cleave S proteins of multiple HCoVs, these protease inhibitors usually have broad HCoV inhibitory activity. The furin inhibitor dec-RVKR-CMK (CMK) can inhibit S protein cleavage to inhibit SARS-CoV-2 infection (Cheng et al. 2020). In addition to inhibiting furin activity, CMK was reported to have TMPRSS2 and Cat L inhibitory activity (Matsuyama et al. 2018).

In vitro studies proved that camostat mesylate and nafamostat mesylate can inhibit SARS-CoV-2 and MERS-CoV entry by inhibiting the activity of TMPRSS2 (Hoffmann et al. 2020a; Yamamoto et al. 2020). Since the expressions of TMPRSS2 in different cell types are different, the inhibitory activities of those inhibitors are varied in different cell types. One advantage of these inhibitors is that their targets are host proteases, and SARS-CoV-2 variants showed no resistance to these host protease inhibitors (Hoffmann et al. 2021a, b). Camostat mesylate and nafamostat mesylate have entered clinical trials for the treatment of COVID-19 (Apaydin et al. 2021). Results of a double-blind randomized clinical trial showed that treatment with camostat mesylate (200 mg t.i.d.) could not improve clinical outcomes or even shorten recovery times of COVID-19 patients (Gunst et al. 2021). Therefore, the dosage and time of camostat mesylate for clinical use may be worthy of further studies to increase its clinical efficacy.

K11777, teicoplanin, and E64D have Cat L/B inhibitory activity, which can block the endosome-dependent entry of HCoVs (Mellott et al. 2021). K11777 was evaluated in clinical trials as an anti-parasite drug, which was reported to effectively inhibit SARS-CoV-2, SARS-CoV, and MERS-CoV in vitro (Mellott et al. 2021). Currently, however, in vivo coronavirus inhibitory activity of K11777 has not been reported. Teicoplanin was reported to broadly inhibit SARS-CoV, MERS-CoV, and SARS-CoV-2 infection in vitro (Zhou et al. 2016), but reported clinical efficacy of teicoplanin in the treatment of critical COVID-19 patients is limited (Ceccarelli et al. 2021). Combinations of inhibitors targeting TMPRSS2 and Cat L/B can simultaneously inhibit two entry pathways of CoVs and show more potent CoVs inhibitory activity (Hoffmann et al. 2020a), which may improve their clinical efficacy.

Hydroxychloroquine (HCQ) and chloroquine (CQ) can change the pH of the endosome to inhibit the activity of endosomal proteases, subsequently inhibiting the entry of HCoVs (Zumla et al. 2016; de Wilde et al. 2014). A recent study demonstrated that HCQ and CQ could inhibit SARS-CoV-2 infection in Vero E6 cells, but could not inhibit SARS-CoV-2 in the lung-derived cell line (Hoffmann et al. 2020b). Inhibitory activity of HCQ and CQ was influenced by TMPRSS2 expression of different cell types. High TMPRSS2 expression contributes to more efficient cell-surface fusion pathway-dependent entry, making endosome-dependent entry less important, and subsequently reducing coronavirus inhibitory activity of HCQ and CQ (Zhou et al. 2021). The clinical applications of HCQ and CQ are still controversial because of their side effects and limited clinical effectiveness (Axfors et al. 2021; Li et al. 2020b).

7.8 Entry Inhibitors Targeting Membrane Fusion

7.8.1 Peptide Entry Inhibitor

As previously described, the interaction between HR1 and HR2 to form 6-HB structure is important for membrane fusion (Du et al. 2009). Both HR1 and HR2 regions can be used as targets for peptide entry inhibitors, but peptides targeting HR1 generally have higher antiviral activity (Liu et al. 2004). The HR1 region is a more suitable target for developing antiviral peptides. HR2-derived peptide entry inhibitors can bind the HR1 region competitively with the original HR2 region, thereby inhibiting the formation of 6-HB and membrane fusion.

In 2003, Prof. Jiang's group reported CP-1, a peptide derived from the HR2 region of SARS-CoV S protein. It was shown to potently inhibit SARS-CoV infection (Liu et al. 2004), and this study laid the foundation for the development of coronavirus peptide entry inhibitors. Later, Lu et al. reported MERS-HR2P, a peptide from the MERS-CoV HR2 region, which can effectively inhibit MERS-CoV infection (Lu et al. 2014). Although the solubility of MERS-HR2P was low, thus restricting its clinical application, an improved MERS-HR2P peptide, termed HR2P-M2, was developed by adding Glu (E) and Lys (K) residues. HR2P-M2 showed improved solubility and MERS-CoV fusion inhibitory activity and could effectively reduce MERS-CoV viral titers (>1000-fold reduction) in human DPP4-transduced mice (Channappanavar et al. 2015). Similarly, peptide inhibitors, such as 2019-nCoV-HR2P and IPB01, based on the SARS-CoV-2 HR2 domain can also inhibit SARS-CoV-2 infection (Xia et al. 2020c; Zhu et al. 2020b).

Lipidation is an important approach to improve the antiviral activity of peptide entry inhibitors (Park and Gallagher 2017). Lipid molecules, such as cholesterol (Chol) and palmitic acid (Palm), can be added to the C-termini of peptides to increase their inhibitory activity on plasma membrane fusion. Unlike lipid-free peptides, these lipopeptides can bind to the lipid membrane and get into the endosome where they can block endosomal membrane fusion (Park and Gallagher 2017). For example, [SARSHRC-PEG4]2-chol is a cholesterol-modified SARS-CoV-2 HR2P peptide, which has a stronger SARS-CoV-2 inhibitory activity than the lipid-free HR2P peptide and possesses potent in vivo protective efficacy (de Vries et al. 2021).

Although the above-mentioned peptide inhibitors showed effective anti-CoV activity, they are specific and cannot inhibit multiple coronavirus infections; therefore, it is important to develop pan-CoV peptide inhibitors which can inhibit multiple coronavirus infections. Sequence analysis revealed that HR1 and HR2 domains are relatively conserved (Xia et al. 2019), but these are two obviously different HR structures in HCoVs. HRs from HCoV-229E and HCoV-NL63 exhibit the insertion of 14 amino acids compared with HRs from SARS-CoV, SARS-CoV-2, MERS-CoV, and HCoV-OC43, making the design of pan-CoV peptide inhibitors more difficult.

To overcome these difficulties, Xia et al. designed a series of peptides from different HR1

and HR2 domains and found a peptide from HR2 of HCoV-OC43, termed OC43-HR2P, which could broadly inhibit the cell-cell fusion mediated by S protein of several CoVs (Xia et al. 2019). Therefore, OC43-HR2P has the potential to be developed as a broad CoV peptide inhibitor. The potent pan-coronavirus fusion inhibitor, EK1, was further developed based on OC43-HR2P. EK1 can broadly and effectively inhibit multiple HCoVs and several infections from SARS-like CoVs (SL-CoVs) in vitro and shows promising coronavirus inhibitory activity and safety in vivo, with the strong likelihood of entering clinical trials (Xia et al. 2019, 2020c). Furthermore, EK1C4, a cholesterol-modified EK1 peptide, shows more potent inhibitory activity against different HCoVs with IC50 values at the nanomolar level (Xia et al. 2020b). Intranasal administration of low-dose EK1C4 can effectively protect newborn mice from HCoV-OC43 infection and protect human ACE2 mice from SARS-CoV-2 infection.

Additionally, EK1 and EK1C4 can potently inhibit infections from several MERS-CoV and SARS-CoV-2 variants without showing resistance (Hoffmann et al. 2021a). This feature is a great advantage of peptide inhibitors targeting HR1. A combination of peptide-targeting HR1 and antibody-targeting RBD (or NTD) exhibited synergism in inhibiting coronavirus entry (Wang et al. 2019a). Fusion proteins consisting of peptide inhibitor and antibody also showed more potent inhibitory activity than antibody or peptide inhibitor alone (Wang et al. 2019b), all of which represent promising approaches to enhance antiviral efficacy of entry inhibitors and reduce the incidence of escape-mutations.

7.8.2 Other Entry Inhibitors Targeting Membrane Fusion

The S2 subunit is more conserved than the S1 subunit; consequently, it is expected to become a target of broad-spectrum HCoV neutralizing antibodies. However, the S2 subunit is located in the stem region of the S protein, and it is generally difficult to induce effective neutralizing antibodies. Therefore, few studies have reported on neutralizing antibodies targeting the S2 subunit (Wang et al. 2015, 2021a; Lip et al. 2006; Amanat et al. 2021).

G4, an identified antibody-targeting the MERS-CoV S2 subunit, was reported to inhibit MERS-CoV infection (Wang et al. 2015), but its inhibitory activity against other HCoVs is poorly studied. Structural studies showed that G4 binding sites are variable among βCoVs (Pallesen et al. 2017), hinting that this epitope is not suitable as a target for broadly neutralizing antibody development. 28D9 was isolated from trimeric spike ectodomains immunized humanized mice and was shown to broadly and effectively neutralize MERS-CoV, SARS-CoV, HCoV-OC43, and SARS-CoV-2 PsV infection, as well as MERS-CoV live virus infection, but its inhibitory activity against SARS-CoV and SARS-CoV-2 live virus is poor (Table 7.1) (Wang et al. 2021a).

7.9 Entry Inhibitors with Other Mechanisms

In addition to the above-mentioned entry inhibitors, some entry inhibitors with other mechanisms, or uncertain mechanisms, can also block the entry of HCoVs. For instance, 8P9R is a dual-function peptide which can cross-link SARS-CoV-2 viral particles and inhibit endosomal acidification to stop SARS-CoV-2 entry (Zhao et al. 2021). 25-Hydroxycholesterol (25-HC), the enzymatic product of cholesterol 25-hydroxylase (CH25H), can broadly inhibit SARS-CoV-2, MERS-CoV, and SARS-CoV-2 entry by reducing cholesterol in the cell membrane (Wang et al. 2020). Apilimod can inhibit the endosome-dependent entry of SARS-CoV-2 by inhibiting an endosomal lipid kinase (PIKfyve kinase) (Kang et al. 2020). These entry inhibitors also show promise and are worthy of further studies.

Table 7.1 Coronavirus entry inhibitors under preclinical and clinical evaluations

Entry inhibitor	Type	Target	Inhibitory activity in vitro (EC50) or therapeutic efficacy in vivo	Developmental stage	Refs
CR3022	Antibody	RBD	SARS-CoV (live): 23.5 μg/ml	Preclinical	ter Meulen et al. (2006)
4C2	Antibody	RBD	MERS-CoV (PsV): 0.71 μg/ml	Preclinical	Li et al. (2015)
			MERS-CoV (live): 6.25 μg/ml		
REGN3048 + REGN3051	Antibody	RBD	MERS-CoV (live): 180 pM, 460 pM, respectively	Phase I	Pascal et al. (2015)
			REGN3048 plus REGN3051 can reduce viral titer in human DPP4 mice		
28D9	Antibody	S2	MERS-CoV (PsV): 0.13 μg/ml	Preclinical	Wang et al. (2021a)
			SARS-CoV (PsV): 60.5 μg/ml		
			SARS-CoV-2 (PsV): 45.3 μg/ml		
			MERS-CoV (live): 0.93 μg/ml		
G4	Antibody	S2	MERS-CoV (PsV): 0.133 μg/ml	Preclinical	Wang et al. (2015)
REGN-COV2	Antibody	RBD	REGN-COV-2 can greatly reduce virus load in rhesus macaques	Emergency use	Baum et al. (2020)
BRII-196 plus BRII-198	Antibody	RBD	BRII-196 plus BRII-198 treatment reduces viral replication	Emergency use	Ju et al. (2020)
4A8	Antibody	NTD	SARS-CoV-2 (live): 0.61 μg/ml	Preclinical	Chi et al. (2020)
			SARS-CoV-2 (PsV): 49 μg/ml		
MERS-27	Antibody	RBD	MERS-CoV (PsV): 63.95 nM	Preclinical	Jiang et al. (2014)
			MERS-CoV (live): 13.33 nM		
Soluble human ACE2	Protein	RBD	SARS-CoV-2 (live): >50 nM	Emergency use	Guo et al. (2021)
ACE2 microbody	Protein	RBD	SARS-CoV-2 (PsV): 0.36 μg/ml	Preclinical	Tada et al. (2020)
Trimeric ACE2	Protein	RBD	SARS-CoV-2 (live): 1.88 nM	Preclinical	Guo et al. (2021)
LCB1	Protein	RBD	SARS-CoV-2 (live): 23.54 pM	Preclinical	Cao et al. (2020)

(continued)

Table 7.1 (continued)

Entry inhibitor	Type	Target	Inhibitory activity in vitro (EC50) or therapeutic efficacy in vivo	Developmental stage	Refs
Dalbavancin	Lipoglycopeptide	ACE2	SARS-CoV-2 (live) in Caco2: 173.06 nM	Repurposed drug	Zhou et al. (2016)
			SARS-CoV-2 (live) in Vero-E6: 12.07 nM		
			MERS-CoV (PsV): 2.99 μM		
			SARS-CoV (PsV): 9.64 μM		
CP-1	Peptide	HR1	SARS-CoV (live): 19 μmol/L	Preclinical	Liu et al. (2004)
MERS-HR2P	Peptide	HR1	MERS-CoV (live): 0.6 μM	Preclinical	Lu et al. (2014)
EK1	Peptide	HR1	SARS-CoV-2 (live): 2468 nM	Preclinical	Xia et al. (2019), Xia et al. (2020c)
			MERS-CoV (live): 802.1 nM		
			HCoV-OC43 (live): 1554 nM		
			HCoV-229E (live): 4375 nM		
			HCoV-NL63 (live): 3693 nM		
EK1C4	Peptide	HR1	SARS-CoV-2 (live): 36.5 nM	Preclinical	Xia et al. (2020b)
			MERS-CoV (live): 4.2 nM		
			HCoV-OC43 (live): 24.8 nM		
			HCoV-229E (live): 101.5 nM		
			HCoV-NL63 (live): 187.6 nM		
Dec-RVKR-CMK (CMK)	Small molecule	Furin	SARS-CoV-2 (live): 0.057 μM	Preclinical	Cheng et al. (2020)
Camostat mesylate	Small molecule	TMPRSS2	SARS-CoV-2 (live) in Calu-3: 0.083 μM	Repurposed drug	Yamamoto et al. (2020)
Nafamostat mesylate	Small molecule	TMPRSS2	SARS-CoV-2 (live): 22.5 μM	Repurposed drug	Yamamoto et al. (2020)
Teicoplanin	Glycopeptide	Cathepsin L	MERS-CoV (PsV): 0.63 μM	Repurposed drug	Zhou et al. (2016)
			SARS-CoV (PsV): 3.76 μM		
K11777 (K777)	Small molecule	Cathepsin L	SARS-CoV (live) in Vero E6: 2.5 μM	Preclinical	Mellott et al. (2021)
			SARS-CoV-2 (live) in Vero E6: 0.62 μM		

(continued)

Table 7.1 (continued)

Entry inhibitor	Type	Target	Inhibitory activity in vitro (EC50) or therapeutic efficacy in vivo	Developmental stage	Refs
			MERS-CoV (live) in Vero E6: 0.62 μM		
Hydroxychloroquine (HCQ)	Small molecule	Endocytosis	SARS-CoV-2 (live) in Vero: 13.3 μM	Repurposed drug	Hoffmann et al. (2020b)
			SARS-CoV-2 (live) in Calu-3: 119 μM		
Chloroquine (CQ)	Small molecule	Endocytosis	SARS-CoV-2 (live) in Vero: 6.5 μM	Repurposed drug	Hoffmann et al. (2020b)
			SARS-CoV-2 (live) in Calu-3: 64.7 μM		
			MERS-CoV (live) in Huh-7: 3.0 μM		
			SARS-CoV (live) in Vero: 4.1 μM		
8P9R	Peptide	Two entry pathways	SARS-CoV-2 (live): 0.3 μg/ml	Preclinical	Zhao et al. (2021)
25-HC	Small molecule	Membrane cholesterol	SARS-CoV-2 (live): 3.675 μM	Preclinical	Wang et al. (2020)
			SARS-CoV (PsV): 2.48 μM		
			MERS-CoV (PsV): 1.22 μM		
Apilimod	Small molecule	PIKfyve kinase	SARS-CoV-2 (live): 0.023 μM	Repurposed drug	Kang et al. (2020)

7.10 Future Perspectives

In this century, mankind has experienced SARS-CoV and MERS-CoV outbreaks. However, humans remain passive in response to the recent SARS-CoV-2 outbreak. Although a variety of vaccines have been successfully developed and administered, the continuous emergence of SARS-CoV-2 variants might reduce the protective effect of vaccines (Hoffmann et al. 2021a; Shen et al. 2021). Therapeutics currently used for COVID-19 treatment mainly includes convalescent plasma, neutralizing antibodies, and repurposed drugs. The clinical effects of convalescent plasma are not as good as previously predicted (Li et al. 2020a; Simonovich et al. 2021). Antibody monotherapies could result in the emergence of escape mutations (Wang et al. 2021c). On the contrary, antibody cocktails may be important methods to solve the resistance of SARS-CoV-2 variants. Some repurposed drugs, such as chloroquine or camostat mesylate, have shown good antiviral activity in vitro or in animal experiments, but their clinical efficacy is unsatisfactory (Axfors et al. 2021; Simonis et al. 2021). Therefore, it is very important to develop broad-spectrum anti-coronavirus drugs with significant clinical efficacy.

The processes of receptor binding, proteolytic activation, and membrane fusion play important roles in coronavirus entry and are, thus, targets for the development of entry inhibitors. Actually, owing to the high variability of the RBD region among different coronaviruses, RBD is an ideal target for single HCoV-specific inhibitors, but not for the broad-spectrum anti-coronavirus drugs. Entry inhibitors targeting proteolytic activation showed broad antiviral activities in vitro, but

their in vivo coronavirus inhibitory activity and clinical efficacy should be further confirmed.

Membrane fusion mediated by the 6-HB formation is an important process for HCoV entry after receptor binding and proteolytic activation. Therefore, the 6-HB formation is an important target for the development of broad-spectrum coronavirus entry inhibitors. Peptide entry inhibitors disrupting the 6-HB formation (e.g., EK1C4, [SARSHRC-PEG4]2-chol) showed potent inhibitory activity against different HCoVs, with promise for clinical application. Other forms of entry inhibitors targeting membrane fusion (e.g., small molecules) should also be developed.

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Influenza Virus Entry inhibitors

8

Jie Yang and Shuwen Liu

Abstract

Influenza is a major challenge to health and the global economy. However, the increasing emergence of drug resistance during influenza pandemics cannot be ignored. It is particularly important to design and develop anti-influenza drugs with novel mechanisms of action for the treatment and prevention of influenza virus infections. Virus entry is the first essential step in the viral life cycle, the prevention of which leads to suppression of viral infectivity and is an attractive antiviral strategy. Here, we review the development of influenza entry inhibitors and their performance in clinical trials, including small molecule, natural product, peptide-based entry inhibitors, and other types of entry inhibitors, herein discussing some open questions. Assay methods of influenza virus entry inhibitors are also outlined.

Keywords

Influenza virus · Virus entry · Hemagglutinin · Therapeutics · Entry inhibitors

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8.1 Introduction

Seasonal influenza is an acute respiratory infection caused by influenza viruses, which continues to be a major cause of mortality worldwide. Until now, four notorious pandemics (Spanish flu of 1918, Asian flu of 1957, Hong Kong Flu of 1968, and the H1N1 virus of 2009) profoundly affected human health and socio-economic. According to the World Health Organization (WHO), about three to five million cases of severe illness, and about 290,000–650,000 respiratory deaths each year are associated with seasonal influenza (WHO 2018).

Vaccination is one of the most effective strategies for the control and prevention of influenza virus infection in both human and animal populations, but there are several challenges to its efficacy. In general, most vaccines will take many years from virus identification to vaccines large-scale application. However, the vaccines are vulnerable to failure because of genetic variation of influenza virus known as genetic drift and genetic shift as well as making targeting optimal antigens difficult. To date, the global scientific community is attempting to develop universal influenza virus vaccines to provide long-lasting and broad protection against multiple IAV strains (Laursen and Wilson 2013). Broadly neutralizing antibodies against influenza viruses have been recently described, but they have their own defects, including limited cross-reactivity to both influenza A and B strains and the need for repeated

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injections. Therefore, anti-influenza agents are indispensable for both the prophylaxis and treatment of influenza. Currently, there are three classes of FDA-approved antiviral drugs for the treatment and prevention of influenza, including M2 ion-channel inhibitors (amantadine and rimantadine), neuraminidase inhibitors (oseltamivir, zanamivir, peramivir, and laninamivir) and endonuclease (PA) inhibitors (baloxavir marboxil). M2 ion-channel inhibitors could block the M2 ion channel and prevent uncoating of the viral genome, but only work against influenza A virus (IAV). In recent years, this class of drugs are clinically less useful for the treatment of influenza virus infection due to widespread resistance and severe side effects (Yang et al. 2019). Influenza virus neuraminidase (NA) plays a critical role in the release stage of influenza virus life cycle. NA inhibitors are the universal application and effective anti-influenza agents against both influenza A and B viruses, especially highly pathogenic H7N9 and H5N1 avian influenza virus. Although NA inhibitors made a large contribution in the battle against influenza viruses with their specific superiorities, continually emerging drug resistance limits their development and effectiveness (Baranovich et al. 2010; Dharan et al. 2009). The inhibitor of influenza cap-dependent endonuclease is considered to be a new class of antiviral drug for influenza for the first time in nearly 20 years. Baloxavir marboxil (Xofluza™), as the first PA inhibitor was approved for the treatment of influenza virus in 2018 and exhibited good anti-influenza virus effect according to its recent clinical trials (Shirley 2020). Single-dose baloxavir marboxil could profoundly decrease viral titers as well as alleviating influenza symptoms. However, with the prolonged baloxavir marboxil treatment, a few resistant mutants have been recorded. The recent clinical studies showed that the PA I38T mutant reduced sensitivity to the drug for the treatment of influenza A and B viruses (Jones et al. 2018). Additionally, some innovative drugs with new targets are developed continually. Pimodivir (JNJ-63623872, VX-787) is an orally bioavailable inhibitor of IAV polymerases through interaction with the viral PB2 subunit. In phase 2b study, pimodivir alone or in combination with oseltamivir demonstrated a significant reduction in IAV viral load in adults with uncomplicated influenza (Finberg et al. 2019). Search for antiviral resistance toward the drug is currently ongoing. Favipiravir (T-705) (Byrn et al. 2015), an RNA-dependent RNA polymerase inhibitor of influenza virus, is currently undergoing phase III clinical trials in the United States (Wang et al. 2020a, b, c). It has been shown to be effective against all three types of influenza virus (A, B and C) and other RNA viruses, including some of the hemorrhagic fever viruses. A recent vitro study has shown that mutations in the K229R subunit of influenza virus polymerase PB1 reduced the susceptibility to farpiravir (Goldhill et al. 2018). A recent report has shown favipiravir exhibited a certain efficacy against SARS-CoV-2 in vitro (Udwadia et al. 2021) However, this needs to be verified by clinical trials. In view of these drugs, the on-market anti-influenza drugs still cannot satisfy the clinical requirements and lack of diversity for antiviral targets. Therefore, more effective antiviral agents, with a novel mechanism of action, are required for the treatment and prevention of influenza virus infections.

Influenza virus belongs to the family of *Orthomyxoviridae*, possessing a single-stranded negative-sense RNA genome that is comprised of eight segments. There are four types of influenza viruses: A, B, C, and D. Influenza A virus is the only influenza virus known to cause flu pandemics because of its high susceptibility to antigenic variation. There are two surface glycoproteins on the virion: hemagglutinin (HA) and NA, that determine both the host immunity and subtype designation. There are 18 distinct HA subtypes, allowing the virus attach to the host cells. NA has 11 different subtypes, shares the same fundamental biological function (Prachanronarong et al. 2019). Influenza virus entry into host cells is initiated by attachment to sialic acid receptors and is followed by important conformational changes of HA, fusion with cellular membranes in endosomes. The process ends with transfer of viral genomes inside host cells. With recent progress in understanding the virus

entry mechanisms, inhibitors of virus entry offer unprecedented opportunities for the development of novel antiviral drugs and therapeutics.

8.2 The Process of Influenza Virus Entry and Antiviral Targets

The process of influenza virus entry is as follows: the attachment of HA1 to sialic acid receptors (α-2,6-SA or α-2,3-SA) on the surface of the target cell, internalization into the endosome, trafficking to the perinuclear region, fusion of the viral envelope with endosomal membranes, uncoating and import of the viral genome into the nucleus. The influenza HA glycoprotein presents in the membrane envelope of influenza virus, plays a critical role in the virus entry (Skehel and Wiley 2000). IAV has 18 different HA subtypes (H1-18), which can be classified phylogenetically into group 1 (H1, H2, H5, H6, H7, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15) according to the ways that they interact with their receptor and the definition of their epitopes (Wu and Wilson 2018). Each virion contains approximately 500 molecules of HA. Mature HA is a homotrimer, each monomer is composed of two disulfide-linked subunits, HA1 and HA2, generated by the proteolytic cleavage of HA0 precursor and modification by multiple glycosylations. The HA globular head (HA1) domain is highly variable in different HA subtypes. The relatively conserved stalk domain is formed by the HA2 together with the N and C terminal HA1 residues, which includes the transmembrane region (Yun et al. 2019). It has been proved that most of the HA1 subunits are responsible for host cell attachment through binding to sialic acid while the HA2 subunit in the stem region of HA leads to viral-cell membrane fusion (Skehel and Wiley 2000). It is generally accepted that HA-mediated recognition of cell surface sialic acid is the first step in initiating IAV infection, which is critical in all subsequent events.

Following binding, the virus particle is taken up by endocytosis and quickly enters endosomes inside the cell. The endosomes, contain virus particles, are then transported to the locations that near the nucleus. With acidification of the endosome (around 5–6), HA undergoes a dramatic conformational rearrangements, resulting in exposure of the fusion peptide, thus subsequently inserting into the lipid bilayer of the endosomal membrane (Carr et al. 1997). After the fusion of viral and cellular membranes, the viral ribonucleoproteins (vRNPs) are released and transported into the nucleus to begin the replication process.

As mentioned above, influenza virus entry process can be targeted by a variety of entry inhibitors including inhibitors of virus attachment, inhibitors of internalization, and inhibitors of fusion. Although a focus has been on viral protein essential for virus entry, more recent data revealed that IAV requires additional host factors for successful attachment and entry into target cells (Edinger et al. 2014). Thus, cellular factors required for IAV entry represents a favorable target, which support to produce novel entry inhibitors. Here, we review small molecular, natural product, peptide-based entry inhibitors, and other types of entry inhibitors and discuss the drugs in clinical development (Table 8.1).

8.3 Assay Methods for Influenza Entry Inhibitors

The preclinical research methods of influenza virus entry inhibitors are roughly divided into four kinds of experiments: cell infection experiments, target confirmation of the compound, the identification of drug-target protein interaction, and animal infection experiments.

A cell-based pseudovirus entry assay to identify entry inhibitors or influenza HA-specific neutralizing monoclonal antibodies was previously established by Jiang's group (Du et al. 2010). They constructed a luciferase (Luc)-expressing pseudovirus bearing HA and NA protein of IAV H5N1 virus strains, based on the HIV-1 backbone to avoid handling of infectious IAV. The generated H5N1 pseudovirus allows single-cycle infection of host cells. The pseudovirus inhibition assay possess safe,

Table 8.1 Influenza virus entry inhibitors currently in clinical trial

Drug	Highest phase	Recruitment status	Antivirus mechanism
Arbidol	Phase IV	Recruiting unknown	Blocking pH-dependent fusion of cellular and viral membranes in endosomes
DAS181	Phase II	Completed	Inactivating the sialic acid receptor
Nitazoxanide	Phase III	Completed	Inhibiting HA maturation and intracellular trafficking in infected cells
CR6261	Phase II	Completed	Binding to the conserved epitope within the HA stem domain
CR-8020	Phase II	Completed	Binding to the conserved epitope within the HA stalk domain
VIS410	Phase II	Completed	Binding to the conserved epitope within the HA stem domain
MEDI8852	Phase II	Completed	Binding to the conserved epitope within the HA stalk domain
MHAA4549A	Phase II	Completed	Binding to the conserved epitope within the HA stem domain
MHAB5553A	Phase I	Completed	Binding to the conserved epitope within the HA globular head (HA1) domain

convenient, and quantitative characteristics, supporting is a very useful tool for rapid screening potential influenza entry inhibitors. Plaque assay and cytopathic effect (CPE) are applied to appraise the potent antiviral activity of entry inhibitors. General experiments such as qRT-PCR, indirect immunofluorescence, and western blot are also employed to evaluate inhibition effect of entry inhibitors (Liu et al. 2018). Of note, live-cell microscopy as a promising tool can visualize virus entry in live cells and monitor the interaction of the virus with cellular factors and compartments by fluorescently-labeled tetracysteine-tagged proteins (Whitt and Mire 2011).

As mentioned above, HA protein can bind to sialic acid receptors under neutral pH conditions, causing virus adhesion/entrance and red blood cells agglutination (chickens red blood cells are generally used, other species such as guinea pigs are also used). Then, HA protein undergoes conformational changes with low pH conditions. Conformational rearrangements of HA exposes trypsin receptors that can be digested by trypsin, and exposes hydrophobic fragments that can cause hemolysis (Tang et al. 2011). Thus, the direct effect of entry inhibitors on HA protein can be evaluated by different experimental methods, such as hemagglutination inhibition (HI) assay and hemolysis inhibition assay. Additionally, high-throughput screening platforms for HA inhibitors are developed base on the HA protein function (Li et al. 2017; Trost et al. 2018).

WaterLOGSY and STD nuclear magnetic resonance (NMR) (Antanasijevic et al. 2013), surface plasmon resonance (SPR) (Chen et al. 2020) and the enzyme linked immunosorbent assay (ELISA) methods (Barnard et al. 2020) are capable of identificating the interactions between drugs and target proteins. Computer-aided molecular docking and X-ray (Kadam and Wilson 2017) crystallography can be used to predict and analyze the binding site of HA protein, respectively.

Balb/c, CD-1, C57BL/6 and other mice strains are generally used in the animal infection experiments (Thangavel and Bouvier 2014). Influenza virus-infected animals would be affected by severe symptoms such as viral pneumonia, edema in lung tissue, or even suffer death. The inhibitory effect of entry inhibitors can be evaluated through the examination of mouse lung index, mouse mortality, mouse survival time, serum cytokines, etc.

8.4 Influenza Entry Inhibitors

8.4.1 Small Molecule-Based Entry Inhibitors

The researchers intend to block the entry of the virus by designing small molecule inhibitors that bind to HA and stabilize its nonfused conformation. Tert-butylhydroquinone (TBHQ) is a well-known fusion inhibitor that could impede the

conformational change required for fusion (Bodian et al. 1993). Another fusion inhibitor is BMY-27709, which acts H1 and H2 subtypes of influenza A virus but not H3 in cell culture (Luo et al. 1997). Our group identified and characterized CL-385139 as an entry inhibitor of influenza A virus that specifically bound to H5N1 influenza virus HA, thereby blocking virus and host cell membrane fusion. Its detailed molecular mechanism highlights the "induced fit" pocket which is formed during their interaction may be a target for structure-based design of more potent influenza fusion inhibitors (Li et al. 2012).

The most well-known and extensively used small molecule-based entry inhibitors is arbidol (Boriskin et al. 2008), which is currently licensed in Russia and China for prophylaxis and treatment of influenza and other respiratory viral infections. The anti-influenza activities of arbidol and its mechanism of action have been extensively studied (Boriskin et al. 2008). The crystal structure study revealed that arbidol bound to the hydrophobic cavity between the two promoters of the HA trimer. This cavity is located at the far end of the conserved epitope. Arbidol and its binding site mainly have hydrophobic interactions, but also cause some conformational rearrangements, thus forming a network of inter- and intraprotomer salt bridges. By playing the role of molecular glue, arbidol can stabilize the pre-fusion conformation of HA, thereby inhibiting the conformational rearrangement associated with membrane fusion (Kadam and Wilson 2017). Furthermore, with the MALDI mass spectrometry, it was confirmed that arbidol bound to residues 104–120 of the HA2 subunit, which contain arbidol resistance mutations (Nasser et al. 2013). Overall, arbidol could stabilize HA and prevent membrane fusion mediated by low pH-induced HA conformational change, thereby preventing virus infection.

Arbidol has a pronounced antiviral infection in both cell culture and animal models (Leneva et al. 2005). Treatment with arbidol (60–120 mg/kg/day) for 5 days before IAV infection could significantly reduce the virus titer in the lungs of mice, and reduce the mortality rate by 70–80%. Clinical studies of arbidol had shown that arbidol was effective and well tolerated in the treatment of early acquired influenza (Kiselev et al. 2015). Phase IV clinical trials of arbidol are currently underway in the United States (NCT number: 01651663). However, the antiviral activity of arbidol against IAV has not been fully recognized worldwide.

Apart from influenza virus, arbidol has also been shown to have wide range and potent antiviral activities against globally prevalent pathogenic viruses, including respiratory syncytial virus, adenovirus, Coxsackie B5, parainfluenza, Ebola virus, Lassa virus, hepatitis B and C, avian coronavirus, etc. (Pécheur et al. 2016). The recent study suggests arbidol is an efficient inhibitor of SARS-CoV-2 in vitro (Wang et al. 2020a, b, c). The mechanism of action against the SARS-CoV-2 involves target the spike protein and block its trimerization, thereby effectively blocking the entry of SARS-CoV-2 into cells. The recent development of arbidol against SARS-CoV-2 infection demonstrated the promising clinical results, which confirmed their safety and efficacy to inhibit SARS-CoV-2 infection. The therapeutic efficacy should be further elevated. The studies on clinical effect of arbidol alone or in combination with lopinavir/ritonavir, chloroquine phosphate or carrimycin have been recently initiated in China (NCT number: 04255017, 04260594, 04273763). However, as a broad-spectrum antiviral drug, arbidol needs to be used in large dose (at least 200 mg) to achieve ideal therapeutic effects. For example, patients with COVID-19 disease need to receive 400 mg arbidol three times a day for 9 days in a clinical pilot trial of China (Wang et al. 2020a, b, c). Therefore, scientists expect to modify the structure of arbidol so as to improve its antiviral efficacy.

Some novel synthetic compounds which specifically inhibits HA-mediated viral entry have become available recently. Nitazoxanide is a synthetic nitrothiazolyl-salicylamide derivative, which was originally developed and commercialized as an antiprotozoal agent. At present, nitazoxanide (Alinia™) is licensed for the treatment of Cryptosporidium and Giardia infections. In addition to its antiparasitic activity, laboratory studies revealed that nitazoxanide via

its circulating metabolite tizoxanide, has broad-spectrum antiviral activity against rotavirus nitrate, norovirus gastroenteritis, chronic hepatitis B and C, etc., and has recently been repurposed for the treatment of influenza virus (Rossignol 2014). Nitazoxanide exhibited potent antiviral activity against all tested influenza A and B viruses. In combination with oseltamivir or zanamivir, there was a synergistic effect when tested against influenza A H1N1/PR8 and avian influenza A/H5N9. The mechanism appears to be selectively blocking the maturation of the influenza virus HA. This impairs hemagglutinin intracellular trafficking and insertion of the protein into the host plasma membrane (Rossignol et al. 2009). Nitazoxanide was subjected to extensive pharmacological testing for efficacy and safety. A Phase IIb/III trial (NCT01227421) has been conducted in the United States (Haffizulla et al. 2014). It is estimated that two dosing regimens of oral nitazoxanide have been effective in treating patients with uncomplicated influenza. To evaluate the efficacy and safety of nitazoxanide and nitazoxanide plus oseltamivir in the treatment of acute uncomplicated influenza, a placebo-controlled Phase III trial has been completed (NCT01610245). More recently, a new pharmaceutical formulation with controlled release of nitazoxanide was developed to deliver the drug systemically. The second-generation thiazoles is also effective against influenza A virus by selectively blocking the maturation of the viral HA, thus impairing HA intracellular trafficking and insertion into the host plasma membrane, a key step for correct assembly and exit of the virus from the host cell (Rossignol et al. 2009). Notably, as a broad-spectrum viral inhibitor, nitazoxanide has also been reported to work against COVID-19, indicating repurposing of nitazoxanide as a new broad-spectrum agent could prove to be important (Lokhande and Devarajan 2021).

In addition, RO5487624 and its derivative compound RO5487624 (Zhu et al. 2011), MBX2329 and MBX2546 (Basu et al. 2017), IY7640 (Kim et al. 2019), CBS1116 (Hussein et al. 2020) and so on have also been reported to have biological activity against influenza viruses by preventing conformational changes of HA under low pH conditions, thus preventing membrane fusion and virus infection.

Taken together, all of these data make it attractive to further characterize and develop small molecules as HA inhibitors, for potential clinical use. However, improvements in the pharmacokinetic properties of these small molecule-based compounds are required for further development. Except for chemical modifications strategies, nanomaterial strategies are extremely valuable to the successful development of new small molecule-base entry inhibitors. For example, drug envelopment of liposomes can extend the plasma half-life of drugs, elevate blood drug level, increase the therapeutic effect, diminish adverse reactions.

8.4.2 Peptides-Base Entry Inhibitors

Enfuvirtide (also known as T-20, Fuzeon™), a 36-amino acid-containing peptide, was approved for treatment of HIV/AIDS by the U.S. FDA. The successful development of enfuvirtide as the first HIV entry inhibitor has brought a new idea for the research of peptide-based entry inhibitors.

EB peptide is a small peptide composed of 20 small amino acids. Preliminary mechanism studies suggested that EB interacted with the receptor-binding pocket of the HA1 protein, thereby blocking the binding of the influenza virus to its receptor (Jones et al. 2006). In vitro, EB peptide was proved to have broad-spectrum antiviral activities against a broad variety of IAV strains (including H5N1, H5N9, H1N1, HK/483) and influenza B viruses. Additionally, a novel family of peptides (FluPep) was reported to effectively inhibit the infection of H1N1, H3N2, and H5N1 at nanomolar concentrations. The prototype peptide (FP1, also known as Tkip) interacted with HA and inhibited the binding of the virus to cell membranes (Nicol et al. 2012). Recent studies have shown that anti-lipopolysaccharide peptides (SALPs) in PEP 19–2.5 have a high binding affinity for influenza virus N-acetylneuraminic acid receptor molecule, impairing viral attachment and entry into cells.

In vitro studies have shown that SALP PEP 19–2.5 could inhibit various strains of influenza virus without toxicity, including H7N9, H3N2, and 2009 pandemic H1N1. The mechanism of action of these peptides as entry inhibitors suggests a potential application for anti-influenza virus therapy (Hoffmann et al. 2014).

Fusion peptides play an important role in the process of membrane fusion. The researchers synthesized positively charged fusion peptides (pFPs) which effectively inhibited the replication of IAV including oseltamivir-resistant strains. The mechanistic study indicated that the antiviral activity might be associated with the interactions between the HA2 subunit and pFP, of which, the nascent pFP exerted a strong effect to interrupt the conformational changes of HA2, thereby blocking the entry of viruses into host cells (Wu et al. 2015a, b).

Although the inhibitors in this category with higher potency and fewer side effects than above small molecules, there are still many problems including pharmacokinetic properties, intravenous or subcutaneous delivery, stability and so on, which is obstacle needing overcome before their successful application in clinic. To date, peptide-drug conjugates for targeted therapeutics have attracted enormous interest due to their significantly enhanced efficacy and reduced side effects.

8.4.3 Natural Product-Based Entry Inhibitors

Natural products as a continuing source of novel drugs play an indispensable role in the development of antiviral drugs. Extensive researches of the biological activities of natural products have been done over the centuries. Literature review affirms natural products possess a distinctive efficacy for viral infections. Glycyrrhizin (Wolkerstorfer et al. 2009), stachyflin (Motohashi et al. 2013), curcumin (Chen et al. 2013), and quercetin (Wu et al. 2015a, b) have been previously demonstrated to exert antiviral activity against influenza viruses by various mechanisms. In particular, many natural products exert a dual antiviral mechanism by direct acting viral protein, as well as anti-inflammatory, highlights the clinical value of natural products as potentially effective new antiviral agents.

The most representative compound is epicallocatechin-3 gallate (EGCG), the most abundant ingredient presents in tea leaves and a well-known antioxidant, both in vitro and in vivo, effectively inhibited IAV infection. Antiviral activities of EGCG were observed by the different modes of action. It has been found that EGCG mainly prevents influenza virus entry by destroying the viral envelope (Kim et al. 2013; Song et al. 2005). EGCG also exerted anti-inflammatory effects through modulating TLR4 (Xu et al. 2017) and MAPK (Mou et al. 2020) signal pathways involved in influenza virus pathogenesis. The structure-activity relationship studies showed that the 3-gallic and 5-OH groups of the catechin derivatives were the keys to the antiviral activity, which was of great significance to enhance the binding affinity between EGCG and virus particles and to improve the virulent activity of the virus (Kaihatsu et al. 2018). Additionally, the antiviral effects of chemically modified EGCG derivatives have also been investigated. At present, the clinical studies of EGCG on anti-influenza virus are in the development stage, and the clinical effect of EGCG on the prevention of influenza virus is still controversial, so large-scale studies are needed to confirm the clinical treatment effect of EGCG. The latest reports demonstrated EGCG could also inhibit replication of SARS-CoV-2. It could suppress ACE2 (a cellular receptor for SARS-CoV-2) and TMPRSS2 by activating Nrf2, and then inhibited the virus entry into cells (Zhang et al. 2021).

Natural products have gained more attention as novel therapeutic agents because of their innate advantages such as less expensive, better patient tolerance, and fewer or no side effects. It is worth note that the acute lung injury induced by influenza virus is widely recognized. Therefore, the dual ability of natural products that concomitantly halt virus replication and dampen proinflammatory mediators might constitute a viable therapeutic option in patients with IAV infection. However, considering the poor

pharmacokinetic parameters and solubility characters of natural products, it may be difficult to apply natural products in clinical use in the present form. Further structural optimization is believed to contribute to the further development of effective and broad-spectrum entry inhibitors. Moreover, natural products may be clinically used in combination with some NA inhibitors such as oseltamivir to treat viral infections and their complications.

8.5 Other Novel Entry Inhibitors

8.5.1 Monoclonal Antibodies (mAb)

As a novel therapeutic application, mAbs are being developed for a broad range of illness, from cancer to cardiovascular diseases to infectious diseases. The development of monoclonal antibodies is currently progressing rapidly. In particular, broadly neutralizing mAb binding conserved epitopes in HA shown great promise for the treatment of influenza, which could provide immunity to the diverse influenza subtypes and protection against future pandemic viruses. The discovery of The first monoclonal antibody C179 leads to the development of a broad spectrum of antibodies targeting the conserved stem domain of HA. Another broadly neutralizing mAb CR6261 was isolated from the immune system of a vaccinated healthy individual in 2009. This mAb could recognize a highly conserved helical region in the membrane-proximal stem of HA1 and HA2, preventing pH-dependent conformational rearrangement which was required for cellular and viral membranes in endosomes (Ekiert et al. 2009). Moreover, CR6261-like epitopes in the HA stem could be applied in targeted vaccine strategy or provide a new idea for the design of the next generation of antivirals. Antibody CR6261 could broadly neutralize several subtype influenza viruses with high affinity, including neutralized almost all influenza A group 1 viruses(H1, H2, H5, H6, H8, and H9) which constituted a breakthrough in the influenza field (Throsby et al. 2008). CR6261 had completed Phase I (NCT01406418) and Phase II (NCT02371668) clinical trials that confirmed its safety and immunogenicity. But the phase III clinical trial evaluating whether CR-6261 reduced viral replication in healthy volunteers infected with H1N1 has been withdrawn because of the business decision.

Another human mAb CR8020 was recently identified and applied as a broadly neutralizing influenza HA stem-specific antibody. CR8020 possessed broad neutralizing activity against most group 2 viruses (Tharakaraman et al. 2014). Giving CR8020 with 3 mg/kg for a week could protect mice against influenza A H3N2 and H7N7 viruses challenges (Ekiert et al. 2011). Thus, if the cocktail of two antibodies which have the activity of neutralizing all influenza A group 1 virus and group 2 virus at the same time, which makes the development of a universal flu vaccine and broad-spectrum antibody therapies promise. CR8020 had completed phase I and phase II clinical trials in 2012 and 2014, respectively. However, CR8020 failed to pass the study which assessed the safety, pharmacokinetics and immunogenicity in Japanese healthy participants, due to an unexpected preliminary result. Later on, several mAbs targeting the highly conserved epitope of HA have been described, such as VIS410 (Sloan et al. 2020), MEDI885262 (Paules et al. 2017), MHAA4549A (Lim et al. 2016).

Besides targeting the conserved HA stem region, an alternative approach is to recognize conserved epitopes in the globular head of HA. The latter type of mAb can inhibit virus attachment to cell surface receptors, such as MHAB5553A. As discussed above, it is feasible to develop monoclonal antibodies against different types of influenza viruses targeting highly conserved sites of HA. However, mAbs presents several disadvantages. On the one hand, these drug products are expensive and have complicated lengthy production processes. On the other hand, their large molecular size limits their biodistribution and efficacy. Moreover, they can also induce immune reactions, which further limits their long-term application.

8.5.2 Novel Influenza Entry Inhibitor Target Host Factor

Virus is believed to exploit cellular machinery to complete the initial stages of infection. Experimental evidences suggested host proteins are potential antiviral targets for drug development because it is not susceptible to resistance by viral mutations. DAS181 (Fludase™) is a novel sialidase fusion protein, which leads to desialylation of both alpha2-6-linked and alpha2-3-linked sialic acid on host cells, preventing influenza virus HA binding to the sialic acid receptors. The previous reports have shown DAS181 and its analogs have strong inhibitory effects against multiple influenza virus A and B strains. In animal models, pretreatment and posttreatment of DAS181 provided effective protection to mice challenged with the highly pathogenic H5N1 influenza virus (Belser et al. 2007). As a novel influenza virus receptor inactivator, DAS181 showed safe and well-tolerated in Phase I clinical trials (NCT00527865) and Phase II clinical trials (NCT01037205). These findings support the potential value of DAS181 as a prophylactic and therapeutic agent against influenza viruses, especially H5N1 (Moss et al. 2012; Zenilman et al. 2015). The Clinical trial (NCT04324489) which evaluates the safety and effects of nebulized DAS181 on hypoxic coronavirus disease 2019 patients is presently underway.

Recent studies revealed sialic acid might not be the sole receptor required for virus entry, several host factors have been proved that contribute to the efficient entry of IAV into the host cells, such as epidermal growth factor receptor pathway substrate 8 (EPS8) (Larson et al. 2019), protein kinase C (PKC) (Sieczkarski et al. 2003), and epidermal growth factor receptor (EGFR) (Eierhoff et al. 2010). The inhibitors are designed to target the host cells rather than the virus can avoid the development of antiviral resistance. PKC inhibition affects endocytosis and specific inhibitor of classical PKCs Gö6976 has been shown to prevents influenza virus entry and subsequent infection of the host cell. Very recently, it has been shown that M85 (O'Hanlon et al. 2019), which targets host kinases, EGFR and PIK3C2β, exhibited a broad spectrum of anti-influenza virus activity with minimal cytotoxicity. The mechanistic studies indicated that interference blocking endocytosis of influenza viruses was the mode of action for this new compound. In vivo, combinations of M85 and oseltamivir have strong synergism. Despite host factors are gaining recognition as a novel drug target, specific entry inhibitors still remain limited so far.

8.6 Conclusion and Prospect

The glycoprotein HA of influenza virus plays a crucial role in the initial stage of virus infection, making it a potential target for anti-influenza therapeutics development. In recent years, some animal-hosted influenza viruses have successively mutated and become human-to-human transmission. Seasonal influenza needs to be monitored every year to confirm whether there are mutants that cause antigenic drift. Influenza virus entry inhibitors target the most variable globular part of HA, making it easier to obtain drug resistance. Furthermore, most currently developed small molecule inhibitors, peptides or vaccines target the specific type of virus or specific HA subunit. The most challenging aspect for the development of entry inhibitors is to develop a broad-spectrum entry inhibitor that is sensitive to a broad range of influenza virus strains. The structure studies demonstrated the stalk region of the HA protein is a highly conserved functional region. The inhibitors that target the stalk of HA would be valuable therapeutics and could guide the development of universal entry inhibitors. Moreover, the unique mechanisms of influenza virus entry inhibitors as compare with existing anti-influenza drugs highlights their potential for future combinations. In fact, current evidences in vitro have supported treatment with the combination therapy is more effective than administering NA inhibitor alone. What's more, host factors involved in viral entry rendering them be potential targets for new therapeutic

interventions. The development of new antivirals targeting host factors rather than viral protein would minimize the occurrence of resistance as observed with available anti-influenza drugs in the clinic. However, no influenza virus entry inhibitor was approved by the U.S. FDA. There are two major factors involved in low potency and poor pharmacokinetics. To conquer these problems, tremendous efforts have been made through both chemical modifications and material science. It is hoped that influenza virus entry inhibitors are licensed in the near future, they will offer a new option to the treatment of influenza.

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Broad-spectrum Respiratory Virus Entry Inhibitors

9

Hanjun Zhao and Kwok-Yung Yuen

Abstract

With the increasing global human population, travel, and socioeconomic activities, more and more novel pathogenic viruses will emerge or re-emerge. While more than 260 viruses are known to infect humans, only a small minority of these viral diseases are treatable by clinically approved antiviral drugs. Apart from these identified viruses, new emerging viruses and drug-resistant viruses are also important challenges to our public health and healthcare systems. The COVID-19 and influenza pandemics remind us the importance of getting broad-spectrum antivirals against emerging and re-emerging respiratory viruses. Broad-spectrum antivirals against different viral families for fighting the currently known viruses and novel emerging viruses are urgently needed. Viral entry is the universal first step for viral infection, and therefore is a promising target for identifying broad-spectrum antivirals. In this chapter, we mainly focus on discussing the risks of respiratory viruses, the challenge of finding broad-spectrum antivirals, the entry processes of respiratory viruses, the current studies on broad-spectrum entry inhibitors for respiratory viruses, and the directions for discovering broad-spectrum antivirals in the future.

Keywords

Antiviral · Broad-spectrum · Entry inhibitor · Respiratory virus · Viral entry

9.1 Risks of Respiratory Virus Infection

9.1.1 Risks from Emerging Viruses

One of the major public health challenges in the future will be respiratory infectious diseases. In the past century, there have been at least four influenza pandemics with more than 50 million of estimated deaths (Monto and Fukuda 2020) and three coronavirus outbreaks including SARS-CoV in 2003, MERS-CoV in 2012, and SARS-CoV-2 in 2019. Besides, the seasonal influenza viruses cause seasonal flu with the estimated global deaths of more than 250,000 every year. The SARS-CoV (Du et al. 2009) and MERS-CoV (Zumla et al. 2015) outbreaks caused more than 770 deaths and 480 deaths with a 10% and 40% mortality, respectively. The SARS-CoV-2, causing the COVID-19 pandemic, has led to more than 0.4 billion cases with over six million deaths. Except for these pandemics of influenza virus and coronavirus (CoV), other

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respiratory viruses, including parainfluenza virus (PIV), rhinovirus, respiratory syncytial virus (RSV), adenovirus (Adv), and human metapneumovirus (hMPV) have caused the most common cold, upper and middle respiratory tract infection associated with huge financial and health burden (Tang et al. 2017; Vandini et al. 2019). With the ease of global traveling, the transmission of respiratory viruses from one country to another country, and from one continent to another continent will be faster than any time in human history. This will increase the difficulties of global epidemic control as global transmission often occurs before the identification of the novel respiratory viruses.

9.1.2 Risks from the Lack of Antiviral Drugs

The severity and mortality of respiratory viral diseases are inevitable in the absence of effective and safe antivirals. Vaccination is the most effective way to prevent viral infection, but if they are not yet available, the antiviral drugs play important roles in alleviating the symptoms and severity. Currently, the most widely available antiviral drugs for treating respiratory viruses are the anti-influenza drugs, such as oseltamivir and zanamivir. However, the suboptimal effectiveness of these anti-influenza drugs given 48 h after symptom onset was reflected by the high mortality rates (30–50%) caused by H5N1 and H7N9 viruses. Moreover, drug-resistant viruses can emerge quickly after specific antiviral treatment with oseltamivir and baloxavir (Hayden et al. 2018; Zhao et al. 2018). Even though the outbreak of SARS-CoV happened in 2003, we still do not have widely available drugs to effectively inhibit coronavirus infection. The COVID-19 has caused more than 0.3 billion infectious cases, which is another reminder of the importance to find anti-coronavirus drugs. Despite the enormous effort from scientists, there is still no promising antiviral for treating SARS-CoV-2. Similarly, rhinovirus, PIV, Adv, RSV, hMPV, and other coronaviruses can cause mild common colds, but also some severe cases or deaths especially in elderlies and those with underlying medical comorbidities (Chemaly et al. 2021; Tang et al. 2017). There is no effective antiviral drug clinically available for treating these viruses.

9.1.3 Risks from Human Activities

Besides the fact that the virulence of respiratory viruses and the lack of antiviral drugs are the two important factors of the risks of respiratory virus diseases, our daily activities also play main roles in spreading respiratory viruses. Most emerging viruses originate from wild animals. They are the reservoirs of most known and novel viruses. There are about half a million unknown viruses that are predicted to be in wildlife reservoirs (Anthony et al. 2013). The jumping of viruses from animals to humans may happen at the human–animal interface by close contact in the wild, farms, market, and game food restaurants. Reducing hunting and eating wild animals is helpful to prevent viral transmission from animals to humans. The non-pharmacological interventions such as wearing masks and washing hands are essential in controlling respiratory viral transmission. However, for some personal or cultural reasons, some populations are reluctant to comply which makes epidemic control difficult.

9.2 Challenges of Broad-Spectrum Antiviral Development

9.2.1 Challenges from the Relative High Mutation Rates of RNA Viruses

Most respiratory viruses are RNA viruses, which have a relative high mutation rate due to the absence of the proofreading of RNA polymerases (Tang et al. 2017) when compared with DNA viruses (Sanjuán et al. 2010). As we mentioned above, the most common and widely used antiviral drugs are anti-influenza drugs including oseltamivir and zanamivir. However, because of the high mutation rates of RNA viruses, even if one or two amino acid mutations occur at the key

binding pockets of the small molecular compounds, antiviral efficacy could be markedly reduced. Thus, the drug-induced viral mutants can quickly emerge during the passages of virus in the presence of drugs (Zhao et al. 2020) and during the treatment for patients (Ikematsu et al. 2020). Especially, when there is a pandemic virus outbreak, such as pandemic 2009 H1N1 and pandemic COVID-19, the mutants emerged more quickly because of the huge numbers of circulating viruses in millions of people. These new mutant viruses may be associated with increased transmissibility during the pandemics (Arora et al. 2021; Hoffmann et al. 2021; Liu et al. 2021).

9.2.2 Challenges from Viruses Without Highly Conserved Proteins

During the evolution of viruses, viruses could gain mutations in key proteins associated with better replication efficiency. There is almost no highly conserved viral protein essential for viral replication. For example, the neuraminidase (NA) inhibitors can inhibit viral release to reduce viral replication, but the mutations located at NA could overcome the antiviral activity of neuraminidase inhibitors (L'Huillier et al. 2015; Zhao et al. 2020). In addition, the entry and release of PIV are mediated by neuraminidase activity of hemagglutinin-neuraminidase (HN) protein. Both influenza virus and PIV can bind to sialic acids for viral attachment. However, PIV is not sensitive to zanamivir with $IC_{50} > 0.25$ mM (Greengard et al. 2000). This finding presents a challenge for developing the broad-spectrum neuraminidase inhibitors. Also, this kind of antiviral drugs may be theoretically suboptimal because neuraminidase inhibitors cannot effectively inhibit viral entry and viral replication. Thus, the infected cells may continue to produce viruses until cell death and then release new virus to infect other cells, which may reduce the antiviral efficiency of the drugs inhibiting viral release. Moreover, once the viral load peaks at 48 h after symptom onset in influenza, these drugs have much lower efficacy which is compatible with the nature of its antiviral mechanism. In addition, RNA polymerases are the key components for RNA viral replication and have been selected as the antiviral targets against many different viruses. However, RNA dependent polymerases are also prone to mutations (Triggle et al. 2021), which often creates obstacles in getting effective RNA polymerase inhibitors with broad-spectrum antiviral activity. The polymerase inhibitors against influenza virus such as baloxavir (Hayden et al. 2018) could induce resistant virus quickly during the clinical trials.

9.2.3 Challenge from Viral Quasispecies

Viral quasispecies is the common characteristics of RNA viruses with high mutation rates and refers to a population of viruses with large numbers of variant genomes. Even in the same infected host, there are multiple viral variants in the viral quasispecies. Recent SARS-CoV-2 studies indicated that SARS-CoV-2 quasispecies was different from one day to the next day in the same patient and different between lower and upper respiratory tracts (Armero et al. 2021; Jary et al. 2020). Intra-host diversity of viruses raises the challenge that specific antivirals may quickly screen out the drug-resistant strains during drug treatment and viral quasispecies in host may speed up the transmission of mutant viruses with high transmission efficiency. Viral quasispecies is also the challenge for discovering the broad-spectrum antivirals because the viral variants may increase the chance of viral escape by mutant quasispecies at the common targets of broad-spectrum antivirals. However, we cannot change the viral quasispecies during the viral infection in host. Thus, the discovery of antiviral acting on multiple viral targets or antiviral combinations acting on different viral targets may be the answer to this important problem.

9.3 The Entry Processes of Respiratory Viruses

The entry process of enveloped virus begins with viral attachment and ends with virus-cell membrane fusion. It is initiated by binding between viral proteins and host receptors. This is the first and important step in the entry process for starting viral infection. Here, we classified viral entries into three types.

First, viral entry is mediated through the endocytic pathway. For example, influenza virus can bind to sialic acid receptors through viral surface glycoprotein HA. After receptor binding, it can trigger the endocytosis pathway. When the virus in the endocytic vesicle is trafficked from the early endosome to the late endosome, the low pH in endosomes triggers the HA conformation change, which will expose the fusion peptide in the C-terminal of HA to insert into endosomal membrane causing membrane fusion for viral RNA release and subsequent replication (Das et al. 2010).

Second, viral entry is mediated by both endocytic pathway and cell surface fusion. For example, coronavirus SARS-CoV-2 could enter cells through spike-ACE2 mediated endocytic pathway and spike-ACE2-TMPRSS2 mediated direct membrane fusion. When SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) receptors of cells without transmembrane protease serine 2 (TMPRSS2) cleavage, virus could enter cells by endocytosis. After undergoing the endosomal trafficking from early endosomes to lysosomes, viral spike protein is activated by the endo-lysosome proteases such as cathepsin L to cleave the S2 cleavage site to expose the fusion peptide (Tang et al. 2021). The fusion peptide can trigger virus-endosome membrane fusion for viral RNA release (Shang et al. 2020). To trigger the spike-mediated fusion, the interaction of fusion peptide of spike of coronavirus with Ca^{2+} is required (Straus et al. 2020). Host cells have increased calcium oscillations with increased activity of the plasma membrane TMEM16 channels, which is required for plasma membrane fusion. The inhibitors of Ca^{2+}-activated TMEM16 family can broadly inhibit spike induced membrane fusion (Braga et al. 2021). When SARS-CoV-2 binds to ACE2 of cells with TMPRSS2 expression, S2 cleavage site in spike protein could be cleaved by TMPRSS2 to expose the fusion peptide and trigger the membrane fusion on cell surface for RNA release and replication (Hörnich et al. 2021), which does not need the S2 cleavage by proteases in endo-lysosomes.

Third, viral entry is only mediated by direct virus-cell membrane fusion. For example, parainfluenza virus binds to sialic acid receptors on cell surface through the membrane glycoprotein hemagglutinin-neuraminidase (HN). Following the receptor binding and engagement, a conformational change in HN activates the fusion protein of PIV to trigger viral membrane fusion with host cell membrane for viral RNA release (Chibanga et al. 2019).

9.4 Broad-Spectrum Entry Inhibitors for Respiratory Viruses

Antiviral drugs can exert antiviral activity by targeting any step in the viral life cycle. Currently there are only antiviral drugs for treating around ten out of more than 260 viruses which are currently known to infect humans (Sorin et al. 2021; Woolhouse et al. 2012). There are a lot of efforts trying to identify specific antiviral drugs mainly for treating influenza virus and coronavirus, but much less so for PIV, rhinovirus, Adv, RSV, and hMPV (Tang et al. 2017). There are challenges for discovering broad-spectrum antivirals (such as different host receptors, entry pathways, enhanced cellular toxicities, and risk of immune deregulation), but there are also many advantages (such as high resistant barrier, broad-spectrum activities for different viruses, and potential effective drugs for novel pathogens before diagnosis) to develop broad-spectrum antivirals (Martinez et al. 2015). Viral attachment is the first step of viral entry. Blocking viral entry can be the effective strategy to stop viral infection. For example, vaccination is the most effective strategy to stop

viral infection and can induce neutralizing antibodies to stop viral infection before viral entry, which can prevent viral replication in the initial step and reduce the competitive pillage from host cellular metabolism and thus can maximumly reduce virulence. However, vaccines are highly strain-dependent without broad-spectrum neutralizing activities. For those using the same host receptor, blocking the receptor binding should be a possible way to develop broad-spectrum viral entry inhibitors. For those using different host receptors, targeting host factors or conserved viral components, which are common elements for viral replication, might be the possible way to develop broad-spectrum antivirals. Here, we will mainly review the possible targets to identify broad-spectrum entry inhibitors for respiratory viruses (Table 9.1).

9.4.1 Targeting Viral Attachment

Influenza virus might be the most familiar respiratory virus to the general public probably due to the global pandemics and annual epidemics. Influenza virus is enveloped, segmented single-stranded, negative-sense RNA virus spiked with multiple copies of hemagglutinin protein (HA), neuraminidase protein (NA), and few M2 ion channel protein. The HA of human influenza viruses binds to α-2, 6-linked sialic acids, which are predominately expressed in the upper respiratory tract in human lungs, to initiate the cell entry of influenza virus (Gambaryan et al. 1997). As far as we know, sialic acids are also the common host receptors for parainfluenza virus, adenovirus, and the co-factors for coronavirus attachment. Parainfluenza virus is enveloped, single-stranded, negative-sense RNA virus. Parainfluenza virus (PIV) is enveloped with hemagglutinin-neuraminidase protein (HN) and fusion protein, which is known to bind to α-2,6-linked sialic acids and also α-2,3-linked sialic acids (Fukushima et al. 2014). Human PIV is the common cause of respiratory diseases in infants, young children, elderly, and immunocompromised individuals. PIV infections may cause more than 20% respiratory tract infection and mortality has been observed in severely immunocompromised population with transplantation (Chemaly et al. 2021). However, currently there is no approved antiviral drug for treating PIV infection. Human adenovirus (Adv) is non-enveloped double-stranded DNA viruses and are classified into more than 100 different serotypes (Greber and Flatt 2019). The Cryo-EM structure of Adv-C capsid showed that Adv is icosahedral which is composed of 12 copies of trimeric hexon. Adv can use a range of receptors and attachment factors for entry with unclear mechanism (Greber and Flatt 2019). Young children are susceptible to Adv infection and normally the infection is self-limiting within 3 weeks. So far, there has been no effective anti-Adv drug available. Studies demonstrated that some types of Advs could use sialic acid residues as the binding receptor for entry (Arnberg et al. 2002). Coronavirus is a positive-sense, single-strained RNA viruses with enveloped proteins including spike, membrane, and envelop proteins. Coronavirus can be divided into four subtypes, in which α-CoVs and β-CoV can infect humans. Because of the COVID-19, antiviral studies on SARS-CoV-2 are speeded up for finding the viral treatment. Previous studies indicated that several β-CoV can interact with sialic acids. However, sialic acids may be not essential for CoV replication (Widjaja et al. 2019).

One of the potentially broad-spectrum antiviral drugs that target sialic acids is DAS181. It is a recombinant neuraminidase analogue which can cleave sialic acid residues on host cells. Theoretically, virus binding to host cells with sialic acids as the receptors will be inhibited by the cellular treatment of DAS181. DAS181 has been identified to show activity against influenza viruses (Chan et al. 2009) and recent clinical trials have shown that inhaled DAS181 is generally tolerated for up to 7 days (Zenilman et al. 2015). Moreover, DAS181 has been shown to effectively inhibit PIV infection in vitro and in vivo (Moscona et al. 2010). Phase II clinical trial showed the antiviral efficacy of DAS181 in immunocompromised patients with PIV infection and the therapeutic efficacy in patients who underwent hematopoietic stem cell

Table 9.1 Broad-spectrum antivirals blocking respiratory virus entry

Antivirals	Targeting respiratory viruses	Mechanism on blocking viral entry
Arbidol	CoV and influenza virus	Block HA conformation changes and endosomal components affecting viral fusion (Kadam and Wilson 2017; Wang et al. 2020b; Zhao et al. 2021)
Cyanovirin-N	Influenza and PIV	Bind to viral glycoproteins to inhibit viral entry (Martinez et al. 2015; O'Keefe et al. 2003)
Chloroquine	CoV and influenza virus	Increase endosomal pH to block viral fusion (Rebeaud and Zores 2020; Zhao et al. 2021)
DAS181	Influenza virus and PIV	Cleave sialic acids to block viral attachment and entry (Chan et al. 2009; Moscona et al. 2010)
Human neutrophil peptide 1	Adv and influenza virus	Multifunctional defensin affects endosomal release and viral aggregation (Smith and Nemerow 2008; Tripathi et al. 2013)
Human defensin 5	Adv and CoV	Block viral attachment and virus-endosomal fusion (Smith and Nemerow 2008; Wang et al. 2020a)
Lipopeptide	CoV and influenza virus	Block conformation change of viral fusion protein (Wang et al. 2018)
LJ001	CoV and influenza virus	Block fusion by affecting membrane flexibility (Vigant et al. 2013; Wolf et al. 2010)
MDL28170	CoV, Hendra virus, and Nipha virus	Cysteine protease inhibitor blocks viral protein cleavage activation (Simmons et al. 2005; Zhou and Simmons 2012)
Mucroporin-M1	CoV and influenza virus	Virucidal peptide disrupts viral membrane (Li et al. 2011)
Niclosamide	Adv, CoV, rhinovirus, and RSV	Multifunctional drug blocks fusion and endosomal acidification (Braga et al. 2021; Niyomdecha et al. 2021; Xu et al. 2020)
P9, P9R, 8P9R	CoV, influenza virus, and rhinovirus	Multifunctional peptides block fusion by clustering viral particles and inhibiting endosomal acidification (Zhao et al. 2021, 2020, 2016)
Tilorone	CoV and influenza virus	The lysosomotropic activity may affect viral entry (Ekins et al. 2020; Puhl et al. 2021)
Tyrphostin A9	Influenza virus, Sendai virus, and murine CoV	Kinase inhibitor blocks Clathrin-mediated viral entry (Kumar et al. 2011; Mazzon et al. 2019)

transplantation (Chemaly et al. 2021). Recently, a phase III clinical trial has recruited participants for evaluating the therapeutic treatment efficacy on human PIV infections in immunocompromised patients (NCT04298060), which also has plan to recruit COVID-19 patients for testing the therapeutic efficacy of DAS181. Because of these developments of DAS181, FDA has designated DAS181 as both a fast-track and breakthrough therapy (Heida et al. 2021). The cleavage of common viral receptors of sialic acids may be a promising strategy with broad-spectrum antiviral activities against different viruses using sialic acids as the receptor or attachment factor. The inhaled DAS181 was thought to minimize the adverse systemic side effects because of the topical administration in lungs (Heida et al. 2021). However, it is uncertain whether the removal of sialic acids from the respiratory epithelial cells will induce unexpected side effects (Heida et al. 2021).

Except for the neuraminidase cleaving sialic acids to block viral binding, the sialic acid analogues could mimic the cellular receptor to bind to viral attaching proteins, which could also be the promising broad-spectrum antiviral candidates to block viral infection when viruses use sialic acids as the receptors. Synthetic sialic acid analogues for blocking viral attachment have been tested to inhibit influenza viruses with limited success (Heida et al. 2021) while the sialic acid analogues (zanamivir and oseltamivir) preventing sialic acid cleavage by neuraminidase to stop viral release have been widely used as clinical drugs for treating influenza infection. At the same time, they do not interfere viral attachment/entry and have low barrier to generate resistance. Synthetic compounds with 6′-sialyl-*N*-

acetyllactosamine could effectively inhibit influenza viruses and sialic acid nanoparticles could also effectively inhibit influenza virus infection in vitro and in vivo (Gambaryan et al. 2005). Although the sialic acid analogues are theoretically worked as the virus binding inhibitor, so far there have been few studies to show the promising results, especially in vivo.

Glycosaminoglycans (GAGs) are expressed on almost all mammalian cell surfaces for the interactions between cell–cell and host–pathogens. They are long, linear, and negatively charged polysaccharides. GAGs can bind to various biological molecules such as growth factors, proteinases, and pathogens, which play major roles during physiological interactions. For viruses, such as RSV, Adv, SARS-CoV-2, rhinovirus, and other viruses (Kamhi et al. 2013; Sorin et al. 2021), the attachment to host cells can be affected by the interaction with cell surface GAGs (Zhou and Simmons 2012). Small molecule antagonists of GAGs or peptides which bind to GAGs showed broad-spectrum antiviral activity by reducing viral entry (Clausen et al. 2020; Donalisio et al. 2010; Krepstakies et al. 2012; Schuksz et al. 2008). Most recently, heparin and enoxaparin derivatives were demonstrated to effectively inhibit SARS-CoV-2 entry (Clausen et al. 2020; Tandon et al. 2020). Cellular interactions related to GAGs might affect cell proliferation, motility, and differentiation with multiple functions (Schuksz et al. 2008). Thus, the off-target interactions of GAGs inhibitors and antagonists on host cells might also cause the systemic side effects. More studies will be needed to evaluate the pros and cons of this kind of host targeting broad-spectrum antivirals.

Host defensins have been identified to show broad-spectrum antiviral activities against respiratory and non-respiratory viruses (Brice and Diamond 2020). Human α defensin 5 (HD5) showed broad-spectrum antiviral activity against SARS-CoV-2 by binding to ACE2 to block spike-based pseudovirus entry (Wang et al. 2020a). Also, HD5 could stabilize adenovirus capsid to block viral uncoating and virus-mediated endosome penetration, resulting in virion accumulation in endosomes (Smith and Nemerow 2008). The antiviral peptide of human neutrophil defensin, namely human neutrophil peptide 1 (HNP1) showed very broad-spectrum antiviral activities (Brice and Diamond 2020). HNP1 could prevent Adv-mediated endosomolysis (Smith and Nemerow 2008) and inhibit influenza virus by causing viral aggregation (Tripathi et al. 2013). The broad-spectrum antiviral activities of host defensins with divergent antiviral mechanism against different viruses implicated that more studies are needed to investigate the antiviral mechanism against different viruses.

9.4.2 Conformation Change Inhibitors

After binding to host receptors, viral binding proteins require conformational changes to trigger the virus-cell membrane fusion. The conformational changes of viral proteins might need the rearrangement of intramolecular disulfide bonds (Vigant et al. 2015). Such rearrangement of disulfide bonds depends on the thiol-disulfide exchanges catalyzed by protein disulfide isomerase (PDI). The cell surface protein disulfide isomerase A1 (PDIA1) had been shown to affect the viral entry of HIV when cells were treated by PDIA1-specific monoclonal antibodies (Elahi et al. 2012). Nitazoxanide, proposed as a potential PDI inhibitor, showed broadly antiviral activity in vitro against influenza virus, PIV, RSV, CoV, HIV, and other viruses with unclear mechanism (Rossignol 2014). The relationship between the antiviral activity of nitazoxanide and PDI inhibitor warrants more studies. The multiple targets of nitazoxanide make it more complicated when used as the antiviral drugs in humans (Lokhande and Devarajan 2021). In addition, viral membrane glycoproteins need the activation by cysteine protease cathepsin B or cathepsin L for inducing membrane fusion. Inhibitors of these proteases showed broad-spectrum antiviral activities against viral entry of coronavirus, Hendra virus and Nipha virus (Zhou and Simmons 2012). Recently, studies indicated that Ca^{2+} played important roles in SARS-CoV-2 and Ebola virus fusion. Drugs, including Niclosamide and

Nitazoxanide which block Ca^{2+} release, could effectively inhibit cell–cell fusion triggered by viral glycoprotein conformation changes (Braga et al. 2021; Das et al. 2020; Nathan et al. 2020), which showed broad antiviral activities against influenza virus, CoV, Adv, and RSV (Niyomdecha et al. 2021; Xu et al. 2020). Thus, this kind of drugs affecting Ca^{2+} flow in cells might have broad-spectrum antiviral activities, which will also need to evaluate the systemic side effects because Ca^{2+} is an essential ion for keeping normal cellular environment and signal transduction.

Small molecules directly binding to viral proteins might be another way to alter the viral protein conformation changes. The broad-spectrum antiviral Arbidol is known for treating flu in China and Russia. Binding structure analysis between HA and Arbidol indicated that it could stabilize HA by binding and block HA conformation change induced by low pH (Kadam and Wilson 2017). Aribidol has been shown to have broad antiviral activities inhibiting other pH-dependent viruses, including CoV and Ebola (Hulseberg et al. 2019; Zhao et al. 2021). Recent studies indicated that it could trap SARS-CoV-2 in endo-lysosomes, which was probably due to the blocking of membrane fusion (Wang et al. 2020b; Zhao et al. 2021). However, the broad-spectrum antiviral activities in vitro have not been observed in animals and clinical studies. The low serum concentration of Arbidol might be one of the possible reasons of lacking effective antiviral activity in vivo.

Nitazoxanide, Niclosamide, and Arbidol with broad-spectrum antiviral activities are promising candidates for immediate use in clinical trials for treating emerging viral infectious diseases because of their good bioavailability with a safe record of oral administration in patients. Nitazoxanide has been approved by FDA for the treatment of Giardia lamblia. Niclosamide has been approved for treating tapeworm infection (Kappagoda et al. 2011). Arbidol has been approved for therapeutic use in Russia and China for treating influenza virus. The broad-spectrum antiviral activities for other viruses will need further validation in vitro and in vivo. Studies to optimize the chemical structure of Arbidol and drug delivery methods might be a possible way to increase the antiviral efficiency in vivo for treating respiratory viruses.

9.4.3 Fusion Peptide Inhibitors

There are supposed to have three classes of fusions between virus and host cell membranes (Vigant et al. 2015). The activation of the conformation changes of class I fusion proteins can be pH dependent, pH independent, or both. Class II fusion proteins have a tightly folded fusion loop located at the Doman II of three-domain architecture (DI, DII, and DIII) for membrane fusion in the representative virus (Dengue virus). For class III fusion proteins, the α-helical domains and β-sheet domains with fusion loops are responsible for membrane fusion in the representative virus (Vesicular stomatitis virus, VSV). Both Class II and Class III fusions are pH dependent. Respiratory influenza virus belongs to the class I fusion and needs low pH to induce conformational change of HA. SARS-CoV, SARS-CoV-2, MERS-CoV, and HIV also belong to the class I fusion family but are pH independent (Wang et al. 2018). These viruses will form a coiled-coil six-helix bundle (6HB) structure in the post-fusion conformation. The six-helix bundles are considered as the broad-spectrum antiviral targets. Previous studies showed that short peptides targeting 6HB of HIV (Jiang et al. 1993) and MERS-CoV (Lu et al. 2014) could effectively inhibit viral fusion and entry. With the modification on C-terminal heptad repeat (CHR), the α-helical lipopeptide IIQ binding to N-terminal heptad repeat (NHR) showed broad antiviral activities against influenza virus and coronavirus by blocking viral entry (Wang et al. 2018). RVFV-6, derived from the membrane-proximal stem region of rift valley fever virus, inhibited not only RVFV (Class II) but also Ebola (Class I) and VSV (Class III) (Koehler et al. 2013), in which the underling mechanisms are needed to study further. These studies showed encouraging ways that targeting the fusion proteins of viruses might be a promising strategy

to develop broad-spectrum antivirals against different viral families.

9.4.4 Targeting Membrane Components

Enveloped virus membranes have similar components with host cell membranes. The lipid components of membranes are essential for virus-cell membrane fusion. The broad-spectrum antiviral peptide C5A derived from the nonstructural protein C5A of HCV (Cheng et al. 2008; Zhang et al. 2013) and mastoparan-derived peptide MP7 (Sample et al. 2013) could interact with membrane lipids to disrupt enveloped viruses, including RSV, HCV, and other viruses. The host defensin peptide from scorpion venom, mucroporin-M1, could disrupt viral membrane against SARS-CoV and H5N1 virus (Li et al. 2011). The lack of a cohesive mechanism of antiviral activity of these virucidal peptides warrants more studies for drug development. Membrane fluidity is another crucial parameter for virus-cell membrane fusion, which is affected by the cholesterol (Vigant et al. 2015). Reducing the amounts of cholesterol in virus or cell membrane by polyunsaturated ER-targeting liposomes (PERLs) could inhibit viral entry (Pollock et al. 2010). Rigid amphipathic fusion inhibitors (RAFIs) have been showed with broad-spectrum, irreversible antiviral activities against enveloped viruses, such as influenza virus, HCV, and so on (St Vincent et al. 2010). The exact mechanism, spectrum of activity, and side effects of PERLs and RAFIs on viruses and host cells will need further investigation.

One of the factors affecting the intactness of virus and cell membranes is the lipid oxidation. There are differences between enveloped virus membrane and host cell membrane, especially the repair capability. Mammalian cells are able to repair and replenish the lipid bilayer but not the viruses (Holthuis and Levine 2005). For example, cells can repair small or large plasma membrane lesions via exocytosis or self-sealing lipid repair. However, viruses do not have the cellular organelles to repair membrane damage to make the membrane intact. According to these differences, small molecule LJ001, the thiazolidine derivatives, showed broad antiviral activity against 11 different families of enveloped viruses, including influenza virus, coronavirus, etc. (Vigant et al. 2013; Wolf et al. 2010). LJ001 might function on viral membrane to affect virus-cell fusion without disrupt viral particles. LJ001 was supposed to effectively inhibit virus-cell membrane fusion but not cell–cell membrane fusion, which was because enveloped viruses do not have the bio-reparative capacity (Wolf et al. 2010). Recently, LJ001 was also identified to inhibit viral replication after viral entry for coronavirus (Zhang et al. 2020) but LJ001 needs light to activate and has a short half-life time, which might limit its application in vivo (Vigant et al. 2013). New compounds overcoming these limitations (Hollmann et al. 2014) and more studies will need to evaluate the underlying mechanisms of inhibiting different enveloped viruses without severe side effects. In addition, phospholipid specific antibodies might be the promising broad-spectrum antivirals. Bavituximab is an immunoglobulin G3 monoclonal antibody targeting the anionic phospholipid phosphatidylserine. It has been proved to be effective for murine cytomegalovirus, VSV and is in Phase II clinical trials for chronic HCV and advanced hepatocellular carcinoma (Mokdad et al. 2019; Soares et al. 2008; Vigant et al. 2015). Its effect on the respiratory viruses warrants further studies.

9.4.5 Targeting Endocytic Pathway

Majority of viruses bind to cell surface receptors for entry through endocytic pathway followed by viral RNA release for viral replication after fusion with endo-lysosome membrane. The receptor-mediated endocytosis (named as clathrin-mediated endocytosis), caveolar-dependent endocytosis, and micropinocytosis exerts the function of viral internalization (Mercer et al. 2010). Thus, inhibitors of endosomal trafficking or acidification are well known antivirals which can broadly inhibit viral entry through the

endocytic pathway, such as cytochalasin D, micropinocytosis Pak1, and protein kinase C (PKC) inhibitors (Hoffmann et al. 2008; Saeed et al. 2010). The trafficking inhibitor (aryl semicarbazone EGA) which interferes with the movement from early endosomes to later endosomes was investigated for antiviral development (Gillespie et al. 2013). In addition, ABMA might serve as a broad-spectrum antiviral against pH-dependent viruses by hampering the late endosomal trafficking (Wu et al. 2020). The pH inhibitors, NH_4Cl and bafilomycin A1, are known to block endosomal acidification to suppress pH-dependent viral replication. NH_4Cl is a weak base that increases endosomal pH in cells. Bafilomycin A1 is the inhibitor of vacuolar-type H^+-ATPase and nonspecifically inhibits H^+-ATPase in mammalian cells. Although the broad-spectrum and potent antiviral activities of these endocytosis and endosomal acidification inhibitors were shown in vitro, they are not regarded as effective therapeutics because of the off-targeted side effects which possibly block the essential cellular metabolizing pathway of endocytosis. Further studies will be needed to find the balance between targeting viruses and host cells.

Another antiviral candidate, chloroquine, is a weak base compound that was shown to inhibit viruses such as influenza virus and coronavirus in some studies (Rebeaud and Zores 2020). Similar to bafilomycin A1 and NH_4Cl, chloroquine nonspecifically increases the pH in endosomes. Even though chloroquine has been used as anti-malaria drug for many years with good tolerance in humans and animals, the in vivo and clinical studies indicated that it did not exhibit clear benefits in influenza virus, SARS-CoV-2 and Ebola treatment (Falzarano et al. 2015; Paton et al. 2011; Yan et al. 2013; Zhao et al. 2021). The reasons for low antiviral efficacy of chloroquine in vivo are not clear and need more studies to identify. The quick accumulation of chloroquine in endosomes and the very low cytosolic concentrations of chloroquine might not provide the effect environment to inhibit the new entry virus in new endosomes (Kaptein et al. 2020), in which there is no effective chloroquine to prevent endosomal acidification. The other possible explanation might be the low efficiency developed in respiratory tracts for inhibiting SARS-CoV-2 and influenza virus while chloroquine was administrated orally. Topical administration methods such as nasal spray may be considered to improve the efficacy of chloroquine as treatment of SARS-CoV-2 or influenza virus infection. Whether an inhibitor affecting cellular endocytic activities could show appropriate therapeutic index in humans remains to be determined.

As mentioned above, off-targeted side effects of host-target antivirals are the core problem for endosomal acidification inhibitors. Blocking virus-endosome acidification without significant effects on the normal cell metabolism is the important issue for drug development. Moreover, Bafilomycin A1 could inhibit autophagy and induce apoptosis which are generally irreversible (Weisz 2003). Chloroquine has a long half-life time in vivo and can exist for more than 1 month (Karunajeewa et al. 2010; Moore et al. 2011). Thus, the short half-life time of these host-target antivirals might be the advantage but not the disadvantage when using them to treat respiratory viruses with limited duration of infection. Besides these known antivirals with endosomal acidification inhibition activity, discovering new compounds with different mechanism to interfere endocytic pathway might be another way for development drugs with less harm to cellular pathways. For instance, broad-spectrum antiviral peptides (P9 and P9R), developed from mouse-beta defensin, could broadly inhibit respiratory virus replication (including influenza virus, coronavirus, and rhinovirus) by binding to viral particles and inhibiting endosomal acidification. Similarly, antiviral peptide EV37 could restrict viral entry by alkalizing acidic organelles against HCV, dengue virus, and other pH-dependent viruses (Li et al. 2019). The viral binding of P9R and its inhibition on virus-endosome acidification increase the antiviral efficiency and reduce the cytotoxicity of non-specific inhibiting endosomal acidification. Moreover, based on the broadly binding activity to different viral particles, the developed 8P9R from P9R could cluster SARS-CoV-2 particles thus stop the viral

entry through endocytic pathway in Vero E6 cells and the viral entry through membrane fusion pathway in Calu-3 cells. The antiviral activity of 8P9R is shown to be more potent than that of P9R, which is probably due to its dual functions of clustering viral particles and inhibiting endosomal acidification. The antiviral mechanism of binding activities to viruses, the broad-spectrum antiviral efficiency, and potential side effects in vivo will be studied in detail for new drug development.

9.5 Future of Broad-Spectrum Entry Inhibitors for Respiratory Viruses

From the initial isolation of virus in 1890s (Woolhouse et al. 2012), more than 260 human-infective viruses have been identified (Sorin et al. 2021) and there will be more and more new viruses to be identified from human viral infectious diseases. We currently have limited antiviral drugs for treating few viral infectious diseases (Chaudhuri et al. 2018). Discovery of broad-spectrum antivirals against viral entry will be a critical and promising strategy to control viral infection diseases, which will provide the advantages of high barriers to drug resistance and broad antiviral activity against different viruses and novel viral pathogens before diagnosis. Insights from following the knowledges of current virology and not following the current knowledges are the two possible ways to discover novel broad-spectrum antivirals.

First, following the available knowledges, as mentioned in above sections, to discover the broad-spectrum antivirals by drug repurposing and blocking the common entry processes used by different viral families should be a wise strategy, even though we have not found the suitable clinically approved drug to broadly inhibit viral entry of different viruses. With further studies of antivirals targeting host factors affecting viral binding and internalization, antivirals targeting viral glycoproteins affecting conformation changes and the side effects of host targeting antivirals, it would be likely to identify the broad-spectrum entry inhibitors in coming future.

Second, there are huge numbers of unknown in our world, especially the functions of biomolecules in human bodies and in other species. If we try to discover new broad-spectrum antivirals through screening natural materials or isolates from species which have high resistance to viral infection, we may identify broad-spectrum entry inhibitors with the widely used pesudovirus entry assay not requiring biosafety level 3 containment.

Third, for studying antivirals against respiratory viruses, we should also pay attention to the route of administration. When drugs are delivered by systemic routes, like oral or intravenous injection, achieving the effective concentration in lungs to inhibit viral replication will require high systemic doses which may cause side effects for patients. Most respiratory virus infections mainly cause airway damages. Although severe coronavirus infection, like SARS-CoV-2, raises multiple organ infections, lung failure is the major cause of deaths. Thus, for some drugs against respiratory viruses, it would be better to test the antiviral efficacy when drugs are administrated via inhalation (Heida et al. 2021), which is likely to be the optimal delivery route because inhalation can increase the local antiviral efficiency and reduce the systemic side effects when compared with systemic drug administration.

9.6 Outlook

Respiratory viruses, such as SARS-CoV-2 causing COVID-19 and pandemic influenza virus 2009, can spread to the whole word in a very short time. It is hard to predict and effectively prevent the transmission of the new emerging viruses to humans. Many respiratory viruses are RNA viruses with many subgroups. The high rates of mutation and genetic recombination provide RNA viruses with many opportunities to acquire important site mutations that increase their fitness in humans and might also increase the possibility of emergence of novel viruses and resistance to drugs or antibodies. Without rapid

diagnosis and broad-spectrum antivirals, a chaotic period in response to the novel viruses will lead to social panic and medical burden, which has been clearly evidenced by COVID-19 and SARS-CoV outbreak in 2003. With the development of biological and telecommunication technologies in the world, we can respond to the novel emerging viruses very quickly after diagnosis. It is now time for us to pay more attention to discover effective, safe and affordable antivirals with broad-spectrum antiviral activities which can be used in various clinical settings to reduce morbidities and mortalities.

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Ebola Virus Entry Inhibitors

10

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Abstract

Ebola virus (EBOV) is one of the most deadliest agents already known, causing periodic epidemic of a severe hemorrhagic fever disease in Africa. Although two monoclonal antibody (mAb) drugs have recently received approval in the USA, additional therapeutics are still needed to combat potential outbreaks of resistance variants and other closely related ebola viruses. In this chapter, we describe the current understanding of the EBOV entry process and summarize the approaches, strategies, and advances in discovery and development of EBOV entry inhibitors, including therapeutic antibodies, peptides, small molecules, natural products, and other chemical structures.

Keywords

Ebola virus · Entry inhibitor · Therapeutic mAb · Drug repurposing · Small molecule

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10.1 Ebola Virus Entry and Potential Drug Targets

Filoviruses from the *Filoviridae* family are enveloped, single-stranded, negative-sense RNA viruses that exhibit a unique heterogeneous filamentous structure (Feldmann et al. 2020). To date, there are 12 filoviruses that can be classified into six established genera, among which the genus *Ebola virus* and *Marburg virus* are the best-known and extensively studied. For example, four ebola viruses (Ebola virus, EBOV; Sudan virus, SUDV; Bundibugyo virus, BDBV; and Taï Forest virus, TAFV) and two marburg viruses (Marburg virus, MARV; and Ravn virus, RAVV) have been identified as etiological agents of severe human hemorrhagic fever diseases, called Ebola virus disease (EVD) and Marburg virus disease (MVD), respectively (Kuhn et al. 2019). Both EVD and MVD are associated with high fatality rates ranging from 40% to 90% (Coltart et al. 2017; Feldmann and Geisbert 2011). In particular, EBOV associated EVD outbreaks have occurred 10 times in history, including the 2013–2016 West Africa epidemic, which resulted in 28,646 reported infections and 11,323 deaths (Coltart et al. 2017). In 2018, the World Health Organization listed EVD and MVD as priority diseases, given their potential to cause a public health emergency and the lack of efficacious drugs and vaccines. Although two monoclonal antibody (mAb) drugs REGN–EB3 and Ansuvimab have received approval for treatment

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of EBOV infection in late 2020 in the USA (Lee 2021; Markham 2021), there is still an urgent need to develop novel antivirals to combat infections of EBOV, as well as other highly pathogenic filoviruses.

As the first step during virus infection, virus entry is an extremely attractive therapeutic intervention point and it is expected that the accumulated knowledge of the entry process may greatly accelerate the research and development of virus entry inhibitors. The entry of filoviruses is a complicated, multi-step process, which is comprehensively orchestrated by both viral and host factors, providing a variety of promising drug targets. Notably, knowledge of filoviruses entry is largely based on studies of EBOV, moreover, most antiviral studies focused on EBOV, because this virus causes epidemics periodically and it is the most concerning public health issue. In this chapter, we discuss advances in the development of EBOV entry inhibitors, as well as potential pan-ebola virus/filovirus inhibitors.

10.1.1 A Brief Description of EBOV Entry

EBOV gains entry into host cells by initial binding of viral surface glycoproteins (GPs) to cell surface receptors, which may be nonspecific and possibly comprise numerous glycosylated proteins, including the folate receptor-alpha (Chan et al. 2001), C-type lectins (Alvarez et al. 2002), integrins β1 and/or αV (Dahlmann et al. 2015; Takada et al. 2000), Tyro3, Axl, Mer (TAM) family tyrosine kinase receptors (Dahlmann et al. 2015; Shimojima et al. 2006), glycosaminoglycans (O'Hearn et al. 2015), T-cell immunoglobulin mucin (TIM) family proteins (Kondratowicz et al. 2011; Rhein et al. 2016), and sialic acid-binding Ig-like lectin 1 (Siglec-1) (Perez-Zsolt et al. 2019).

After binding, the virus enters the cell via macropinocytosis, which is a nonselective process of engulfment, and subsequently trafficks through early and late endosomes (Mulherkar et al. 2011; Saeed et al. 2010). During the maturation of endosomal vesicles into late endosomes, activation of endosomal cysteine proteases Cathpsin L and B occurs in the increasingly acidic and reducing environment. These cathepsins sequentially process GP into a smaller, fusion competent form (Chandran et al. 2005; Schornberg et al. 2006).

Unlike many other enveloped viruses, acidification and protease cleavage are not sufficient for inducing EBOV GP mediated membrane fusion, a process required for the virus to release genetic materials. Strikingly, an intracellular receptor Niemann-Pick C1 (NPC1) is required (Carette et al. 2011; Côté et al. 2011). Upon NPC1 binding, the smaller form of GP continues to undergo a conformational change that mediates fusion between viral and endosomal membranes. In addition to NPC1, Ca^{2+} is also a critical factor for the GP mediated fusion process (Das et al. 2020).

10.1.2 EBOV GP and GP Mediated Fusion Events

EBOV GP mediates both receptor binding and membrane fusion during the entry process. Mature GP is a typical class I fusion protein, composed of dimerized subunits GP_1 and GP_2, with $GP_{1,2}$ existing as trimers on the surface of virions (Pallesen et al. 2016). GP_1 is composed of three distinct domains: the receptor binding domain (RBD), the glycan cap, and a heavily *O*-linked glycosylated mucin-like domain, while the membrane-anchored GP_2 contains the fusion machinery: a fusion loop near the amino terminus followed by a N-terminal heptad repeat region (HR1), a C-terminal heptad repeat region (HR2), a transmembrane region and a short cytoplasmic tail (Pallesen et al. 2016). The glycan cap of GP_1 is extensively N-linked glycosylated and interacts with two regions of GP_2, including the internal fusion loop (Lee et al. 2021). As the fusion loop is critical for GP_2-mediated membrane fusion, the glycan cap-GP_2 interaction protects the fusion peptide from premature fusion events. No evidence has suggested that the mucin-like domain is obligated for virus entry, although it may shield

key residues of the RBD from immune recognition (Lee et al. 2021). In addition, the mucin-like domain causes cell cytotoxicity in vitro and tissue damage in animals, suggesting a critical role in EBOV pathogenicity (Ning et al. 2018).

As described above, after virus uptake into host cells, $GP_{1,2}$ undergoes proteolytic processing, generating a smaller $GP_{1,2}$ that lacks both the glycan cap and mucin domain of GP_1 (Chandran et al. 2005). The N-terminal fusion loop was consequently exposed to the acidic environment within endosomes, undergoing a change from the original flattened extended loop to a more hook-like structure with subsequent insertion of the fusion loop to endosomal membranes (Gregory et al. 2011). Upon NPC1 binding, a collapse of the highly ordered, alpha-helical structures HR1 and HR2 is triggered, forming a six-helix bundle containing three HR1 and HR2 domains. This conformational rearrangement into a coiled-coil structure draws the viral and endosomal membranes into close proximity, thereby allowing partial fusion (hemifusion) and eventual formation of a pore (Salata et al. 2019), through which the viral RNA and its associated proteins can be released into the host cytoplasm, where the viral life cycle continues.

10.1.3 Unresolved Puzzles of EBOV Entry

Although the means by which EBOV enters a host cell have been becoming less mysterious, a series of puzzles remain to be unraveled. First, since diverse cell surface proteins have been identified to facilitate EBOV entry, which may partially contribute to the broad tropism of EBOV for a variety of different cell types, one question remains about whether there is a definitive cell surface "entry" receptor for EBOV to directly mediate virus internalization (Falzarano and Feldmann 2015). This is very important since specific receptors often act like the "door" for a virus to enter host cells, e.g., sialic acid for influenza virus (Du et al. 2019), and ACE2 for SARS-CoV and SARS-CoV-2 (Chen et al. 2021). Upon closing the door, virus entry can be subsequently halted. Interestingly, one study demonstrated that EBOV entry is an asynchronous process, and the cell-bound state of the virus can stay for an extended period of time before internalization occurs (Reynard and Volchkov 2015). This is unusual for viruses, and combined with the fact that EBOV is internalized by a nonselective macropinocytosis, it seems unlikely for EBOV to adopt a specific cell surface "entry" receptor.

Instead, EBOV recognizes a unique intracellular receptor, NPC1, at the late stage of entry in endosomes/lysosomes, in contrast to the more frequent observation that virus receptors act at early stages on the cell surface (Carette et al. 2011; Côté et al. 2011). NPC1 is primarily involved in the architecture and trafficking of endosomal/lysosomal compartments, and plays an essential role in cholesterol absorption and homeostasis. Although it has been clearly demonstrated that binding of NPC1 to the fusion competent form of $GP_{1,2}$ is necessary and that the cholesterol transport activity of NPC1 is not critical for EBOV entry (Côté et al. 2011), details about the GP-NCP1 interaction and mechanism of membrane fusion are missing. On the other hand, a recent report suggested that cholesterol in EBOV GP-containing membranes enhances fusion (Lee et al. 2021), emphasizing a potential role of cholesterol during EBOV entry. Future studies exploring the cholesterol homeostasis and changes in lipid composition within the endosomal pathway may further improve our understanding of the role of NPC1 in EBOV entry.

Moreover, it was reported that EBOV entry into host cells also requires the endosomal calcium channels called two pore channels (TPCs), and the authors observed that cellular $TPC2^+/NPC1^-$ compartments could allow EBOV GP mediated fusion (Sakurai et al. 2015). Therefore it was argued that NPC1 may be not required for fusion itself (Falzarano and Feldmann 2015). Controversially, a recent study demonstrated that lower pH, Ca^{2+}, and NPC1 binding is required and sufficient to induce conformational changes in the EBOV fusion machine using a virus-liposome lipid mixing model (Das et al.

2020). It will be important to elucidate this discrepancy in future.

TPCs are triggered by nicotinic acid adenine dinucleotide phosphate (NAADP) to release Ca^{2+} from endosomes and lysosomes (Sakurai et al. 2015). It is not known whether TPCs act directly or by maintaining the Ca^{2+} homeostasis within endosomes/lysosomes. If the latter is true, it is interesting to further investigate how TPCs induced Ca^{2+} equilibrium contributes to the EBOV GP mediated fusion process.

10.1.4 Potential Therapeutic Targets During EBOV Entry

The complex set of entry events described above provides numerous promising targets for the development of EBOV entry inhibitors. These potential avenues include: (1) interfering with attachment to permissive cells by blocking interactions between EBOV GP and attachment factors on cell surface; (2) preventing virus internalization by blocking macropinocytosis; (3) interfering with the host cathepsins activities; (4) inhibiting the EBOV GP-NPC1 interaction directly or inducing NPC1 phenotypes that disrupt viral entry; (5) disrupting Ca^{2+} homeostasis in endosomes/lysosomes; (6) blocking conformational changes of GP_2, particularly, blocking the formation of the six-bundle helix.

Recently, an increasing number of host factors have been shown to play key roles during EBOV entry, providing additional potential targets for the development of antiviral therapies against EBOV infection. For example, using a genome-wide CRISPR screen, Flint et al. identified *N*-acetylglucosamine-1-phosphate transferase (GNPTAB) to be important for EBOV infection. Since GNPTAB is required for the correct intracellular localization of lysosomal proteins such as cathepsins, disruption of GNPTAB function may represent another strategy for a host-targeted therapy for EBOV (Flint et al. 2019). The potential antiviral host targets also include the phosphoinositide-3 kinase-Akt (PI3k) pathway, which mediates virus uptake via endocytosis (Saeed et al. 2008), the homotypic fusion and protein sorting (HOPS) tethering complex as well as its regulator, UV radiation resistance-associated gene (UVRAG), which facilitates trafficking of EBOV to $NPC1^+$ cellular compartments (Bo et al. 2020), and so on.

The model of EBOV entry and avenues for targeted antiviral development are summarized in Fig. 10.1.

10.2 Established EBOV Models for Entry Inhibitor Development

Virus models provide important tools for antiviral drug discovery and development, and a simple and robust assay can significantly facilitate antiviral research. Considering EBOV is a biosafety level 4 (BSL-4) agent and can only be handled in laboratories with BSL-4 containment, only a few scientific institutions can conduct basic virology and antiviral research using the authentic EBOV, since the number of BSL-4 laboratories worldwide is limited. This has been one of the major challenges for studies of EBOV as well as other BSL-4 pathogens.

To solve this issue, several "surrogate" systems that mimic the entry process of authentic EBOV were developed as alternatives, including EBOV GP-based pseudotyping systems (Rumschlag-Booms et al. 2011), a recombinant vesicular stomatitis virus (rVSV) encoding EBOV GP in place of the VSV glycoprotein G (Wong et al. 2010), and Ebola virus-like particles (eVLPs) (Martinez et al. 2010). As these systems are safe and can be handled in BSL-2 conditions, dissecting of EBOV entry steps and discovery of entry inhibitors have been greatly accelerated.

10.2.1 EBOV GP-Based Pseudotyping System

Unlike authentic viruses, a pseudovirus carries a core or backbone of one vector virus but incorporates surface proteins derived from another virus. The genome inside a pseudovirus is usually modified to abolish native surface

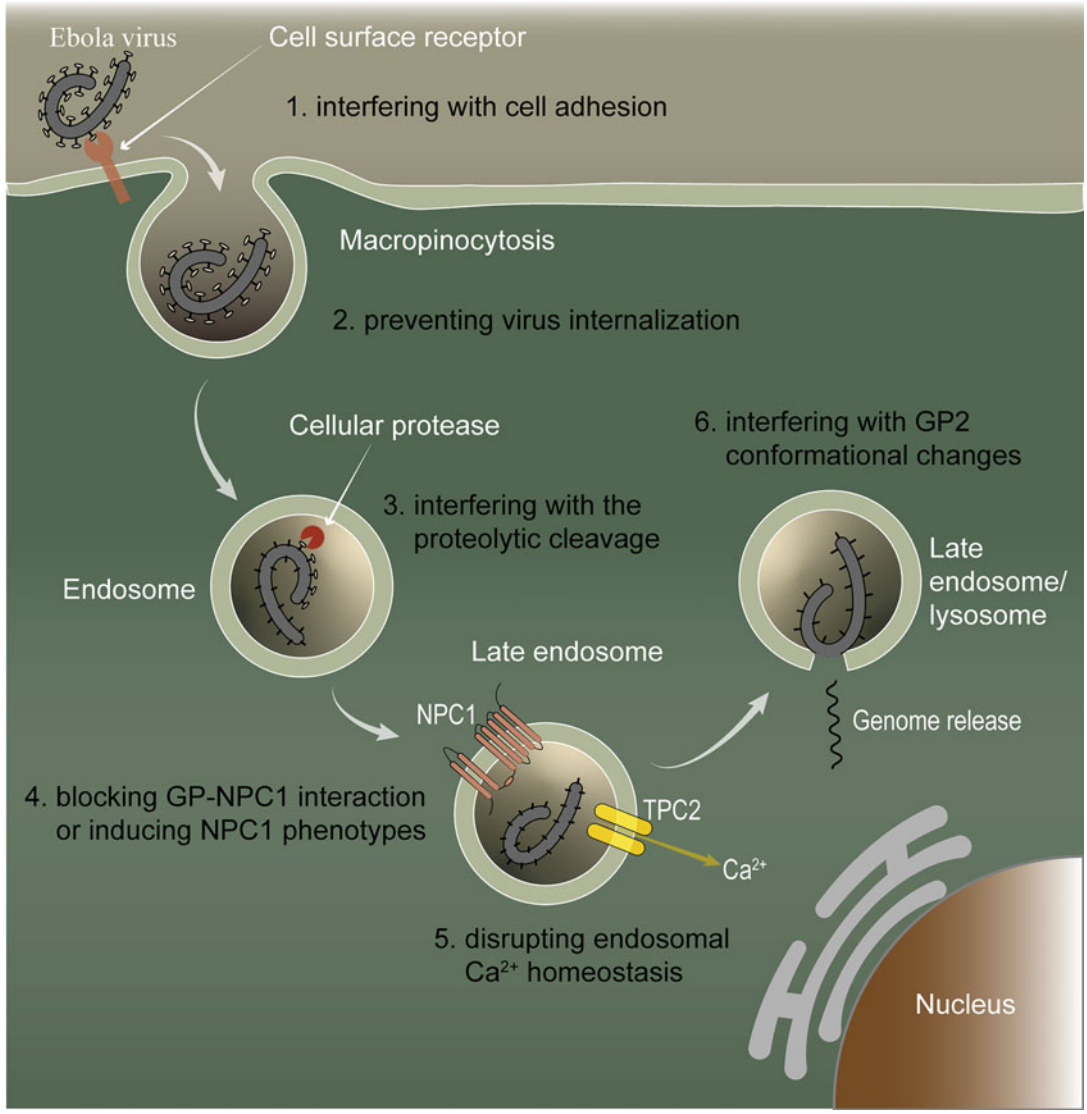

Fig. 10.1 Ebola virus entry and targeted antiviral therapeutics. Upon adhesion to cell surface receptors, the virus is internalized via macropinocytosis, followed by endosomal proteolysis of viral glycoproteins and NPC1 binding, subsequently inducing fusion between the viral and endosomal membranes. In addition, TPC2-mediated Ca^{2+} homeostasis is also a critical factor to induce membrane fusion. Potential avenues toward EBOV entry inhibitors include targeting: cell adhesion, internalization, proteolysis process, NPC1 binding, TPC2 and Ca^{2+} homeostasis, and GP2 conformational changes, separately

protein expression. Consequently, in cells transiently or stably expressing alternative surface proteins, the pseudovirus can replicate and produce progeny pseudoviruses. While in native susceptible cells, only one single round of infection can occur for pseudoviruses (Rumschlag-Booms et al. 2011).

Since viral surface proteins are pivotal during entry process, and the pseudoviral surface proteins have similar conformational structures with the native viral proteins, pseudoviruses can

be therefore used as a powerful tool to dissect mechanisms of virus entry, evaluate neutralizing antibodies, and discover novel viral entry inhibitors (Rumschlag-Booms et al. 2011). Moreover, the backbone of a pseudovirus can be further modified by insertion of reporter genes, such as fluorescent proteins, luciferases or the chloramphenicol acetyltransferase (CAT). The expression level of these reporter proteins can be easily monitored to indirectly reflect the pseudovirus infection.

Three viruses including HIV-1, Murine leukemia virus (MLV), and VSV have been explored as pseudotyping vectors, among which the HIV-1 based lentiviral packaging system is currently most widely used for EBOV studies. Using EBOV GP-based HIV-1 pseudoviruses (EBOVpp), we and others have discovered numerous entry inhibitors (Basu et al. 2011, 2015; Gaisina et al. 2020; Yermolina et al. 2011). However, although the GPs on surface of EBOVpp can largely mediate cell entry in a fashion similar to that of the authentic viruses, several issues should be considered. For example, HIV-1 and MLV pseudoviruses are spherical viruses, while VSV pseudoviruses are bullet shaped, both of which are remarkably distinct from the filamentous authentic EBOV. Thus, the pattern of GP distribution and density on EBOVpp may not reflect its "natural" state, and results obtained from assays using EBOVpp should always be compared to and validated using authentic EBOV-based assays, which remain the gold standard. Encouragingly, based on our own experience, most entry inhibitors identified by EBOVpp-based screens become validated using authentic EBOV, suggesting the feasibility and robustness of EBOVpp system as a screening approach for EBOV entry inhibitors (Basu et al. 2011, 2015; Gaisina et al. 2020; Yermolina et al. 2011).

10.2.2 Replication-Competent rVSV Encoding EBOV GP

VSV is a member of *Vesiculovirus* genus, *Rhabdoviridae* family, which is also an enveloped, single-stranded negative-sense RNA virus. Since human infection of VSV is a rare event, and usually causes mild influenza-like illness, VSV can be handled in laboratory with BSL-2 containment. Moreover, VSV has been used as a model to study many aspects of negative-strand RNA viral replication (Whitt 2010).

As mentioned above, VSV pseudoviruses can incorporate EBOV GP on its surface and mimic the entry of authentic EBOV. While in the VSV pseudotyping system, EBOV GP is provided in trans. This system can be further engineered, by inserting the EBOV *gp* gene into the VSV genome by replacement of the VSV-G coding region, generating a rVSV that encodes EBOV GP in place of the VSV-G (rVSV-ΔG-eGP) (Wong et al. 2010). Unlike pseudoviruses that can undergo only a single round of infection, rVSV-ΔG-eGP is replication competent. Indeed, the pathogenic mucin-like domain of EBOV GP was deleted to avoid potential biosafety risks (Wong et al. 2010). In addition to the applications discussed above for EBOVpp, rVSV-ΔG-eGP allows further characterizations, e.g., to select escape mutants under pressure of EBOV entry inhibitors.

10.2.3 Ebola Virus-Like Particles

As aforementioned, both the pseudotyping systems and recombinant VSV are inherently flawed for mimicking EBOV entry, partially due to the morphological differences as compared to authentic EBOV. To establish a more reliable entry-mimicking system, eVLPs were produced by co-expressing of EBOV major matrix proteinVP40 with GP (Martinez et al. 2010). As VP40 promotes the formation and budding of eVLPs which resemble the authentic filamentous virions, GP can be subsequently incorporated into the VLPs and allows the particles to be able to infect susceptible host cells, providing a valuable tool to study virus entry and identify entry inhibitors in a more authentic context as compared to EBOVpp and rVSV-ΔG-eGP. Furthermore, VP40 can be easily engineered with, e.g.,

the beta-lactamase enzyme or fluorescent tags, so that the entry of eVLPs can be easily traced (Martinez et al. 2010).

As an extension of eVLP system, transcription- and replication-competent eVLPs (tr-eVLPs) have been developed (Watt et al. 2014). By introducing an EBOV minigenome system, which expresses a minigenome and the ribonucleoprotein complex (RNP) proteins, to the eVLP producing cells, novel tr-eVLPs can be generated. Besides resembling the filamentous authentic virions, incorporating GP on the surface, these tr-eVLPs also harbor minigenome-containing nucleocapsids. When entering a cell, tr-eVLPs can deliver the minigenome to cytoplasm, where the minigenome can undergo transcription, translation, and replication. The tr-eVLP system is also known as a life cycle modeling system, which allows the study of almost all aspects of the viral life cycle.

10.3 REGN–EB3, Ansuvimab, and Other mAb Therapeutics

Therapeutic mAb has become one of the most valuable treatments for emerging infectious diseases, especially for those where no other therapy is yet available. Compared to chemical drugs, mAbs possess attractive advantages, including a rapid discovery process, remarkable specificity, and low toxicity. For EBOV, the main target of therapeutic mAbs is the GP, mainly via neutralizing mechanisms, and possibly additional antibody-dependent cellular cytotoxicity (ADCC) effects.

10.3.1 REGN-EB3

REGN-EB3 (Inmazeb) is developed by Regeneron Pharmaceuticals for the treatment of EBOV infection. It is a combination of three fully human monoclonal antibodies, consisting of atoltivimab (REGN3470), maftivimab (REGN3479), and odesivimab (REGN3471).

Originally, mice encoding fully human antibody variable region gene segments (VelocImmune mice) were immunized with a DNA expression vector for EBOV GP and purified EBOV GP protein as immunogens, followed by isolation and selection for neutralizing mAbs. Eventually, mAbs REGN3470, REGN3479, and REGN3471 were generated (Pascal et al. 2018). Both the REGN3470 and REGN3479 can neutralize pseudovirus particles efficiently, with half maximal inhibitory concentration (IC_{50}) values of 0.27 nM and 0.17 nM, respectively. However, the neutralizing capacity of REGN3471 is much weaker. On the other hand, REGN3470 and REGN3471 but not REGN3479 can induce ADCC in vitro. In a guinea pig model of EBOV infection, monotherapies of REGN3470, REGN3479, and REGN3471 can confer protection from disease and/or lethality (Pascal et al. 2018).

In addition, the three mAbs can recognize non-overlapping epitopes and do not compete with each other for GP binding. In order to improve the therapeutic efficacy and delay the emergence of possible escape mutants, the three mAbs were therefore mixed as a cocktail (REGN-EB3) (Pascal et al. 2018). During the 2018 EVD outbreak in the Democratic Republic of Congo, clinical trials of REGN-EB3 were conducted (Mulangu et al. 2019), and based on the results, the U.S. Food and Drug Administration (FDA) authorized approval of REGN-EB3 on October 14, 2020 for the treatment of EBOV infection (Markham 2021).

10.3.2 Ansuvimab

Ansuvimab (also known as mAb114) is a human monoclonal IgG1 antibody developed by Ridgeback Biotherapeutics for the treatment of EBOV infections. Initially, Corti et al. observed EBOV GP-specific antibodies circulating in a human survivor of the 1995 Kikwit EVD outbreak, therefore they subsequently isolated a series of EBOV mAbs and selected those that were able to neutralize both the EBOV variants from the 2013–2016 and 2018 outbreaks (Corti et al. 2016). Among these mAbs, Ansuvimab

exhibits the most potent neutralizing capacity, by a mechanism targeting the LEIKKPDGS epitope located in the RBD of GP and blocking the GP-NPC1 interaction. In addition, Ansuvimab also mediates ADCC and offers high protective efficacy in rhesus macaques infected with a lethal dose of EBOV (Corti et al. 2016). Along with REGN-EB3, Ansuvimab also passed the clinical trials during 2018 EDV outbreak, and thus received approval for the treatment of EBOV infection on December 21, 2020 in the USA (Lee 2021).

10.3.3 Other mAb Therapeutics

Historically, ZMapp was the first mAb therapy against EBOV that entered clinical trials, at the time shortly after the 2013–2016 West African Ebola epidemic struck. ZMapp consists of a cocktail of three highly purified mAbs. Notably, all three mAbs are humanized chimers and produced in a plant-based system involving transgenic *Nicotiana benthamiana*. Humanized mAbs usually do not elicit an immune response and are safe for human use, while *N. benthamiana* produced mAbs contain afucosylated/agalatosylated glycans, which can enhance the ADCC activity (Qiu et al. 2014). Unfortunately, although ZMapp is able to achieve 100% protection of rhesus macaques from EBOV infection, it did not show significant efficacy in clinical trials. Nonetheless, the formulated mAb cocktail deserves further optimization and reevaluation.

Notably, EBOVs may keep evolving (Holmes et al. 2016), and other highly pathogenic ebola viruses, e.g., Sudan virus (SUDV) and Bundibugyo virus (BDBV), may unpredictably emerge or re-emerge, causing scattered outbreaks. Since ZMapp as well as REGN-EB3 and Ansuvimab can provide protection against EBOV alone, novel pan-ebola virus active therapeutic mAbs are urgently needed. Encouragingly, preclinical success with broadly neutralizing ebola virus mAbs, including MBP134, FVM04, and CA45, has shown protection against diverse ebola viruses (Bornholdt et al. 2019; Brannan et al. 2019). Moreover, when supplemented with MARV neutralizing mAb R191, the FVM04 and CA45 cocktail might provide a pan-filovirus protection (Brannan et al. 2019).

10.4 Repurposing Old Drugs as New EBOV Entry Inhibitors

Repurposing screens of existing drugs for new indications is a practical approach to rapidly discover and develop antivirals to combat infections of emerging or reemerging viruses, since the "old" drug can directly enter clinical trials without delay. On the other hand, as the life cycle of a virus becomes well studied, increasing numbers of viral/host factors that play critical roles during virus infection are identified, and thus existing inhibitors/modulators targeting these critical viral/host proteins or relative functions can be repurposed as potential antiviral therapeutics. We would like to refer to the two methods as "forward" and "reverse" drug repurposing screens, after the terms of "forward genetics" and "reverse genetics," respectively.

10.4.1 Forward Repurposing Screen of EBOV Entry Inhibitors

In order to more expeditiously grow the arsenal of drugs against the highly pathogenic virus, the FDA-approved drug library, as well as other existing drug libraries, were screened for potential repurposing as EBOV inhibitors (Cheng et al. 2015; Johansen et al. 2013; Kouznetsova et al. 2014; Zhang et al. 2020; Zhou et al. 2016). As most screening approaches were established using the aforementioned surrogate systems, many of these repurposed inhibitors act upon the entry stages of EBOV infection (Cheng et al. 2015; Kouznetsova et al. 2014; Zhang et al. 2020; Zhou et al. 2016). Interestingly, more than 100 compounds have been reported to have potential inhibitory effects against EBOV entry, which may be correlated with the much more complicated entry process of EBOV with respect to other viruses, as many more viral/host factors

and pathways are involved, thereby providing much more druggable targets.

Among these repurposed candidates, some act upon EBOV entry via their biological activities. For example, Johansen et al. identified a set of selective estrogen receptor modulators (SERMs) from FDA- and ex-U.S.-approved drugs as potent EBOV inhibitors (Johansen et al. 2013), that act by interfering with the fusion at the late-entry stage. Although it turns out SERMs do not inhibit EBOV entry through the estrogen receptor pathway, SERMs have incidental biological activities that induce cholesterol accumulation and Ca^{2+} upregulation in endosomes (Johansen et al. 2013). Since both cholesterol and Ca^{2+} homeostasis are critical during EBOV entry, SERMs may block EBOV entry by interfering with the endosomal pathway. Interestingly, another study, using a thermal shift assay combined with co-crystalization, demonstrated that the two most potent inhibitors, clomiphene and toremifene, can bind to a fusion loop-associated cavity of EBOV $GP_{1,2}$ at the GP1/GP2 interface and drastically destabilize the target protein, triggering a premature conversion of $GP_{1,2}$ to the post-fusion conformation, and subsequently preventing the membrane fusion (Zhao et al. 2016). These results suggest that SERMs may inhibit EBOV entry by a dual-mechanism of action (MOA) in both a chemical structure-based and biological activity-based manner.

In the case of most other repurposing candidates, the targets themselves appear to be altered. Previously, we have identified a series of G protein-coupled receptor (GPCR) antagonists that inhibit EBOV entry, including those targeting histamine receptors, 5-HT (serotonin) receptors, muscarinic acetylcholine receptor, and adrenergic receptor (Cheng et al. 2015, 2017). We therefore asked if GPCR pathways are involved during EBOV entry. However, increasing evidence suggested that the GPCR antagonists block EBOV entry by binding to EBOV GP. Using histamine receptor inhibitors as an example: first, only the first generation of H1-specific antihistamines but not newer drugs can inhibit EBOV entry; second, prior saturation of the cell surface H1-receptors with histamine or H1-receptor knockout do not affect EBOV entry and the sensitivity to antihistamines; third, antihistamines block filovirus entry in the endosome rather than at cell surface; fourth, docking analysis predicts that antihistamines can bind to GP with high affinity. Taken together, it can be concluded that these antihistamines inhibit EBOV entry by directly targeting viral GP (Schafer et al. 2018). Furthermore, using one of the histamine receptor antagonist compounds, CP19, as a starting point, we and colleagues carried out a comprehensive SAR analysis and synthesized a CP19 derivative compound, compound 32, that shows improved inhibitory capacity against EBOV entry (Gao et al. 2020).

Notably, a challenge in the repurposing strategy is attaining therapeutic drug levels of protection for a new indication. In actuality, most of the repurposed EBOV inhibitors could hardly reach the required blood concentration to prevent or ameliorate symptoms in patients with EVD. Consideration of drug combinations can help to lower the required dose of each drug, improving the chances of reaching therapeutic levels, and minimizing adverse effects (Lehár et al. 2009). For example, further screening approaches for synergistic pairs and/or three-drug combinations of approved drugs against EBOV have been developed (Dyall et al. 2018; Sun et al. 2017). Using these methods, several synergistic drug pairs (clomiphene-sertraline, clomiphene-apilimod, sertraline-toremifene, and toremifene-apilimod), and three-drug combinations (toremifene-clarithromycin-posaconazole, toremifene-mefloquine-posaconazole, and chloroquine-maprotiline-azithromycin) have been identified to effectively block EBOV entry, providing potent therapies to treat EBOV infections (Dyall et al. 2018; Sun et al. 2017).

10.4.2 Reverse Repurposing Screen of EBOV Entry Inhibitors

Along with the illustration of many viral/host factors and cellular pathways that play critical roles during EBOV entry, existing inhibitors/modulators targeting these factors or pathways

were tested for the anti-EBOV activity. Host-derived cysteine cathepsins cleave native GP into fusion competent form during EBOV entry, and have become an attractive target for EBOV entry inhibitors. The well known cysteine cathepsins inhibitors, K-11777 and E-64d, therefore become promising candidates of anti-EBOV drugs targeting these enzymes (Chandran et al. 2005; Zhou et al. 2015). Moreover, another cathepsins inhibitor, AMS36, was also selected, followed by chemical modifications and the generation of a focused library of cysteine cathepsin inhibitors. Subsequently, the library was screened and several compounds with potent activity against EBOV entry have been reported (van der Linden et al. 2016).

Considering that both NPC1 and TPC2 are critical host factors involved in EBOV entry, although the underlying mechanisms remain elusive, the existing NPC1 inhibitor U18666A and the TPC2 inhibitor tetrandrine have been proposed as antiviral candidates and they represent ideal starting points for the development of EBOV drugs (Lu et al. 2015; Sakurai et al. 2015). Unraveling and investigation of new factors/pathways that facilitate or hinder EBOV entry can provide additional targets for repurposing, as well as new strategies for antiviral therapeutics (Kuroda et al. 2020; Sarute et al. 2021).

10.5 Development of Novel EBOV Entry Inhibitors

The mAbs and repurposed drugs provide potential therapies as a quick response to the outbreak of an emerging virus that lacks efficacious vaccines or antivirals. However, as the virus may evolve to escape therapeutic mAbs, and repurposed drugs are usually suboptimal and require further improvement, it is necessary to develop novel therapies to address future periodic epidemics and probable pandemics. Encouragingly, efforts to develop specific treatments against EBOV have begun shortly after its discovery in 1976, and more recently efforts have been accelerated, due to the devastating 2013–2016 West African epidemic. To date, diverse entities of novel potent EBOV entry inhibitors have been reported, including varieties of small molecules, natural products, peptides, and nano-structures.

10.5.1 Small Molecule Inhibitors

For the past two decades, we and others have discovered a number of small molecule EBOV entry inhibitors, mainly via the EBOVpp-based high-throughput screening approach (Basu et al. 2011, 2015; Cui et al. 2018a; Gaisina et al. 2020; Yermolina et al. 2011). However, for a very long time the MOA of these small molecule inhibitors was not known, although they are presumed to mainly target EBOV GP (Schafer et al. 2018; Zhao et al. 2016).

Since understanding the binding mode of a small molecule to the target can advance its further optimization as anti-EBOV agents, we and colleagues have been devoted to dissecting the MOA of these small molecule EBOV inhibitors, by developing a chemical probing approach. Initially, we noticed that most of the entry inhibitors that bind EBOV GP also show inhibition against MARV, albeit with less potency (Basu et al. 2011, 2015; Yermolina et al. 2011). However, EBOV and MARV GP show just 25% sequence homology in GP1 and 43% in GP2, suggesting that the cross-reactive molecules may target some conserved portion of the two GPs. Moreover, mutation in one single site of these portions of EBOV GP may produce a MARV-like dose-response curve for a batch of EBOV inhibitors, indicating the possible binding sites of these molecules (Schafer et al. 2021).

Comprehensive probing profiles for the identified small molecule EBOV entry inhibitors to wildtype/mutant EBOV GP and MARV GP were subsequently generated, and at least three distinctive MOA were thereafter identified. First, the molecules may directly bind to the internal fusion loop region of EBOV GP; second, some of the molecules may bind to the HR2 domain as a secondary binding site; third, some molecules with basic side chains can be trapped in the acidic

endosomes/lysosomes, increasing compound concentrations and subsequently improving the viral inhibition (Schafer et al. 2021). The molecular basis provides profound mechanistic insights into EBOV entry and can guide future rational design for the development of novel EBOV entry inhibitors.

Notably, inspired by the discovery of a set of SERMs as EBOV entry inhibitors from a repurposing screen, we and colleagues further carried out a focused screen of ER ligands with various functions and diverse chemical scaffolds for potent anti-EBOV inhibitors. Encouragingly, 20 out of the 61 assayed compounds exhibited inhibitory potency against EBOV entry, among which Ridaifen-B is one of the most potent inhibitors (Cooper et al. 2020). Using the aforementioned chemical probing method against wildtype/mutant EBOV GP, we demonstrated that Ridaifen-B can also target the toremifene-binding pocket of EBOV GP, suggesting a MOA by inducing GP destabilization (Cooper et al. 2020). It is noteworthy that the SERM-like activity is not only undesired for an anti-EBOV agent, but also has side effects, for example, ER agonists may have feminizing actions, whereas ER antagonists may cause chemical menopause. Therefore, reverse engineering of the ER activity was carried out for Ridaifen-B, in addition to chemical modifications to increase affinity for the EBOV GP, eventually leading to a novel anti-EBOV compound (XL147) that exhibits improved activities against EBOV, as well as MARV, at sub-micromolar levels (Cooper et al. 2020). Moreover, as mentioned above, most of the small molecule inhibitors, including both repurposed and newly identified ones, exhibit broad activity against diverse Ebola virus species as well as MARV. These small molecules provide promising leads for the development of valuable pan-ebola virus or pan-filovirus therapeutics.

10.5.2 Natural Product Derived Inhibitors

Natural products are considered to be a natural combinatorial chemical source because they contain structurally diversified bioactive chemicals, providing a valuable reservoir for new drug discovery. Recently, numerous compounds derived from medicinal plants or other natural materials have been identified as potent EBOV entry inhibitors, including ellagic acid from the traditional Chinese medicinal plant *Rhodiola rosea* L. (Cui et al. 2018b), sclareol and sclareolide from *Salvia sclarea* (Chen et al. 2020), and a flavonoid derivative quercetin 3-β-*O*-D-glucoside (Q3G) (Qiu et al. 2016). Notably, the prophylactic efficacy of Q3G against EBOV infection has been validated using a mice model of infection (Qiu et al. 2016).

10.5.3 Peptide-Based Inhibitors

Peptide-based inhibitors represent an attractive alternative entity as virus inhibitors, and possess promising advantages, e.g., peptides can usually be well tolerated due to improved efficacy and specificity. Many peptide-based inhibitors have been designed as entry inhibitors of diverse viruses, including the approved peptidic drug, T20, for treatment of HIV-1.

Both HIV-1 Env and EBOV GP are class I fusion proteins. T20 mimics the HR2 domain of HIV-1 Env protein (corresponding to HR2 of EBOV GP), and exerts its inhibitory inhibition by preventing the formation of the six-helix bundle necessary for fusion. A similar strategy was therefore used to design EBOV GP mediated fusion inhibitors. However, the peptide derived from HR2 of EBOV GP (C-peptide) initially exhibits only modest inhibitory activity (Watanabe et al. 2000). Interestingly, Miller et al. further engineered the C-peptide by conjugation with an arginine-rich sequence from HIV-1 Tat (Tat-Ebo). As the Tat sequence can facilitate endosomal accumulation and subsequently increase the intracellular concentration of the peptide, Tat-Ebo shows improved anti-EBOV activity, providing a potential candidate for EVD treatment (Miller et al. 2011).

Since the binding of the fusion competent form of EBOV GP (GPcl) to NPC1 is required to induce membrane fusion, the crystal structure

of GPcl in complex with NPC1 has been determined (Wang et al. 2016). The crystal structure shows that NPC1 binds to a hydrophobic cavity on the head of GPcl, and thus this cavity represents a potential target for virus entry inhibitors. However, the cavity is large and flat, representing a typical protein-protein interaction interface; therefore it is not suitable for the binding of a small molecule, while a rational designed peptide may better fit and occupy the binding interface. With the aid of computer modeling, an eight-residue cyclic peptide (Pep-3.3) was designed, synthesized and eventually shown to exhibit specific inhibitory effects against EBOV entry. It will be interesting to further develop this cyclic peptide as a potential drug against EBOV infection (Li et al. 2018).

10.5.4 Nanotechnology-Based Inhibitors

Besides small molecules, natural products and peptides, the advances in material chemistry, have aroused increasing interests for the development of new artificial chemicals as novel antiviral agents. Recently, various nanostructures displaying multivalent moieties, e.g. carbohydrates, were prepared and shown to have high inhibitory effects against the entry of diverse viruses, including EBOV (Illescas et al. 2017; Muñoz et al. 2016; Nie et al. 2021). Although additional studies are still required for chemical optimization and efficacy evaluation, the nanotechnology-based structures provide remarkable leads for practical applications in future.

10.6 Conclusions

Despite the recent approval of two EVD therapeutics and advances of numerous entry inhibitors, our countermeasures against an EVD/MVD outbreak are still limited and far from satisfactory. Continuing efforts should be engaged in dissecting the filovirus entry steps as well as preparing more drug candidates, especially those that possess pan-ebola virus or even pan-filovirus activities.

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Flavivirus Entry Inhibitors

11

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Abstract

Flaviviruses, including Dengue virus, Zika virus, Yellow fever virus, Japanese encephalitis virus, West Nile virus, cause thousands of deaths and millions of illnesses each year. The large outbreak of ZIKV in 2016 reminds us that flaviviruses can pose a serious threat to human safety and public health as emerging and re-emerging viruses. However, there are no specific drugs approved for the treatment of flavivirus infections. Due to no need to enter the cells, viral entry inhibitors have the unique advantage in suppressing viral infections. Flaviviruses bind to receptors and attach to the cell surface, then enter the endosome in a clathrin-dependent manner and finalizes the viral entry process after fusion with the cell membrane in a low pH environment. Small molecules, antibodies or peptides can inhibit flavivirus entry by targeting the above processes. Here, we focus on flavivirus entry inhibitors with well-defined target and antiviral activity. We hope that our review will provide a theoretical basis for flavivirus treatment and drug research and help to accelerate the clinical application of flavivirus entry inhibitors.

Keywords

Flavivirus · Entry inhibitors · Small-molecule · Antibody · Peptide

11.1 Introduction

The flaviviruses include more than 70 single positive-stranded RNA enveloped viruses, such as Dengue virus (DENV), Zika virus (ZIKV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), etc. Flaviviruses are mainly transmitted by blood-sucking arthropods (mosquitoes or ticks). DENV has four serotypes (DENV1, DENV2, DENV3, and DENV4) with 30–35% variation in amino acid sequence (Dejnirattisai et al. 2015). Initial infection with one serotype of DNEV may cause self-limited Dengue fever (DF), while secondary infection with another serotype of DENV may lead to fatal capillary leak syndrome (Dengue hemorrhagic fever [DHF]/Dengue shock syndrome [DSS]) (Kyle and Harris 2008). In addition, children under age 15 are at high risk of progression to DHF/DSS (Kyle and Harris

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2008). Currently, there are hundreds of millions of DENV infections and up to 5.2 million cases of DHF/DSS worldwide annually (WHO 2021). ZIKV infection of pregnant women results in the birth of infants with microcephaly and other congenital malformations. ZIKV also causes Guillain–Barré syndrome, neuropathy, and myelitis in some adults or children (Christian et al. 2019). Mosquito-borne ZIKV infection has been reported in more than 80 countries or tribes worldwide. About 15% of YFV infection patients develop to severe visceral disease, of which 20–50% die (Monath 2008). JEV is the leading pathogen of viral encephalitis worldwide, causing 67,900 cases per year, with 20–30% of deaths and 30–50% of survivors suffering from neurological sequelae (Wang and Shi 2015). Normally, WNV infects humans with no obvious symptoms, but the risk of brain infection and death for people over the age of 50 increases 20-fold (Diamond 2009). The ZIKV outbreak in 2016 reminds us that the threat of flaviviruses to humans has never ceased. However, there are no approved drugs for flavivirus infections. Therefore, effective anti-flavivirus drugs are urgently needed.

The flavivirus genome is ~11 kb, which encodes three structural proteins (precursor membrane/membrane protein, envelope, capsid) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Knipe and Howley 2013). The non-structural proteins are responsible for viral replication, assembly, and escape from host immunity, while the structural proteins form the viral particles. The prM protein is cleaved by a furin-like protease into pr peptide and the 8 kDa M protein during viral maturation (Wang AY et al. 2017a). Envelope (E) protein is approximately 53 kDa and is involved in viral attachment, membrane fusion, assembly, and budding. E protein is a typical class II membrane fusion protein consisting of three domains linked by short flexible hinges. Domain I (EDI), located in the middle of the E protein, consists of eight β-folds; Domain II (EDII) is a long structure mediating the formation of E protein dimers, the top of which is the membrane fusion loop (FL) region consisting of 13 conserved glycine-rich amino acids; Domain III (EDIII), an immunoglobulin-like structure located in the C terminal region, is mainly responsible for binding to cell surface receptors and facilitating viral attachment and entry (Knipe and Howley 2013). 180 M and E proteins form 90 heterodimers lying flat on the surface of the mature viral particles (Kuhn et al. 2002). After E protein binding to cell surface receptors or co-receptors (acetyl heparan sulfate, DC-SIGN, AXL/Cas6, Fc receptors, etc.), the virus subsequently enters the endosome via clathrin-mediated endocytosis. The E protein dimer undergoes dissociation in the acidic environment of the endosome. EDII extends outward and the exposed fusion loop inserts into the endosomal membrane, facilitating subunit reaggregation to form an extended trimeric intermediate conformation (Modis et al. 2004). Subsequently, EDIII folds inversely, driving the viral envelope and endosomal membrane in close proximity to each other, and thus membrane fusion occurs. During this process, the E protein stem region near the viral membrane changes from an α-helical structure to an irregularly coiled structure, facilitating the membrane fusion and stabilizing the E protein trimeric structure (Schmidt et al. 2010a). Due to the important role in the viral entry, E protein has become a major target for the development of flavivirus entry inhibitors.

With the rapid development of transportation and tourism, outbreaks of viral infections are no longer a local event, but a global human concern. Humans need to build capacity to deal with emerging and re-emerging virus outbreaks. And research on antiviral drugs is critical. Viral entry inhibitors block virus outside cells, eliminating the series of subsequent events caused by viral infection of cells. This chapter discusses flavivirus entry inhibitors with well-defined targets and efficacy, and provides strategies for the treatment and drug development of flaviviruses.

11.2 Small Molecule Entry Inhibitors

11.2.1 Inhibition of Viral Binding to Receptors

Drugs blocking or interfering with the viral binding to cell receptors inhibit the viral entry. Heparan sulfate (HS), a highly sulfated polysaccharide, is a cellular attachment receptor for multiple flaviviruses, including DENV, ZIKV, JEV, etc. The positively charged E protein with two mucopolysaccharide binding sites interacts with heparan sulfate by electrostatic interactions or receptor binding (Liu and Thorp 2002; Chen et al. 1997). Heparin or HS analogs can inhibit flavivirus infections (Chen et al. 1997). λ-carrageenan (Talarico and Damonte 2016), curdlan sulfate (Ichiyama et al. 2013), sulfated *Escherichia coli* K5 polysaccharide derivatives (Vervaeke et al. 2013) inhibit DNEV infection, chondroitin sulfate E suppresses DENV1-4 and JEV infections (Kato et al. 2010), and polysulfonate suramin restrains ZIKV infection (Tan et al. 2017). Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is mainly expressed on the surface of DC cells and works as an important receptor for flaviviruses. Carbohydrate-binding agents, including Hippeastrum hybrid (HHA), Galanthus nivalis (GNA), and Urtica dioica (UDA), inhibit DENV1-4 infection of Raji/DC-SIGN+ and primary immature monocyte-derived DC (MDDC), with IC_{50} values as low as nanomolar level (Alen et al. 2011). Bovine lactoferrin inhibits DENV1-4 (Chen et al. 2017) or JEV (Chien et al. 2008) infections by interfering with viral binding to the cell surface receptors, including HS, DC-SIGN, and low-density lipoprotein receptor (LDLR), and decreases morbidity in Balb/C suckling mice infected with DENV2 (Chen et al. 2017). DENV and ZIKV recognize AXL receptor through the binding of viral surface phosphatidylserine to growth arrest-specific 6 (Gas6), the natural ligand of AXL (Meertens et al. 2017). The modified AXL decoy receptor MYD1 and R428, an inhibitor of AXL kinase, both inhibit ZIKV infection of human glial cells (Meertens et al. 2017). A flavivirus may have multiple cellular receptors, and different flaviviruses prefer different receptors. Therefore, a single receptor inhibitor may not be able to completely inhibit the infection of flaviviruses, and it is better to be used in combination with other drugs.

11.2.2 Inhibition of Viral Endocytosis

Both the process of endocytosis and the low pH environment in endosome are targets for flavivirus entry inhibitors. Nanchangmycin is aglycone polyether produced by Streptomyces nanchangensis and has insecticidal activity against silkworms and anti-bacterial activity in vitro (Sun et al. 2002). Nanchangmycin suppresses ZIKV infection of a wide range of cells, including biologically relevant progenitor cells, through inhibiting clathrin-mediated endocytosis. It also inhibits the entry of WNV, DENV, Kunjin, and chikungunya virus (Rausch et al. 2017). Chlorpromazine, a cationic amphipathic drug, prevents clathrin-mediated endocytosis by inhibiting the assembly of clathrin-coated pits in the cell membrane and inducing the assembly of clathrin lattice on endosomes (Wang et al. 1993). Chlorpromazine was shown to repress ZIKV infection of human glioblastoma cells (Li et al. 2020), DENV infection of myeloid cells (Carro et al. 2018) and JEV infection of Vero cells (Nawa et al. 2003). Chloroquine, an FDA-approved drug for the treatment of malaria, is used prophylactically in pregnant women exposed to Plasmodium vivax. Due to its alkalinity to elevate the pH of endosomes, chloroquine has been reported to inhibit infection of DENV, ZIKV, JEV, and WNV (Delvecchio et al. 2016). Bafilomycin A1 (Sabino et al. 2019), 6-deoxyglucose-diphyllin (DGP) (Martinez-Lopez et al. 2019), and niclosamide (Kao et al. 2018) inhibit membrane fusion of ZIKV or DENV by suppressing acidification of endosomes. Additionally, another research reported that niclosamide reduced viral RNA load and protein expression even at 4 h after ZIKV infection, suggesting that it may also inhibit viral replication (Xu et al. 2016).

Niclosamide also reduces DENV-induced mortality in ICR sucking mice (Kao et al. 2018). Flaviviruses share a similar endocytosis process, and endocytosis inhibitors may be developed into broad-spectrum anti-flavivirals.

11.2.3 Inhibition of Membrane Fusion

11.2.3.1 Drug Screening Using Membrane Fusion Models

Since it is difficult to directly observe the membrane fusion process between virus and cell, cellular models are used to study the membrane fusion of flaviviruses. Yamamoto and colleagues constructed a flavivirus dual reporter cell membrane fusion model in C6/36 cells using plasmids, including pUb-prME, pUb-DSP1-7 (RL1-155-Ser-Gly-Gly-Gly-Gly-GFP1–156) or pUb-DSP8-11 (Met-GFP157–231-Gly-Gly-Gly-Gly-Ser-RL156–311) (Yamamoto et al. 2020). Atovaquone, an FDA-approved antimalaria drug, was screened using this model. The drug inhibits the E protein-mediated membrane fusion of ZIKV, DENV, YFV, WNV, and JEV, and represses DENV1-4 infection of C6/36 cells and ZIKV infection of Vero cells (Yamamoto et al. 2020). Peptides from DENV E protein stem region bind the stem-less E trimer. Based on the above phenomenon, Schmidt and colleagues set up a competition binding assay and obtained 1662G07 and its analogs. These small molecules bind to E dimer, block the fusion of virus with liposome, and inhibit infections of DENV1-4 (Schmidt et al. 2012).

11.2.3.2 Occupying the EDI-EDII β-OG Pocket Region

A hydrophobic pocket region in the hairpin structure (residues 268–280) between DENV2 EDI and EDII region, can be occupied by a molecule of β-*N*-octyl-D-glucoside (β-OG), known as the β-OG pocket region (Modis et al. 2003). Small molecule inhibitors occupying this site inhibit the membrane fusion of flaviviruses through blocking the conformational changes between EDI and EDII (Wang and Shi 2015) in low pH environment. The combination of molecular docking and in vitro experiment facilitates efficient screening of drugs targeting the β-OG pocket region. Based on the structural modification of P02, M02, and D03, which were screened from 142,000 compounds (Zhou et al. 2008), compound 36 was obtained to inhibit YFV infection at the nanomolar level (Li et al. 2008) and compound 23 was shown to inhibit DENV2 infection at the micromolar level (Jadav et al. 2015). Other small molecule compounds targeting the β-OG pocket region, such as compound 6 (Wang et al. 2009), A5 (Kampmann et al. 2009), and SA-17 (a derivative of the antibiotic adriamycin) (Kaptein et al. 2010), exhibit excellent inhibitory activity against DENV, JEV, WNV, or YFV-17D. Lian W and colleagues constructed a high-throughput screening model based on the binding of 4,6-disubstituted pyrimidines (GNF2) to the β-OG pocket region (de Wispelaere et al. 2018) and competitive neighborhood luminescence analysis (Lian et al. 2018). They harvested eight compounds, including S4105, K786-9739, C200-9144, S7337, etc. with inhibitory effects on DENV1-4 and ZIKV infections (Lian et al. 2018). Therefore, the β-OG pocket region may serve as one of the targets for high-throughput screening of flavivirus membrane fusion inhibitors.

11.2.3.3 Inhibiting the Formation of E Protein Trimer

The formation of E protein trimer is necessary for viral membrane fusion and can serve as one of the flavivirus entry inhibitor targets. Compound F1065-0358 binds to region between ZIKV EDI and EDIII, including residues H144 in EDI, K301 and Y305 at linker and N362 in EDIII, inhibiting EDIII reverse folding and E protein trimer formation (Sharma et al. 2020). Epigallocatechin gallate (EGCG) inhibits DENV, ZIKV, WNV or JEV infections (Sharma et al. 2017; Vazquez-Calvo et al. 2017; Wang et al. 2018), by binding to the linker (residues 298–308) between EDI and EDIII and disrupting E protein trimer formation. In addition, the polyphenolic drugs delphinidin and EGCG were found to inactivate WNV particles directly and the activity was stronger than the activity of membrane fusion inhibition

(Vazquez-Calvo et al. 2017). Drugs targeting EDIII alone can also exert antiviral activity. For example, gossypol inhibits the infection of ten ZIKV strains, including six epidemic strains, by targeting viral EDIII (Gao et al. 2019).

11.2.4 Small Molecule Viral Inactivators

Some small molecule compounds inactivate viral particles directly by disrupting the integrity of envelope, causing the internal genome released. Erythromycin estolate inhibits ZIKV, DENV2, and YFV infections of BHK-21 cells, protects A129 mice from lethal ZIKV infection, and reduces vertical transmission of ZIKV in C57BL/6 pregnant mice (Wang X et al. 2019b). Keggin-type niobium-substituted-heteropolytungstate $Cs_2K_4Na[SiW_9Nb_3O_{40}].H_2O$ (POM-12) inactivates viral particles of DENV, JEV, and ZIKV (Qi et al. 2020). Montelukast, a drug for asthma and safe for pregnancy, inactivates ZIKV directly (Chen et al. 2019). Baicalein, antiviral bioflavonoid, inactivates JEV and inhibits JEV adsorption (Johari et al. 2012). Porphyrins (amphiphilic lipid molecules) bind to membrane, absorb light, and are mainly used in photodynamic therapy. Co-protoporphyrin IX (CoPPIX) and Sn-protoporphyrin IX (SnPPIX) directly deprive the viral envelope, disrupt the viral morphology, and inhibit attachment and entry of flaviviruses, including DENV, YFV, ZIKV, etc. (Neris et al. 2018). CLR01, which promotes the degradation of amyloid and prevents its assembly, is mainly used in the treatment of neurodegenerative diseases, such as Parkinson's disease (Rocker et al. 2018). CLR01 selectively binds to lipid raft-rich regions of viral membrane, leading to viral membrane damage and infectivity reduction, including HIV-1, herpes virus and ZIKV (Rocker et al. 2018). In summary, small molecule viral inactivators have the potential to become broad-spectrum antiflaviviral agents.

11.2.5 Other Types of Viral Entry Inhibitors

Viral capsid protein is also a target for small molecule inhibitors. ST-148 targets viral capsid protein and inhibits viral assembly, release, and entry, with IC_{50} ranging from 0.012 to 8.9 μM, and reduces viremia and cytokine levels in AG129 mice infected with DENV (Byrd et al. 2013; Scaturro et al. 2014). The host cell membrane also acts as a target for viral entry inhibitors. Cholesterol 25-hydroxylase (CH25H) and 25-hydroxycholesterol (25HC) inhibit viral entry by altering the stability and integrity of the cholesterol-enriched region of the cell membrane (Zhao et al. 2020). 25HC inhibits the entry of ZIKV and attenuates the viremia in mice and rhesus monkeys (Li et al. 2017). In addition, CH25H also inhibits viral infection by modulating the immune response (Zhao et al. 2020).

11.3 Antibody Entry Inhibitors

Antibodies bind to E protein to block viral attachment or prevent E protein conformational rearrangement, thus inhibiting flavivirus entry. A large number of antibodies have been isolated from convalescent patients, mice or phage display libraries, targeting quaternary epitopes or epitopes on EDI, EDII, or EDIII. Here, we will review these antibodies according to their epitopes.

11.3.1 Structural-Based Quaternary Epitopes

Antibodies isolated from flavivirus infected patients mostly recognize the structural-based quaternary epitopes (also named envelope dimer epitope, EDE) consisted of two E monomers from the same or adjacent E protein dimer, with broad-spectrum and high antiviral activity. Antibody SIgN-3C isolated from the plasmablasts of a

patient shows preventive and therapeutic effects against DENV infection in adult AG129 mice (Xu et al. 2017) and reduces mortality in ZIKV-infected mice (Kam et al. 2017). SIgN-3C recognizes G100 and W101 on one monomer and K310 and R323 on the other monomer in the E dimer, and its remarkable long CDR3 region suggests that SIgN-3C may recognize more than one E dimer (Xu et al. 2017). Antibody C10, recognizing the intra-dimer interface of E protein, inhibits DENV (Barba-Spaeth et al. 2016) and ZIKV infection without ADE (Zhang et al. 2016). At pH 6.5 or 5.5, C10 locks E protein or E protein raft structure across the viral surface, thereby preventing E protein conformational rearrangement during viral membrane fusion (Zhang et al. 2016). Another antibody ZIKV-195, targeting EDI and EDII within an E dimer, potently inhibits ZIKV infection (Long et al. 2019). ZIKV-195 may prevent the depolymerization of the E dimers by cross-linking the E monomers within dimers, thus suppressing formation of fusogenic trimers. 2D22 recognizes EDII and EDIII on adjacent E monomer from different E dimers to prevent E protein conformational changes during viral membrane fusion (Fibriansah et al. 2015). However, 2D22 was also reported to recognize a quaternary epitopes centered on EDIII (Gallichotte et al. 2018). And 2D22 has preventive and therapeutic effects against DENV infection in AG129 mice (Fibriansah et al. 2015). HM14c10 exhibits good inhibitory activity against all DENV1 genotypes and reduces viral load in AG129 mice under both prophylactic and therapeutic conditions (Teoh et al. 2012). It was reported that 120 copies of Fab HM14c10 bind to all of the available 180 copies of E protein, by targeting the quaternary epitopes of the E dimer junction. Besides, human monoclonal antibody (HMAb) ADI-30056 (Sevvana et al. 2020) and ZIKV-117 (Hasan et al. 2017) were demonstrated to interact with E monomer not only in an E dimer but also in adjacent E dimer, thus preventing the reorganization of E protein monomers into fusogenic trimers. Importantly, the multi-site interactions reduce the number of antibodies occupying the entire flaviviral surface, which potently promotes the neutralizing activity of antibodies. Comparing with antibodies binding FL region, antibodies targeting the EDE exhibit better cross-neutralizing activities and weaker ADE, with 50% plaque reduction neutralization titer ($PRNT_{50}$) lower to picomolar level (Dejnirattisai et al. 2015).

11.3.2 EDI/EDII Epitopes

EDI/EDII is an important target in E protein. HMAb 1F4 recognizes EDI and EDI-EDII hinge, thus preventing viral attachment. It inhibits DENV1 infection of U937 and Vero cells, and reduces viral load in serum and bone marrow of DENV1-infected AG129 mice (de Alwis et al. 2012; Fibriansah et al. 2014). Antibody 5F8, obtained from mice immunized with ZIKV E (residues 1–409), recognizes the glycan loop of EDI, a conserved region within ZIKV, and completely protects ICR mice from lethal ZIKV infection (Qu et al. 2020).

In addition to quaternary epitopes, the FL is also a major epitope for monoclonal antibodies isolated from patients in the acute or recovery phase of viral infection. HMAb 4.8A, D11C and 1.6D, which recognize FL, neutralize DENV1-4 as well as WNV (Costin et al. 2013). 2A10G6, isolated from immunized mice with inactivated DENV2, also targets FL and can neutralize DENV1-4, WNV as well as ZIKV (Deng et al. 2011; Dai et al. 2016). The crystal structure of ZIKV-E with 2A10G6 reveals that a highly conserved peptide 98DRXW101 is the epitope of 2A10G6 (Dai et al. 2016). Antibodies targeting FL may block the insertion of E protein into the endosomal membrane during fusion. Due to the high conservation of FL in DENV, or even in the entire genus of flavivirus, antibodies targeting FL generally exhibit broad-spectrum anti-flavivirus activity. However, subneutralizing levels of these antibodies might cause antibody-dependent enhancement (ADE).

11.3.3 EDIII Epitopes

The EDIII of flavivirus is an immunoprotein-like region. Antibodies targeting EDIII peak early

after infection, last for several months, and have less cross-reactivity to other flaviviruses (Yu L et al. 2017a). While, antibodies targeting EDI/II peak and decay quickly in the early period of infection and have strong cross-reactivity (Yu L et al. 2017a). Therefore, the EDIII tends to be the preferred region for flavivirus antibody preparation.

Antibody ZK2B10, targeting the lateral ridge of EDIII, completely protects mice from ZIKV infection and significantly reduces the tissue damage in mice infected by ZIKV (Li C et al. 2018a; Yu L 2017a; Wang L et al. 2019a). Antibody 7B3 and 1C11, derived from sera of convalescent patients, recognize the lateral ridge of EDIII with K394 as its key amino acid site. And 7B3 obtains a broader epitope, which recognizes residues T335, G337, E370, N371, and K394 (Niu et al. 2019). 7B3 and 1C11 showed potent and broad neutralization activities against ZIKV in different strains and protected neonatal fetal mice against lethal ZIKV infection (Niu et al. 2019). After immunization of AG129 mice with DENV1 and EDIII, monoclonal antibody (mAb) DENV1-E105 and DENV1-E106 targeting EDIII were isolated and showed neutralization activity against all four different genotypes of DENV1, with $PRNT_{50}$ ranging from 0.5 to 20 ng/mL, and completely protected against DENV1 infection in AG129 mice (Shrestha et al. 2010). The crystal structure of DENV1-E106 with EDIII reveals that DENV1-E106 recognizes the lateral ridge of EDIII, including the A-strand (K307 and K310), the B-strand terminal (K325 and Y326), BC-loop (E327, T329, and D330), and DE-loop (K361 and E362) (Edeling et al. 2014). Murine mAb DENV1-E111 recognizes the CC′ loop of EDIII and exhibits different neutralization activities to different genotypes of DENV1 (Austin et al. 2012). Murine mAb 4E11 recognizes DENV EDIII A-strand and subsequently disrupts the viral structure, leading to premature exposure of FL, which in turn inactivates the virions and inhibits infections of DENV1-4 (Cockburn et al. 2012). Fifteen murine mAbs, isolated from Balb/C mice immunized with EDIII, cross-recognize four serotypes of DENV-infected C6/36 cells and their epitopes mainly locate at 309–320 of EDIII (Li et al. 2013). Among them, 3E31 recognizes the quaternary epitope of EDIII and prevents the formation of E protein trimers, thus inhibiting infections of all four serotypes DENV (Li J et al. 2018b). The myeloid mAb m366.6, screened from a natural antibody library, showed strong affinity to EDIII of all four serotypes of DENV, inhibits DENV1-4 infections and reduces the mortality of neonatal mice infected with DENV1-4 (Hu et al. 2019). Importantly, m366.6 showed no ADE effect against different DENV serotypes.

Antibodies to EDIII show high specificities and inhibitory activities, especially against viruses of EDIII origin. Concomitantly, generation of viral escape mutants may lead to ineffectiveness of antibodies. The combination of antibodies can solve this problem. Z004 and Z021 both recognize the lateral ridge of EDIII (Van Rompay et al. 2020; Keeffe et al. 2018) with key sites at E393 and K394. When used in combination, they can inhibit viral immune escape and attenuate the damage to the rhesus fetus in infected rhesus monkeys. Another strategy is construction of bispecific antibody targeting multiple sites. 1A1D and 2A10, which target EDIII and EDII, respectively, were constructed into the bispecific antibody DVD1A1D-2A10, which targets both EDII and EDIII (Shi et al. 2016). DVD1A1D-2A10 shows neutralization activity against all four serotypes of DENV and exhibits both preventive and therapeutic effects against DENV1-4 infection in suckling mice (Shi et al. 2016). In addition, ZKA190 recognizes the lateral ridge of EDIII as well as the DI-DIII linker, which is conserved in 217 known ZIKV strains, except for V341I and E393D substitution in MR766 strain (Wang J 2017b). But ZKA190 at sub-neutralizing levels lead the production of resistant mutants at E370K after three round of selection in vitro (Wang J et al. 2017b). Then, bispecific antibody FIT-1 is constructed according to ZKA190 and ZKA185, which binds DIII, E, and VLPs and preserves the original antiviral activity of the antibodies in vivo and in vitro, without viral escape (Wang J et al. 2017b).

11.3.4 Strategies to Eliminate ADE

ADE is a challenge in the development of flavivirus therapeutic antibody. Neutralizing antibodies at sub-neutralization levels or non-neutralizing antibodies bind to viral particles to form an immune complex and mediate viral entry into host cells with Fc receptors, thus promoting viral proliferation, diffusion, and the progression of clinical disease. The above phenomenon is called ADE. To eliminate the ADE, researchers have investigated various strategies. Removal of the entire Fc region of antibodies reduces ADE, but the antiviral activity may be compromised (Wang J et al. 2017b). L234A and L235A (LALA) mutations in the Fc region of antibodies reduce or even eliminate the ability to bind to the Fc receptors and complements (Hessell et al. 2007). The introduction of LALA mutations into antibodies yields neutralizing antibodies without ADE, such as SIgN-3C-LALA (Xu et al. 2017), 2D22-LALA (Fibriansah et al. 2015), and ZKA 190-LALA (Wang J et al. 2017b). And LALA mutations can also inhibit ADE induced by other antibodies (Wang J et al. 2017b). Besides, deletion of nine amino acids (positions 231–239) in the Fc can also eliminate ADE (Goncalvez et al. 2007). For example, deletion of nine amino acids completely removes the ADE of antibody 1G5 (Lu et al. 2018). The N297 site in Fc region also has an effect on ADE, e.g. N297A (Ramadhany et al. 2015) or N297Q (Injampa et al. 2017). The N297 mutation significantly reduces the ADE, but also decreases the neutralizing activity of antibodies. These strategies enable the promising application of neutralizing antibodies for flavivirus therapy.

11.4 Peptide Entry Inhibitors

11.4.1 Peptide Membrane Fusion Inhibitors

Peptides or proteins derived from the virus itself inhibit membrane fusion by interfering with E protein conformational changes. EDIII of JEV or WNV expressed in vitro was verified to inhibit JEV (Fan et al. 2013) or WNV (Chu et al. 2005) infection. Two peptides (WN53 and WN83) from the overlapping regions of EDI/EDII junction specifically inhibit infection of WNV (Hrobowski et al. 2005). WN53 and WN83 may be involved in E protein structural rearrangements during fusion (Hrobowski et al. 2005). DN57opt from the EDII hinge region and 1OAN1 from the first β-sheet connection of EDI and EDII of DENV, inhibit DENV attaching to LLM-CK2 cells (Costin et al. 2010). P4 and P7, from DENV EDIII, inhibit viral entry into human umbilical vein endothelial cells (HUVECs) though interacting with β3 integrin (Cui et al. 2018). A series of peptides from the membrane proximal "stem" of the DNEV E protein bind viral particles, enter endosomes, and inhibit viral infection by suppressing membrane fusion (Schmidt et al. 2010a, b). The peptide MLH40 from the extracellular domain of DENV M protein has inhibitory activity against DENV1-4 (Panya et al. 2015). The molecular mimicry tests showed that the N-terminal loop of the MLH40 peptide may bind to E protein and change the dimeric structure of E protein (Panya et al. 2015).

Peptides derived from animals and plants or designed by humans also inhibit flavivirus entry. Peptide Ev37 from scorpion inhibits infections of DENV2, HCV, ZIKV, and HSV-1 though alkalinizing the acidic endosomes and inhibiting membrane fusion (Li et al. 2019). Peptide HS-1 secreted by Anuran Hypsiboas semilineatus skin inhibits viral adhesion and endocytosis and represses DENV2 and DENV3 infections of Vero cells (Monteiro et al. 2018). The antimicrobial peptide LL-37, from human neutrophil specific granules, inhibits DENV2 infection of Vero E6 cells through binding to E protein dimer (Alagarasu et al. 2017). Pep-RTYM extracted from the Asian medicinal plant Acacia catechu inhibits DENV infection of Vero cells through binding to EDIII of viral particles (Panya et al. 2020). Peptides DET4 and DET2, designed by BioMoDroid algorithm targeting EDIII, inhibit DENV2 infection of LLC-MK2 cells (Alhoot et al. 2013). TEM imaging showed that the structure and arrangement of E proteins of DENV

were altered upon these peptides binding, thus disturbing viral binding and entry into cells (Alhoot et al. 2013). Phage display library screening technique is used to obtain peptide entry inhibitors of flaviviruses, such as peptides P1 and P3 inhibiting infection of JEV (Wei et al. 2020; Zu et al. 2014), and P9 inhibiting infection of WNV (Bai et al. 2007). A cyclic 19-amino acid peptide duramycin, which binds phosphatidylethanolamine on the viral surface, prevents virus binding to TIM1 and inhibits ZIKV infection of placental cells and chorionic villus explants (Tabata et al. 2016).

11.4.2 Peptide Viral Inactivators

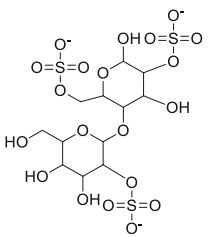
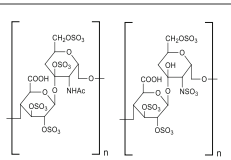
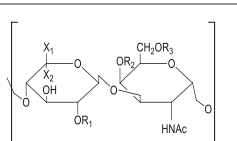
Peptides can also work as viral inactivators. DN59 from the stem region of DENV E protein inhibits infections of DENV1-4 with an IC_{50} of 2–5 μM as well as other flavivirus, including YFV, Central European encephalitis virus, forest encephalitis virus (Lok et al. 2012). DN59 binds to viral particles and punches holes at the five-fold vertices, resulting in viral genome release (Lok et al. 2012). Peptide Z2 from stem region of the ZIKV E protein also exerts antiviral effects by inactivating ZIKV viral particles (Yu et al. 2017b). Yodha from the skin of Indosylvirana aurantiaca inhibits both ZIKV and DENV1-4 though binding to viral particles and destroying the viral structure (observed by electron microscopy) (Lee et al. 2021). Yodha reduces ZIKV viral load in sera, eyes, and spleens of the anti-IFNAR1 monoclonal antibody-treated C57BL/6 mice (Lee et al. 2021). ZY13, a peptide analogue of BF-30 (antimicrobial peptide from Bungarus fasciatus snake venom), inhibits ZIKV infection in vitro and in vivo by direct inactivation of viral particles (Xing et al. 2020). Moreover, ZY13 enhances antiviral immune response through the AXL-SOCS (suppressor of cytokine signaling protein) signaling pathway (Xing et al. 2020). Peptide families with membrane interface activity, including VS peptides, BS peptides, It1b peptides, bind to viral surface proteins, resulting in viral aggregation or viral self-fusion, thus inhibiting viral entry into target cells (Hoffmann et al. 2020).

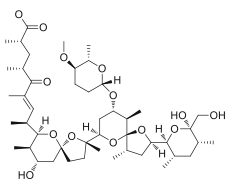
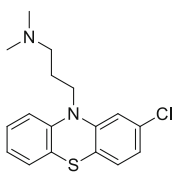
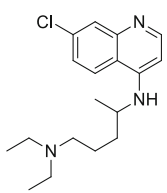
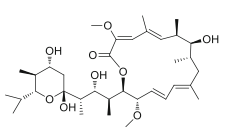
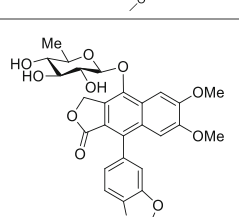
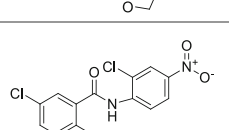
11.5 Conclusion and Prospect

Viral entry inhibitors, on the one hand, reduce the stimulatory effect of viral gene amplification and protein expression in cells; on the other hand, they can inhibit viral infection in body fluids such as blood, lymphatic, and cerebrospinal fluid without the need to enter the cells (Wang and Shi 2015), unlike inhibitors targeting viral RNA synthase or protease. Progress has been made in the study of flavivirus entry inhibitors, including small molecules, antibodies, and peptides, as described above (Table 11.1). However, there are no clinically approved flavivirus entry inhibitors and clinical trials are needed to facilitate their clinical applications.

Emerging and re-emerging flaviviruses may pose a serious threat to humans. Stockpile drugs are needed to fight the outbreaks of flavivirus unfamiliar to humans as well as to treat the endemic flaviviruses, such as DENV, ZIKV, JEV, and YFV. The wide variety of flavivirus requires a stockpile of broad-spectrum anti-flaviviral drugs. It is promising to develop the broad-spectrum anti-flavivirals targeting viral endocytosis, β-OG pocket region, and E protein trimer formation. Antibodies to the quaternary epitopes or the highly conserved fusion loop are expected to become broad-spectrum antivirals. The ADE of antibodies can be eliminated by LALA mutation or nine amino acid deletion in the Fc region. In addition, some viral inactivators can be broad-spectrum anti-flavivirus entry inhibitors, such as erythromycin estolate, CoPPIX and SnPPIX, montelukast, etc. In contrast, specific antiviral drugs may be less toxic and more effective against known or human familiar flaviviruses. Flavivirus entry inhibitors in combination with the RNA synthase and protease inhibitors may achieve better clinical outcomes, just as cocktail therapy for HIV.

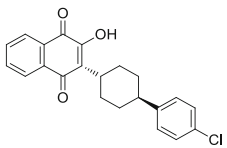
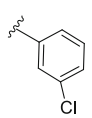
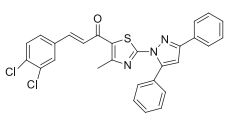
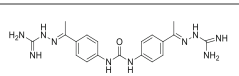
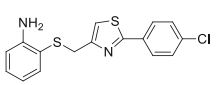
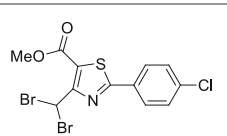
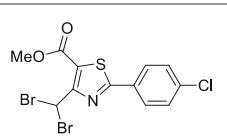
Table 11.1 Flavivirus entry inhibitors

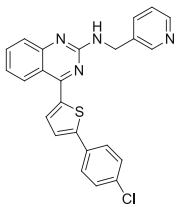
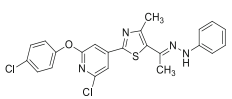
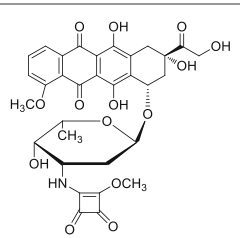
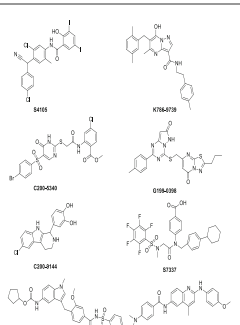
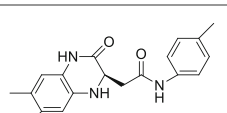
Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
λ-carrageenan	Compound		Attachment and internalization	DENV2	C6/36, Vero	0.08 μg/mL[a]	Talarico and Damonte (2016)
Curdlan sulfate	Compound		Attachment and fusion	DENV1-4	LLC-MK2, HL-60, THP1, BHK-21, C6/36	7 μg/mL (DENV1)[b] 0.262 μg/mL (DENV2)[b] 0.010 μg/mL (DENV3)[b] 0.069 μg/mL (DENV4)[b]	Ichiyama et al. (2013)
Sulfated *Escherichia coli* K5polysaccharide derivatives	Compound		Attachment and entry	DENV2	HMEC-1 or HMVEC-d	0.11 μM (K5-OS(H))[c], 0.11 μM (K5-N,OS(H))[c]	Vervaeke et al. (2013)
Chondroitin sulfate E	Compound		Attachment	DENV1-4, JEV	BHK-21, Vero	0.53 μM (DENV1)[d] 3.8 μM (DENV2)[d] 1.38 μM (DENV3)[d] 0.3 μM (DENV4)[d] 0.93 μM (JEV)[d]	Kato et al. (2010)
Carbohydrate-binding agents	Plant lectins	—	Attachment	DENV1-4	Raji/DC-SIGN$^+$ cells, Primary MDDC	4.6–92 nM (HHA)[c] 5.6–560 nM (GNA)[c] 0.29–7.0 nM (UDA)[c]	Alen et al. (2011)
Bovine lactoferrin	Protein	—	Attachment	DENV1-4	Vero/suckling Balb/C mice	165.8 μM (DENV1)[a] 40.7 μM (DENV2)[a] 166.7 μM (DENV3)[a] 164.5 μM (DENV4)[a]	Chen et al. (2017)

Nanchangmycin	Compound		Endocytosis	DENV, ZIKV, WNV	U2OS, HBMEC, JEG-3	0.1–0.4 μM (ZIKV)[e]	Rausch et al. (2017)
Chlorpromazine	Compound		Endocytosis	DENV, ZIKV, JEV	U937, K562, T98G, Vero	—	Li et al. (2020), Carro et al. (2018), Nawa et al. (2003)
Chloroquine	Compound		Endocytosis	ZIKV, DENV, JEV, WNV	Vero, HBMEC, Neural stem cells/mouse neurospheres	9.82–14.20 μM (ZIKV)[c]	Delvecchio et al. (2016)
Bafilomycin A1	Compound		Endocytosis and maturation	ZIKV	A549, SH-SY5Y	—	Sabino et al. (2019)
6-Deoxyglucose-diphyllin (DGP)	Compound		Endocytosis	ZIKV, DENV1, JEV, TBEV, WNV, EBV	Vero, HT1080, CHME3/ Ifnar1$^{-/-}$ C57BL/6 mice	0.01–0.07 μM[c]	Martinez-Lopez et al. (2019)
Niclosamide	Compound		Endocytosis	DENV2	BHK-21, Neuro-2a, A549/ICR suckling mice	10 μM[a]	Kao et al. (2018)

(continued)

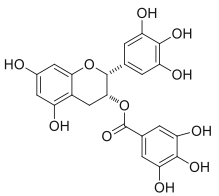
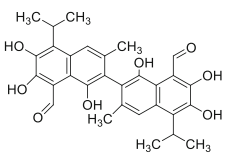
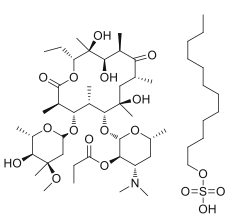
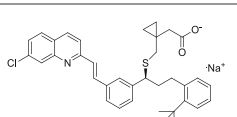
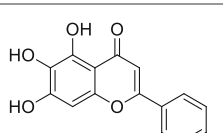
Table 11.1 (continued)

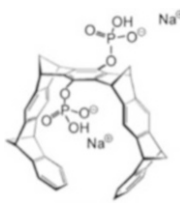
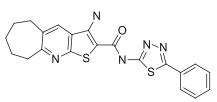
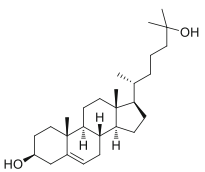
Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
Atovaquone	Compound		Fusion	DENV1-4, ZIKV, YFV, WNV, TBV, JEV	C6/36, Vero	2.1 μM (ZIKV)[f,l], 2.5 μM (DENV1)[f,l], 1.7 μM (DENV2)[f,l], 1.6 μM (DENV3)[f,l], 2.5 μM (DENV4)[f,l]	Yamamoto et al. (2020)
1662G07	Compound		Fusion	DENV1-4	BHK-21	15 μM[g]	Schmidt et al. (2012)
M02	Compound		β-OG pocket	YFV-IRES-Luc	BHK-21	51 μM[h]	Li et al. (2008), Zhou et al. (2008)
P02	Compound		β-OG pocket	YFV-IRES-Luc	BHK-21	13 μM[h]	Li et al. (2008), Zhou et al. (2008)
D03	Compound		β-OG pocket	YFV-IRES-Luc	BHK-21	31 μM[h]	Li et al. (2008), Zhou et al. (2008)
Compound 36	Compound		β-OG pocket	YFV	BHK-15	0.9 μM[h]	Li et al. (2008)
Compound 23	Compound		β-OG pocket	DENV2	BHK-21	1.32 μM[k]	Jadav et al. (2015)

Compound 6	Compound		β-OG pocket	DENV2	A549	0.119 μM[i]	Wang et al. (2009)
A5	Compound		β-OG pocket	DENV2, WNV, YFV	Vero	1.2 μM (DENV2)[d] 3.8 μM (WNV)[a] 1.6 μM (YFV)[a]	Kampmann et al. (2009)
SA-17	Compound		β-OG pocket	DENV2, YFV	BHK-21, Vero-B	0.52 μM (DENV2)[j] 4.8 μM (YFV)[j]	Kaptein et al. (2010)
S4105, K786-9739, C200-5340, G199–0398, C200-9144, S7337, S1633, C066-4182	Compound		β-OG pocket	DENV2	BHK-21	0.8 μM (S4105)[a,l], 3.1 μM (K786-9739)[a,l], 7.2 μM (C200-5340)[a,l], 8.8 μM (G199–0398)[a,l], 1.2 μM (C200-9144)[a,l], 2.7 μM (S7337)[a,l], 7 μM (S1633)[a,l], 17 μM (C066-4182)[a,l]	Lian et al. (2018)
F1065-0358	Compound		Formation of E trimers	ZIKV	Vero	14 μM[k]	Sharma et al. (2020)

(continued)

Table 11.1 (continued)

Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
EGCG	Compound		Formation of E trimers	DENV, ZIKV, WNV, JEV	Vero E6	7 μM (JEV)[k]	Vazquez-Calvo et al. (2017), Sharma et al. (2017), Wang et al. (2018)
Gossypol	Compound		EDIII	DENV1-4, ZIKV	Vero E6	1.87 μM (DENV1)[a] 1.89 μM (DENV2)[a] 3.7 μM (DENV3)[a] 2.6 μM (DENV4)[a] 0.21–4.31 μM (ZIKV)[a]	Gao et al. (2019)
Erythromycin estolate	Compound		Viral inactivator	ZIKV, DENV2, YFV	BHK-21, Vero, U251/A129 mice and pregnant C57BL/6 mice	3.22–7.65 μM (ZIKV)[a] 1.22 μM (DENV2)[a] 2.70 μM (YFV)[a]	Wang X et al. (2019b)
POM-12	Compound	—	Viral inactivator	DENV2, JEV, ZIKV	Vero	1.16 μM (DENV2)[f] 1.9 μM (JEV)[f] 0.64 μM (ZIKV)[f]	Qi et al. (2020)
Montelukast	Compound		Viral inactivator	ZIKV, DENV2, YFV	BHK-21, Vero, U251/A129 mice and pregnant C57BL/6 mice	1.13–1.88 μM (ZIKV)[a] 0.98–1.03 μM (DENV2)[a] 1.11–1.42 μM (YFV)[a]	Chen et al. (2019)
Baicalein	Compound		Viral inactivator	JEV	Vero	14.28 μg/mL[d]	Johari et al. (2012)

CoPPIX and SnPPIX	Compound	—	Viral inactivator	ZIKV, DENV, YFV	BHK-21, Vero, HeLa	2.49–10.68 μM (CoPPIX against ZIKV)[a] 0.12–7.78 μM (SnPPIX against ZIKV)[a]	Neris et al. (2018)
CLR01	Compound		Viral inactivator	ZIKV, EBOV	Vero E6	8.2 μM (ZIKV)[b]	Rocker et al. (2018)
ST-148	Compound		Targeting C protein	DENV1-4	Vero, C6/36, Huh-7, BHK-21, L929/ AG129 mice	0.012–8.918 μM (DENV)[j]	Byrd et al. (2013), Scaturro et al. (2014)
25HC	Compound		Entry	ZIKV, DENV, YFV, WNV	Vero, Balb/C mice and Rhesus Monkeys	188 nM (ZIKV)[f] 406 nM (DENV)[f] 526 nM (YFV)[f] 1109 nM (WNV)[f]	Li et al. (2017)
SIgN-3C, SIgN-3C-LALA	Human mAb	Quaternary epitope: G100 and W101 on one monomer and K310 and R323 on the other monomer in the E dimer	Fusion	DENV1-4, ZIKV	BHK-21/Adult AG129 mice, IFNAR$^{-/-}$mice (for ZIKV)	0.06 μg/mL (DENV1)[a] 0.001 μg/mL (DENV2)[a] 0.47 μg/mL (DENV3)[a] 0.075 μg/mL (DENV4)[a]	Xu et al. (2017), Kam et al. (2017)
C10	Human mAb	Quaternary epitope: intra-dimer interface of E protein	Fusion	DENV1-4, ZIKV	Vero	0.013 μg/mL (ZIKV PF13)[d] 0.063 μg/mL (ZIKV HD78788)[d] 0.54 μg/mL (DENV1)[a] 0.18 μg/mL (DENV2)[a] 1.89 μg/mL (DENV3)[a] 0.08 μg/mL (DENV4)[a]	Zhang et al. (2016), Barba-Spaeth et al. (2016)
ZIKV-195	Human mAb	Quaternary epitope: across two adjacent E monomers and fusion loop	Fusion	ZIKV	Vero/C57BL/6 male mice	77–600 ng/mL[d]	Long et al. (2019)
2D22, 2D22-LALA	Human mAb	Quaternary epitope: EDII and EDIII on adjacent E dimers or quaternary epitope centered on EDIII	Fusion	DENV2	AG129 mice	—	Fibriansah et al. (2015), Gallichotte et al. (2018)

(continued)

Table 11.1 (continued)

Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
HM14c10	Human mAb	Quaternary epitope: the adjacent surface of E dimers	Attachment and fusion	DENV1	BHK-21/ AG129 mice	5 ng/mL–1.505 mg/mL[a]	Teoh et al. (2012)
ADI-30056	Human mAb	Quaternary epitope: across the E dimers on the viral surface	Fusion	ZIKV	Vero E6	1.78 ng/mL[d]	Sevvana et al. (2020)
ZIKV-117	Human mAb	Quaternary epitope: across the E dimers on the viral surface	Fusion	ZIKV	Vero/Adult and pregnant C57BL/6 mice	5–25 ng/mL[d]	Hasan et al. (2017)
1G5, 1G5-LALA, 1G5-9del	Human mAb	Quaternary epitope: E protein of intact or bioactive DENV-1	Pre and post-attachment	DENV1-2	BHK-21/Balb/C suckling mice	0.07 μg/mL (DENV1)[a], 13.84 μg/mL (DENV2)[a]	Lu et al. (2018)
3E31 etc.	Murine mAb	Quaternary epitope: 309–320 region of EDIII	Fusion	DENV1-4	N.A	NA	Li et al. (2013), Li J et al. (2018b)
1F4	Human mAb	EDI/EDII epitope: EDI and EDI-EDII hinge	Pre- or post-attachment	DENV1	U937, Vero/ AG129 mice	0.03 μg/mL (U937)[c] 0.37–0.41 μg/mL (Vero)[a]	Fibriansah et al. (2014), de Alwis et al. (2012)
5F8	Murine mAb	EDI epitope: Glycan loop of EDI	Post-attachment	ZIKV	Vero	5.7 μg/mL (SZ-WIV01)[a] 27.1 μg/mL (PRVABC-59)[a] 41.6 μg/mL (FLR)[a]	Qu et al. (2020)
4.8A, D11C, and 1.6D	Human mAb	EDII epitope: Fusion loop	Fusion	DENV1-4, WNV	LLC-MK2	2.1– >40 μg/mL (4.8A)[d] 1.0–10.2 μg/mL (D11C)[d] 0.2–2.7 μg/mL (1.6D)[d]	Costin et al. (2013)
2A10G6	Murine mAb	EDII epitope: Fusion loop	Fusion	DENV1-4, YFV, WNV, ZIKV	BHK-21/ suckling mice, Balb/C mice	2 μg/mL (DENV1)[a] 1.5 μg/mL (DENV2)[a] 2.1 μg/mL (DENV3)[a] 1.8 μg/mL (DENV4)[a] 3.6 μg/mL (YFV)[a] 46 μg/mL (WNV)[a] 249 μg/mL (ZIKV)[a]	Deng et al. (2011), Dai et al. (2016)
ZK2B10	Human mAb	EDIII epitope: Lateral ridge of EDIII	Entry	ZIKV	Vero/ICR pregnantand AG6 mice	0.04 μg/mL[a]	Li C et al. (2018a), Yu L et al. (2017a), Wang L et al. (2019a)

7B3 and 1C11	Human mAb	EDIII epitope: Lateral ridge of EDIII	Entry	ZIKV (7B3 and 1C11), DENV1 (1C11)	Vero/neonatal SCID mice	0.0116–0.176 μg/mL (7B3 against ZIKV)[c] 0.0833–0.805 μg/mL (1C11 against ZIKV)[c] 1.72 μg/mL (1C11 against DENV)[c]	Niu et al. (2019)
DENV1-E105, DENV1-E106, etc.	Murine mAb	EDIII epitope: Adjacent of DIII; DENV1-E106 recognizes the lateral ridge of EDIII	N.A	DENV1	BHK-21, Vero/AG129 mice	0.5–20 ng/mL[a]	Shrestha et al. (2010), Edeling et al. (2014)
DENV1-E111	Murine mAb	EDIII epitope: CC′ loop of EDIII	N.A	DENV1		4–15,200 ng/mL[a]	Austin et al. (2012)
4E11	Murine mAb	EDIII epitope: EDIII A-strand	Fusion	DENV1-4	Huh7.5	0.16 μg/mL (DENV1)[c] 0.13 μg/mL (DENV2)[c] 8.0 μg/mL (DENV3)[c] 15.0 μg/mL (DENV4)[c]	Cockburn et al. (2012)
m366.6	Human mAb	EDIII epitope: EDIII	N.A	DENV1-4	BHK-21	12.74 μg/mL (DENV1)[a], 4.57 μg/mL (DENV2)[a], 5.23 μg/mL (DENV3)[a], 23.31 μg/mL (DENV4)[a]	Hu et al. (2019)
Z004 and Z021	Human mAb	EDIII epitope: Lateral ridge of EDIII	N.A	ZIKV, DENV1	Ifnar1$^{-/-}$ mice, macaques	0.7 ng/mL (Z004 against ZIKV)[a] 1.6 ng/mL (Z004 against DENV)[c] 1 ng/mL (Z021 against ZIKV) [a]	Van Rompay et al. (2020), Keeffe et al. (2018)
DVD1A1D-2A10	Murine mAb	EDII and EDIII	Attachment and fusion	DENV1-4	BHK-21/suckling mice	16.4 nM (DENV1)[a], 7.4 nM (DENV2)[a] 11.4 nM (DENV3)[a] 29.6 nM (DENV4)[a]	Shi et al. (2016)
ZKA190, ZKA190-LALA	Human mAb	Lateral ridge of EDIII and the DI-DIII linker	Attachment or fusion	ZIKV	Vero/AG129 mice and A129 mice	0.004–0.05 nM[a]	Wang J et al. (2017b)
ZKA185	Human mAb	ZKV VLP and recombinant ZIKV E	Fusion	ZIKV	Vero	0.02–0.62 nM[a]	Wang J et al. (2017b)
FIT-1	Human mAb	EDIII, E and VLPs	N.A	ZIKV	Vero/A129 mice	Similar to ZKA190 and ZKA185[a]	Wang J et al. (2017b)
D23-1G7C2-IgG-N297A	Human mAb	E	N.A	DENV2	Vero	1.18 μg/mL[d]	Ramadhany et al. (2015)
N297Q-B3B9	Human mAb	EDII epitope: Fusion loop	N.A	DENV1-4	Vero	0.125–12 μg/mL[d]	Injampa et al. (2017)

(continued)

Table 11.1 (continued)

Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
WN53 and WN83	Peptide	WN53:TFLVHREWFMDLNLPWS SAGSTVWR WN83:TFLVHREWFMDLNLPWSSA	Entry	WNV	LLC-MK2	10 μM[d]	Hrobowski et al. (2005)
DN57opt	Peptide	RWMVWRHW FHRLRLPYNPGKNK QNQQWP	Attachment	DENV2	LLC-MK2	8 μM[d]	Costin et al. (2010)
1OAN1	Peptide	FWFTLIKTQ AKQPARYRRFC	Attachment	DENV2	LLC-MK2	7 μM[d]	Costin et al. (2010)
P4	Peptide	CKIPFEIM DLEKRHV	Entry	DENV2	HUVEC	19.08 μM[k]	Cui et al. (2018)
P7	Peptide	VEPGQLK LNWFKK	Entry	DENV1 DENV2	HUVEC	12.86 μM[k]	Cui et al. (2018)
$DV2^{413-440}$	Peptide	AILGDTAWDF GSLGGVFTSIGK ALHQVF	Fusion	DENV2	BHK-21	3–4 μM[k,l]	Schmidt et al. (2010b)
$DV2^{413-447}$	Peptide	AILGDTAW DFGSLGGVFTSIGK ALHQVFGAIYGAA	Fusion	DENV2	BHK-21	1 μM[k,l]	Schmidt et al. (2010b)
$DV2^{419-447}$	Peptide	AWDFGSLGG VFTSIGKALHQ VFGAIYGAA	Fusion	DENV2	BHK-21	0.25 μM[k,l]	Schmidt et al. (2010b)
$DV2^{419-440}$	Peptide	AWDFGSLG GVFTSIGKALHQVF	Fusion	DENV2	BHK-21	3–4 μM[k,l]	Schmidt et al. (2010b)
$DENV1^{419-447}$	Peptide	AWDFGSIGG VFTSVGKLVHQ VFGTAYGVL	Fusion	DENV1-4	BHK-21	1.5 μM (DENV1)[k,l], 2 μM (DENV2)[k,l], >6 μM (DENV3)[k,l], >6 μM (DENV4)[k,l]	Schmidt et al. (2010b)
$DENV3^{419-447}$	Peptide	AWDFGSVGG VLNSLGKMV HQIFGSAYTAL	Fusion	DENV1-4	BHK-21	0.1 μM (DENV1)[k,l], 2 μM (DENV2)[k,l], 4 μM (DENV3)[k,l], 1.5 μM (DENV4)[k,l]	Schmidt et al. (2010b)
$DENV4^{419-447}$	Peptide	AWDFGSVGG LFTSLGKAVH QVFGSVYTTM	Fusion	DENV1-4	BHK-21	5 μM (DENV1)[k,l], 6 μM (DENV2)[k,l], >6 μM (DENV3)[k,l], 6 μM (DENV4)[k,l]	Schmidt et al. (2010b)
MLH40	Peptide	SVALVPHVGM GLETRTETWMS SEGAWKHVQ RIETWILRHPG	Attachment	DENV1-4	Vero, A549, Huh7	30.35 μM (DENV1)[d] 31.41 μM (DENV2)[d] 27.59 μM (DENV3)[d] 24.45 μM (DENV4)[d]	Panya et al. (2015)

Ev37	Peptide	GLINEKKVQQY LDEKLPNGVVKG ALKSLVHKA AKNQNLCA FNVDTVGMCD ADCKRQGK AKGVCHG TKCKC DVELSYKK	Endocytosis and fusion	DENV2, HCV, ZIKV, HSV-1	BHK-21	NA	Li et al. (2019)
HS-1	Peptide	FLPLILPSIV TALSSFLKQG	Attachment and endocytosis	DENV2, DENV3	Vero	NA	Monteiro et al. (2018)
LL-37	Peptide	LLGDFFRKS KEKIGKEFKRIV QRIKDFLRN LVPRTES	Entry	DENV-2	Vero E6	NA	Alagarasu et al. (2017)
Pep-RTYM	Peptide	DHVTPDIA YNPRTMY	Entry	DENV2	Vero	7.9 μM (Vero)[d]	Panya et al. (2020)
DET2	Peptide	PWLKPGDLDL	Attachment and entry	DENV2	LLC-MK2	NA	Alhoot et al. (2013)
DET4	Peptide	AGVKDGKLDF	Attachment and entry	DENV2	LLC-MK2	NA	Alhoot et al. (2013)
P1	Peptide	TPDCTRWWCPLT	Attachment	JEV	BHK-21/ C57BL/6 mice	35.9 μM[k]	Wei et al. (2020)
P3	Peptide	SENRKVPFYSHS	Attachment	JEV	BHK-21	1 μM[k]	Zu et al. 2014)
P9	Peptide	CDVIALLACHLNT	Attachment	WNV	Vero	2.6 μM[a]	Bai et al. (2007)
Duramycin	Peptide		Attachment	ZIKV, DENV	Primary placental cells	<0.1 μM[d]	Tabata et al. (2016), Richard et al. (2015)
DN59	Peptide	MAILGDTA WDFGSLGGVFT SIGKALHQVFGAIY	Viral inactivator	DENV, WNV, YFV	LLC-MK2	2 μM (DENV1)[d] 5 μM (DENV1)[d] 2 μM (DENV3)[d] 5 μM (DENV1)[d] 20 μM (YFV)[d]	Hrobowski et al. (2005), Lok et al. (2012)
Z2	Peptide	MAVLGDTA WDFGSVGGAL NSLGKGIH QIFGAAF	Viral inactivator	DENV, ZIKV, YFV	BHK-21,Vero	4 μM (DENV2)[a] 1.75–13.91 μM (ZIKV)[a] 5 μM (YFV)[a]	Yu Y et al. (2017b)

(continued)

Table 11.1 (continued)

Inhibitor	Type	Structure/Epitope/Sequence	Target	Virus	Cell line/animal model	IC_{50}/EC_{50}	Reference
Yodha	Peptide	SMLLLFFL GTISLSLCQD DQERC	Viral inactivator	DENV1-4, ZIKV	Vero/C57BL/6 mice	25 μM (DENV1)[d] 2 μM (DENV2)[d] 70 μM (DENV3)[d] 30 μM (DENV4)[d] 20 μM (ZIKV)[d]	Lee et al. (2021)
ZY13	Peptide	VKRWKKWR WKWKKWV	Viral inactivator	ZIKV	U251, Vero/ Balb/C suckling mice	1.06 μM (U251)[k] 1.81 μM (Vero)[k]	Xing et al. (2020)
VS/BS/It1b peptides	Peptide	ARVA:RRGWA LRLVLAY NATT:RRGWNLAL TLTYGRR It1b:RRGFSLK LLLSYRGWALLR LGYGRR	Viral aggregation or self-fusion	DENV2	Vero E6	0.2–2 μM[a]	Hoffmann et al. (2020)

IC_{50}/EC_{50}: half maximal inhibitory concentration (IC_{50}) and half maximal effective concentration (EC_{50}) refer to the concentration of drug causing 50% reduction of virus replication in cell based assays. The data obtained by different methods were listed as follows:
[a]Plaque reduction assay
[b]MTT assay
[c]Flow cytometry
[d]Foci forming assay
[e]Microscopy-based assay
[f]Real-time PCR
[g]Fluorescence polarization assay (FPA)
[h]Reduction of luciferase activity
[i]Cell-based flavivirus immunodetection assay (CFI)
[j]CPE reduction assay
[k]Viral yield reduction
[l]Data showed as IC_{90}
NA Not available

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Entry Inhibitors of Hepatitis B and D Viruses

12

Yang Yang and Youhua Xie

Abstract

Human hepatitis B virus (HBV) and hepatitis D virus (HDV) cause acute and chronic infections. The latter poses a serious public health threat as it is the major cause of chronic hepatitis, liver failure, cirrhosis, and hepatocellular carcinoma (HCC). In nature, HBV and HDV have a narrow host range and highly hepatotropic, only infecting the hepatocytes of humans and a few primates. The elucidation of cell entry mechanism by HBV has made great progress in recent years, which strongly promotes the establishment of new HBV infection models and the development of viral entry inhibitors. The study of HBV entry inhibitors has culminated in the first direct antiviral treatment for HDV. This review provides a concise introduction on the progress of HBV/HDV entry inhibitors in the recent years.

Keywords

HBV · Envelope proteins · Host receptor · Entry inhibitors

12.1 Introduction

Human hepatitis B virus (HBV) causes acute and chronic infections. The latter poses a serious public health threat as it is the major cause of chronic hepatitis, liver failure, cirrhosis, and hepatocellular carcinoma (HCC). It is estimated that more than 250 million people worldwide are chronically infected with HBV. Hepatitis D virus (HDV) is a satellite virus of HBV. HDV coinfection and superinfection (infection of HBV-infected persons) often lead to chronic HDV infection and manifest more serious liver diseases than HBV mono-infection.

HBV belongs to the family *hepadnaviridae* whose members are small DNA viruses that replicate through a RNA intermediate via reverse transcription. The life cycle of HBV requires non-specific and specific cellular receptors as well as cellular microenvironment suitable for viral entry, uncoating, genome repair, gene expression, replication, assembly, and secretion. In nature, HBV has a narrow host range and highly hepatotropic, only infecting the hepatocytes of humans and a few primates, which makes the selection of in vivo and in vitro infection models difficult. The host and

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tissue tropism of HDV, a RNA virus, is similar to HBV, mainly because the envelop of HDV virion is derived from HBV surface proteins. Human primary hepatocytes are terminally differentiated cells and quickly lose susceptibility to HBV infection in vitro, though new methods to prolong HBV susceptibility have been reported (Li et al. 2020). Human HCC cell lines such as Huh7 and HepG2 support HBV replication but not infection (König et al. 2019; Le et al. 2021). Differentiated HepaRG cells can be infected by HBV, but the induction of differentiation is lengthy and complex (Hantz et al. 2009). The identification of NTCP (sodium taurocholate cotransporter) by Li Wenhui's team as a functional receptor of HBV and HDV has clarified the first step of HBV/HDV entry and filled an important puzzle in the virus life cycle (Yan et al. 2012). Cell lines stably expressing NTCP, such as HepG2-NTCP or Huh7-NTCP, serve not only an effective in vitro infection model but also a platform for the development of HBV/HDV entry inhibitors. Here we will concisely review the progress of HBV/HDV entry inhibitors in recent years.

12.2 HBV/HDV Envelope Proteins

HBV encodes large (L), medium (M), and small (S) envelope glycoproteins (Fig. 12.1a). They differ at the N-terminal but share the carboxyl end (i.e., the S protein). The antigenic loop (AGL) region of S protein can bind to heparin sulfate proteoglycan (HSPG) with low affinity (Sureau and Salisse 2013a; Gripon et al. 1995). The N-terminal of L protein PreS1 (48 or 59 residues depending on genotypes) forms a high affinity binding site for hepatocyte NTCP (Schulze et al. 2010; Blanchet and Sureau 2007). The myristoylation of the Pre-S1 N-terminal is crucial for HBV infection(Schulze et al. 2010). The infection efficiency of different subtypes of HBV is affected by their PreS1 structure (Ou et al. 2021). The deletion of the 11 residues from the N-terminal of PreS1 in genotypes B and C can enhance the efficiency of virus infection (Murayama et al. 2021). After entry, HBV uncoating is carried out through unknown mechanism. HBV rcDNA (relaxed circular DNA) genome is then converted into cccDNA (covalently closed circular DNA) in the nucleus which serves as the template for viral gene expression. The newly synthesized envelop proteins can self-assemble to form spherical and rod-like subviral particles (SVP) which are secreted. L protein is absent in spherical particles and presents in low amount in rod-like SVPs. On the other hand, L proteins are crucial for the formation of infectious virions (Dane particles). The secreted SVPs outnumber the infectious virions by 1000–10000 times in the serum of HBV infected persons (Seitz et al. 2007). The assembly of HDV virion relies on HBV envelop proteins. Isoprenylation of HDV ribonucleoprotein helps its encapsidation by HBV envelope proteins. SVP formed by S protein can also encapsulate HDV nucleocapsids to form non-infectious particles (Le Duff et al. 2009; Glenn et al. 1992).

12.3 Host Receptor

HSPGs exist on various cell surfaces, which can mediate the adsorption of many viruses such as human papillomavirus, dengue virus, and SARS-CoV2 (Sarrazin et al. 2011; Nie et al. 2021; Giroglou et al. 2001; Chen et al. 1997). Two positively charged residues (Arg122 and Lys141) of AGL in the S domain can interact with negatively charged HSPGs to mediate HBV retention and promote the binding of virions to NTCP (Sureau and Salisse 2013b). Studies have shown that HBV/HDV can interact with HSPGs on the surface of hepatocytes and preferentially bind glypican 5 (Verrier et al. 2016; Lamas Longarela et al. 2013). The ensuing interaction with NTCP is thought to be the decisive factor for viral entry. NTCP is mainly expressed in the basolateral membrane of hepatocytes, and its expression is reduced in many situations such as inflammatory or proliferative state, therefore, HCC cells are largely not susceptible to HBV infection, making the cells resistant to HBV infection (Yan et al. 2019; Kang et al. 2016). In addition, NTCP expression is maintained

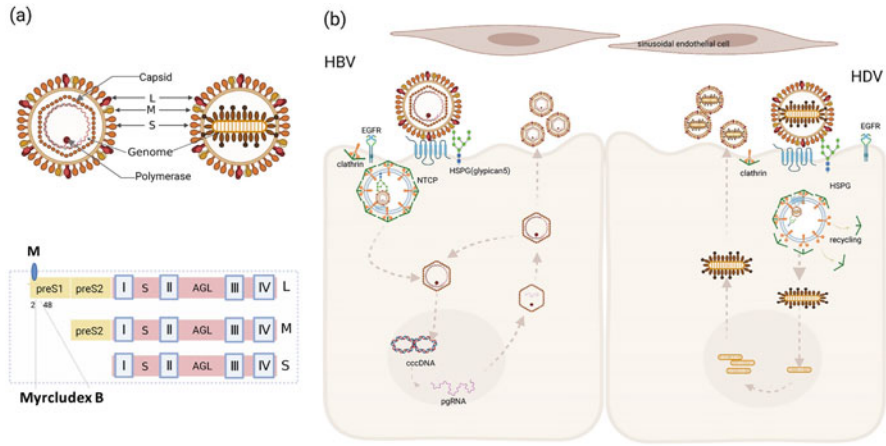

Fig. 12.1 Schematic presentation of HBV envelop proteins (**a**) and entry pathways (**b**) of HBV and HDV. (**a**) HBV encodes three envelop proteins, L, M, and S proteins. L contains preS1 and preS2 at its N-terminal while M contains only preS2. The roman numbers (*I–IV*) represent the four transmembrane regions. M, the N-terminal myristoyl moity. The myristoyl 2–48 residues of the preS1 are used as a lipopeptide inhibitor of HBV entry. (**b**) The entry pathways of HBV (*left*) and HDV (*right*)

temporarily in cultured primary hepatocytes and in HepaRG cells after prolonged induction of differentiation. Recent studies have shown that the activation of farnesoid X receptor (FXR) can inhibit the function of NTCP and then inhibit HBV infection in vivo and in vitro (Baghdasaryan et al. 2011). In vitro study of cyclosporine A (CSA) showed that the high-frequency mutation S267F of NTCP, which leads to the loss of HBV receptor function, in East Asian population had obvious ethnic differences. This may be the result of positive evolutionary selection (Peng et al. 2015; Hu et al. 2016a). However, other studies found that HBV could infect persons carrying the NTCP S267F mutation, suggesting that the virus might enter the cell through other receptors or has adapted to this host mutation (Hu et al. 2016b).

At present, little is known about the follow-up steps after HBV binding to NTCP (Fig. 12.1b). The infection efficiency of cell lines expressing hNTCP is low and successful infections require high MOI (multiplicity of infection). Some studies have shown that EGFR may be a co-factor involved in HBV internalization, and its low expression level in HepG2 may be the reason for its low infection efficiency (Iwamoto et al. 2019). E-cadherin may affect NTCP membrane localization and induce polarization by binding glycosylated NTCP (Hu et al. 2020; Schulze et al. 2012). Several studies have shown that clathrin may be involved in virus entry. HBV can be introduced into cells through the interaction among the PreS1 domain, connexin 2 and clathrin. There is still controversy about caveolin-1's role in HBV entry. Some studies have shown that HBV can initiate replicative infection through caveolin-1 pathway in HepaRG cells (Macovei et al. 2010). Another study showed that HBV may use clathrin mediated endocytosis to invade HepG2-NTCP cells, independent of macropinocytosis or caveolin mediated endocytosis (Herrscher et al. 2020a). Silencing caveolin-1 in HepG2-NTCP cells did not reduce HBV infection, while silencing clathrin heavy chain, dynamin-2 and adaptor protein 2 reduced HBV infection, and clathrin encapsulated HBV particles were observed under electron microscope(Herrscher et al. 2020b).

12.4 HBV/HDV Entry Inhibitors

Entry inhibitors targeting HBV virion: highly effective hepatitis B immunoglobulin (HBIG) is derived from vaccine-immunized healthy donor blood. HBIG is mainly used in high-risk HBV

infection situations such as exposure to HBV, mother to child transmission, liver transplantation, and so on. HBIG preparation is difficult to scale up due to limited blood supply and there exists the potential contamination of blood-borne pathogens. Whole human HBV neutralizing antibodies have the advantages of low immunogenicity and high neutralization activity. However, overcoming escape mutants and high cost are problems await solutions (Hong et al. 2004; Wi et al. 2017; Li et al. 2017; Zhang et al. 2016). Short peptides binding to HBV PreS1 also prevent HBV infection of primary human and tupaia hepatocytes (Ye et al. 2016).

Entry inhibitors targeting HSPG: HSPG is widely distributed in various cell membranes. HSPG-interacting molecules can block the parking of a variety of viruses. Studies have found that synthetic lipopolysaccharide peptides can inhibit the entry of several enveloped viruses such as HIV, HSV, HBV, and HCV, with good tolerance and few adverse effects (Krepstakies et al. 2012). Human lactoferrin derived peptides 1–23 contain GRRRR cationic clusters, which can neutralize the negative residues of the virus envelope and prevent the adhesion and invasion of HBV(Florian et al. 2013).

Entry inhibitors targeting NTCP: the myristoylated peptide composed of the N-terminal 47 residues of the HBV preS1 domain (aa 2–48 of genotype D Pre-S1) serves as an inhibitor that blocks HBV interaction to NTCP, which is the only drug approved for clinical treatment of HBV/HDV co-infection (commercial name Bulevirtide or Myrcludex B). Myrcludex B is also about to undergo a clinical trial for the treatment of chronic hepatitis B single infection (Masetti and Aghemo 2021; Kang and Syed 2020, Trial No. nct02888106). The inhibitor can compete with the natural hepatitis B L protein to bind NTCP and inhibit the entry of the virus. The decrease in serum HBsAg level in previous Myrcludex B treatment is not obvious. The reason may be that the extracellular inhibition effect of the inhibitor is not sufficient or lasting and/or the inhibitor has little effect on the production of SVPs. Myrcludex B showed antiviral effect on HDV infection. After 48 weeks of treatment, the level of HDV RNA decreased and the biochemical indexes of patients could be improved (Loglio et al. 2019). The use of Myrcludex B may affect the transport of bile acids. Animal experiments showed that long-term use of Myrcludex B resulted in elevated serum bile acid, elevated bilirubin, gallstone, thickening, and solidification (Mao et al. 2021). There are also studies on blocking NTCP receptor function using human monoclonal antibody hh-003 (Chinese clinical registration No. ctr20191480). Screening chemicals that targeting NTCP identified several compounds, including irbesartan, rosiglitazone, zafirlukast, triac, sulfasalazine (Donkers et al. 2017), ezetimib (Lucifora et al. 2013) and cyclosporin A derivatives, that can bind to NTCP and inhibit bile acid transport. Some derivatives of cyclosporin A can irreversibly bind to NTCP to inhibit HBV entry, which may also inhibit NTCP related transporters, resulting in hyperbilirubinemia (Crabbé et al. 2009). Future studies on new cyclosporin A analogues aim to identify new compounds that inhibit HBV entry while do not significantly affect bile acid metabolism.

12.5 Perspectives

The elucidation of cell entry mechanism by HBV has made great progress in recent years, which strongly promotes the establishment of new HBV infection models and the development of viral entry inhibitors. The study of HBV entry inhibitors has culminated in the first direct antiviral treatment for HDV in 2020. Nevertheless, the entry and follow-up steps postentry of HBV/HDV are still largely unknown and needs to be clarified. Since HBV cccDNA can be replenished through traffick of newly formed nucleocapsid back into the nucleus, it is unlikely that entry inhibitors alone can eliminate cccDNA. Combination therapy targeting multiple steps in the life cycle of HBV will probably generate the best antiviral efficacy.

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Entry Inhibitors of Hepatitis C Virus

13

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Abstract

Hepatitis C virus (HCV) infection affects approximately 1% of the world's population and is a major cause of chronic liver diseases. Although antiviral therapy consisting of direct-acting antivirals (DAAs) can cure the majority of HCV patients, it is still limited by viral resistances, drug–drug interactions, and high costs. Moreover, the role of DAAs in the prevention of occurrences of graft reinfection in HCV patients who receive liver transplantations is still under comprehensive clinical investigation, bringing the risk of recipient reinfection. HCV entry is composed of initial non-specific attachment and binding, post-binding interactions with essential host factors, internalization, and virion-cell membrane fusion to release viral RNA to cytosol. Thus, a number of novel and promising targets from either virion or cellular factors of these processes become optimal interfering elements for antiviral therapy, eliminating viral infection at the very beginning. Therefore, entry inhibitors can be supplemented into the future treatment regimens to optimize and widen the prevention and therapeutics of HCV infection. This chapter introduces the basic HCV entry processes and summarizes molecular mechanisms and research status of the current antiviral agents targeting HCV entry in preclinical and clinical study.

Keywords

Hepatitis C virus · Antiviral agent · Entry factor · Entry inhibitor · Hepatocyte

13.1 Introduction

Hepatitis C virus (HCV) belongs to the family Flaviviridae, and is a positive sense single-stranded RNA virus with a genome of 9.6 kb which is replicated in host cytoplasm. The viral capsid is surrounded with envelop glycoprotein E1 and E2, together with serum-derived lipoproteins. HCV causes about 71.1 million chronic infection cases worldwide according to WHO's estimation, and is one of the major public health problems globally which consumes large amounts of medical expenses every year (Spearman et al. 2019). HCV has seven identified genotypes and more than 60 subtypes, along with millions of quasispecies. Since the virus genome is highly variable, it is difficult to manufacture effective vaccines to prevent the disease from spreading. Of all acutely infected patients, about 70% will finally develop into chronic infections even they are rendered with advanced medical care. Therefore, HCV infection becomes one of the major causes of liver-associated diseases, including steatosis, fibrosis, cirrhosis, or even

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hepatocellular carcinoma after a long period of disease progression. The end-stage HCV patients of liver failure need liver transplantation to maintain life (Liang and Ghany 2013). Unfortunately, graft reinfection is very common in clinics due to the lack of blockage strategies (Mederacke and Von Hahn 2011). WHO has launched a global program aiming to eliminate HCV before 2030, but only a minority of countries have implemented proper measurements. Of all HCV infected patients, only 20% get their diagnosis, and approximately 15% of diagnosed patients have been well treated.

The recommended therapy for HCV infection decades ago was a regimen composed of PEGylated interferon alpha and ribavirin with a cure rate only covering 70–80% of genotype 2 or 3 HCV patients and 45–70% of genotype 1, 4, 5, or 6 HCV patients (Ghany and Liang 2013). The long treatment durations and combined adverse reactions further result in unsatisfactory treatment efficacy. In recent years, application of direct-acting antivirals (DAAs) increases the cure rate of HCV patients to more than 95% with less side effects and treatment durations, making hepatitis C (HC) become a potentially curable disease (Lazarus et al. 2021). Licensed regimens include sofosbuvir/daclatasvir (Hepcinat Plus), sofosbuvir/ledipasvir (Harvoni), elbasvir/grazoprevir (Zepatier), sofosbuvir/velpatasvir (Epclusa), glecaprevir/pibrentasvir (Mavyret), and sofosbuvir/velpatasvir/voxilaprevir (Vosevi), and these all-oral DAAs are well tolerated and widely accepted by patients. The current marketed DAAs basically target viral nonstructural proteins such as NS3/4A protease inhibitor, NS5A inhibitor, or NS5B polymerase inhibitor. Although the DAA-contained therapies significantly elevate the cure rate of HCV patients, the remarkably high medical expenses, drug–drug interactions (DDIs), and potential emergence of viral resistance are still major limitations that must be resolved (Gao et al. 2021). DAAs can cause DDIs with several drugs, since most DAAs take part in common metabolic pathways, influencing the efficacy and toxicity of these drugs. Meanwhile, HCV resistant strains are easy to develop under antiviral treatments since the RNA virus replicates in an error-prone manner. Besides, the ability of DAAs to prevent HCV infection especially in liver graft reinfection remains to be determined, since they only act after established HCV infection now.

HCV entry is the initial step of the virion to interact with the host cells, and consists of multiple processes which are important to start viral infection and spread. The main processes include the initial low-affinity attachment of the virus to be concentrated on the surface of hepatocytes, the following specific interactions between virus and host entry factors during post-binding period, viral endocytosis through clathrin-mediated route, and a virion-cell membrane fusion triggered by low pH to release HCV RNA for subsequent post-entry life cycle (Fig. 13.1) (Qian et al. 2016), providing a number of potential targets for HCV treatment. Inhibition of HCV infection at the very beginning before viral genomes amplification is an effective prophylactic measurement to restrain both viral spread and cell-to-cell transmission. Entry inhibitors include antibodies, small molecular compounds of either natural or synthesized products and peptides, most of which target conserved host receptors or enzymes essential for HCV entry. Commonly speaking, these targets have relatively higher genetic barriers, avoiding drug resistance more easily. Therefore, most entry inhibitors cover pan-genotypic virus infection and resistant strains, and are able to possess synergistic effect when combined with anti-HCV drugs of other classes. However, these entry inhibitors might cause strong cytotoxicity potentially during the application**.** Viral components especially the glycoproteins E1 and E2 also serve as potential targets for HCV entry inhibitors. Polyclonal immunoglobulin Civacir®, targeting E1/E2, has completed a phase III trial (NCT01804829), and is confirmed to be efficient in preventing HCV reinfection in 80 liver transplant patients. It could broadly neutralize HCV pseudoparticles and cell culture-derived virus variants of all the main

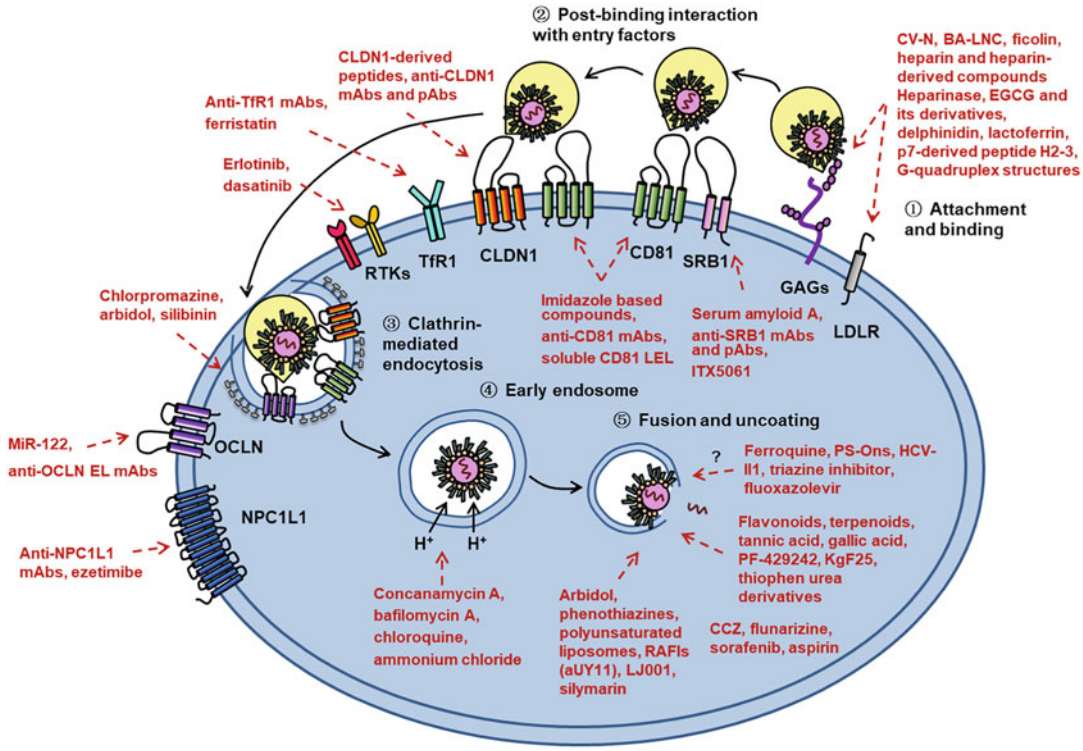

Fig. 13.1 The specific progress of HCV entry into host cells and related anti-HCV drugs targeting essential host factors or key entry steps (Qian et al. 2016). HCV LVPs are recruited and concentrated on the surface of host cells by binding to non-specific receptors such as GAGs and LDLR. Subsequent to binding, LVPs interact with multiple entry factors and co-factors including SRB1, CD81, CLDN1, OCLN, RTK family EGFR and EphA2, TfR1, as well as NPC1L1. The virions are then internalized via clathrin-mediated endocytosis and fused with cellular endosome membrane in a low pH-dependent environment. The representative antivirals and their targeted factors or effecting steps during HCV entry are indicated in *red*

genotypes isolated from patients (Tawar et al. 2016). Additionally, entry inhibitors are able to block viral infection at the initial step of life cycle, rendering them unique properties of protecting naïve host cell from infection, and they might also possess synergistic effect in the combination therapy with current marketing anti-HCV drugs especially DAAs due to their different therapeutic targets from other drugs (Qian et al. 2016)**.** The development of HCV entry inhibitors will expand the existing area of antiviral therapy and provide a great deal of optimal targets and insights into anti-HCV interventions, and finally accomplish the clearance of viral infection. This chapter summarizes the current developing entry inhibitors.

13.2 HCV Entry into Host Cells

13.2.1 Initial Non-specific Attachment and Binding

In vivo, HCV virion particles circulate as "lipo-viro particles" (LVPs) and reach the basal layer of host hepatocytes. The virus here binds to several non-specific host receptors, which allows the virions to be concentrated on the surfaces of hepatocytes and primed to interact with subsequent specific entry receptors (Fig. 13.1). The non-specific receptors consist of glycosaminoglycans on heparan sulfate (GAGs-HS), low-density lipoprotein receptor (LDLR), and newly identified human hepatitis A virus cellular

receptor 1 (HAVCR1). The interaction of GAGs with HCV is mediated at least partially by apolipoprotein E (apoE) on virion surface. LDLR is capable of interacting with apolipoproteins on LVPs as well as viral envelope proteins, and facilitate further binding to the host cells. HCV-HAVCR1 interaction is important for efficient viral entry. This protein may stabilize viral attachment and promote subsequent post-entry interaction with other entry factors. LDL and VLDL are wrapped outside HCV virions to form LVPs in the circulation of infected patients, which may contribute to viral evasion from neutralizing antibodies of host immune system. Besides, LDLR also helps HCV to attach on the surface of target cells. Small interfering RNAs (siRNAs) targeting this receptor reduce virus infectivity significantly, and a soluble LDLR is able to block virus binding by interacting with virion particles before they reach host cells (Albecka et al. 2012). The long-chain fatty acyl-coenzyme A is also reported to have inhibitory effect on viral attachment by targeting virus-associated lipoproteins (Li et al. 2020). Although HCV pseudoparticle (HCVpp) which is free of lipid or cholesterol can accomplish efficient viral entry, productive HCV entry and replication still need the important participation of LDLR. Recent studies have also suggested that HCV-HAVCR1 interaction especially with phosphatidylserine (PS) exposed on HCV envelope may stabilize HCV attachment and promote subsequent post-entry interaction with other entry factors. Therefore, HAVCR1 and its binding ligand, PS may serve as novel targets to interfere viral cell attachment and subsequent HCV infection (Wang et al. 2017).

The lectin cyanovirin-N (CV-N), proteins griffithsin (GRFT), and scytovirin (SVN) are types of carbohydrate-binding agents which can suppress HCV infection efficiently. The high-mannose oligosaccharides of CV-N, GRFT, and SVN can interact with viral envelop proteins and impede virus attachment (Table 13.1) (Helle et al. 2006). Boronic acid-modified lipid nanoparticles (BALNCs) are also recognized by researchers to be effective inhibitors of HCV entry in a manner similar to lectins so that they are usually utilized as pseudolectin-based agents in the development of therapeutic HCV entry inhibitors (Khanal et al. 2015). Ficolins are one type of serum collectins protein which can be found in the circulatory system of chronically-infected HCV patients with neutralizing concentrations. Additionally, researchers also find recently that HCV particles can be neutralized directly by recombinant human L-ficolin thus inhibiting viral binding through the blockage of HCV glycoproteins E1 and E2 (Hamed et al. 2014). Heparin mimics the structure of HS and suppresses virus attachment by competitive binding to host cells before the virus. A number of heparin-derived compounds are under careful investigation to evaluate their potential capacity of serving as potent HCV entry inhibitors. An enzyme compound, heparanase, which can dissemble HS on host cell surfaces also hinders the attachment of HCV E2 as well as HCVcc to be concentrated on target cells (Table 13.1) (Barth et al. 2006).

Epigallocatechin gallate (EGCG) and its derivatives are natural polyphenol compounds that are abundant in green tea extracts and have long been considered to regulate lipid metabolism, thereby having the potential to affect a variety of diseases. Studies suggest that EGCG and its derivatives impair virus binding to the host cell by interfering with virion E1/E2 function and simultaneously blocking cell-to-cell transmission in vitro (Table 13.1) (Ciesek et al. 2011; Calland et al. 2015). Limited sampling estimates of EGCG in HCV patients suggest that a single oral dose of up to 400 mg of this green tea extract is safe and well tolerated (Halegoua-De Marzio et al. 2012). Further data are needed to investigate the clinical application potential of this compound for anti-HCV therapy. Additionally, delphinidin, a polyphenol plant pigment is reported to reduce HCV attachment by inducing conformational changes on viral E1 and E2 glycoproteins against all genotypes (Calland et al. 2015).

The iron-binding glycoprotein lactoferrin (LF) is also found to be an efficient HCV entry inhibitor. LF is a member of the transferrin family, and abundant especially in milk and other biological body fluids. The antiviral function of

Table 13.1 Effecting steps, targets, and development stages for entry inhibitors (Qian et al. 2016)

Effecting step	Exact target	Representative drugs	Development stage	References
Attachment		Lectin cyanovirin-N	Cell culture	Helle et al. (2006)
		BA-LNC	Cell culture	Khanal et al. (2015)
		Ficolin	Cell culture	Hamed et al. (2014)
		Heparin and heparin-derived compounds	Cell culture	Barth et al. (2006)
		Heparanase	Cell culture	Barth et al. (2006)
		EGCG and its derivatives	Cell culture	Ciesek et al. (2011)
		Delphinidin	Cell culture	Calland et al. (2015)
		Lactoferrin	Phase I	El-Fakharany et al. (2013)
		A p7 ion channel-derived peptide H2-3	Cell culture	Hong et al. (2015)
		Lipoquads forming G-quadruplex structures	Cell culture	Koutsoudakis et al. (2017)
Post-binding interactions with entry factors	SRB1	Serum amyloid A	Cell culture	Cai et al. (2007)
		Anti-SRB1 pAb and mAb	Mouse model	Catanese et al. (2007), Lacek et al. (2012)
		ITX5061	Phase I/IIa	Zhu et al. (2012)
	CD81	Imidazole-based compounds	Cell culture	VanCompernolle et al. (2003)
		Anti-CD81 mAbs	Mouse model	Vanwolleghem et al. (2008), Ji et al. (2015)
		Soluble CD81 LEL	Mouse model	Molina et al. (2008), Kapadia et al. (2007)
	CLDN1	CLDN1-derived peptide	Cell culture	Si et al. (2012)
		Anti-CLDN1 mAb and pAb	Mouse model	Fofana et al. (2010), Mailly et al. (2015)
	OCLN	MiR-122	Cell culture	Sendi et al. (2015)
		Anti-OCLN EL mAb	Cell culture	Shimizu et al. (2018)
	EGFR	Erlotinib	Phase I/IIa	Lupberger et al. (2011)
	EphA2	Dasatinib	Cell culture	Lupberger et al. (2011)
	TfR1	Anti-TfR1 mAbs	Cell culture	Martin and Uprichard (2013)
		Ferristatin	Cell culture	
	NPC1L1	Anti-NPC1L1 mAbs	Cell culture	Del Campo et al. (2012)
		Ezetimibe	Mouse model	Sainz et al. (2012)
Clathrin-mediated endocytosis		Chlorpromazine	Cell culture	Blaising et al. (2013b)
		Arbidol	Cell culture	Blaising et al. (2013b)
		Silibinin	Cell culture	Blaising et al. (2013a)
Fusion and uncoating	Endosome acidification	Concanamycin A	Cell culture	Meertens et al. (2006)
		Bafilomycin A	Cell culture	Meertens et al. (2006)
		Chloroquine	Cell culture	Blanchard et al. (2006)
		Ammonium chloride	Cell culture	Blanchard et al. (2006)
	Lipid composition of virus or host cell	Arbidol	Cell culture	Boriskin et al. (2008)
		Phenothiazines	Cell culture	Chamoun-Emanuelli et al. (2013), Uddin and Downard (2018)
		Polyunsaturated liposomes	Cell culture	Pollock et al. (2010)
		RAFIs (aUY11)	Cell culture	Colpitts et al. (2013)
		LJ001	Cell culture	Wolf et al. (2010)
		Silymarin	Cell culture	Wagoner et al. (2010)
	Compounds with possible	Ferroquine	Cell culture	Vausselin et al. (2013)
		PS-ONs	Mouse model	Matsumura et al. (2009)

(continued)

Table 13.1 (continued)

Effecting step	Exact target	Representative drugs	Development stage	References
	or unclear mechanisms	HCV-II1	Cell culture	Bush et al. (2014)
		Triazine inhibitor	Cell culture	Baldick et al. (2010)
		Fluoxazolevir	Mouse model	Ma et al. (2020)
Natural compounds and small molecules		Flavonoids, terpenoids, tannic acid, gallic acid, PF-429242, KgF25, thiophen urea derivatives	Cell culture	Haid et al. (2012), Lin et al. (2015), Yu et al. (2013), Blanchet et al. (2015), El-Tantawy and Temraz (2020), Ryu et al. (2021)
Virion components		Anti-E1/E2 pAb	Phase III	Meuleman et al. (2011)
		Anti-apoE Ab	Cell culture	Liu et al. (2012)
		ApoE-derived peptides	Cell culture	Liu et al. (2012)
Marketing drugs		CCZ	Phase Ib	Hu et al. (2020)
		Flunarizine, sorafenib, aspirin	Cell culture	Perin et al. (2016), Descamps et al. (2015), Yin and Zhang (2016)

LF is believed to be through direct interaction with virion particles so as to interfere viral attachment to target cells both in vitro and animal models (Redwan et al. 2014b). Bioactive peptides, such as the N-lobe or C-lobe of LF, also inhibit virus infection (Redwan et al. 2014a). Among all species, camel lactoferrin (cLF) shows the most effective antiviral property and is now being evaluated in a clinical trial (Table 13.1) (El-Fakharany et al. 2013).

The p7 is a polypeptide encoded by HCV genome and located in the membrane of endoplasmic reticulum. Due to its essential function in prolific viral infectious production, p7 becomes a promising target for anti-HCV agents. A peptide named H2-3 which is derived from ion channel of p7 is found recently to block HCV entry potently by directly inhibiting viral binding to host cells and interfering virus–host interaction (Table 13.1) (Hong et al. 2015). Besides, lipoquads, G-quadruplex DNA structures fused to cholesterol, are reported to be efficient HCV pan-genotypic entry and cell-to-cell transmission inhibitors (Koutsoudakis et al. 2017).

13.2.2 Post-Binding Interactions with Essential Host Entry Factors

Subsequent to viral attachment and binding which are likely quite unspecific events, the concentrated LVPs start to interact with multiple specific entry factors on host cells with their envelope glycoprotein domains. Entry inhibitors targeting these host factors with relatively high genetic barriers and indispensable roles in the early life cycle of virus infection extend the development of prosperous anti-HCV agents. HCV post-binding interaction needs participation of several host factors in a rather regulated manner, including the four main host factors of scavenger receptor class B type 1 (SRB1), tetraspanin molecule CD81, the tight junction (TJ) proteins claudin-1 (CLDN1) and occludin (OCLN), and co-factors of transferring receptor 1 (TfR1), the receptor of tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2), as well as cholesterol uptake receptor Niemann-Pick C1-like 1 (NPC1L1) (Qian et al. 2016) (Fig. 13.1). Expression of these main host factors one or more can confer cell susceptibility to HCV infection. At the same time, co-factors also play important roles in HCV entry. These factors bind viral glycoproteins and act sometimes in the form of co-receptor complex to trigger the subsequent entry process.

SRB1 is a glycoprotein with horseshoe shape and close relation in host lipid metabolism. It binds several lipoproteins including HDL, LDL, and oxLDL and plays important roles in cellular cholesterol uptake, mediating viral entry into host cells (Baugh et al. 2013). The extracellular loop

of SRB1 is believed to interact with the hypervariable region 1 (HVR1) of HCV E2 and is indispensable for efficient viral entry during binding and post-binding steps. SRB1 is also thought to prime HCV to interact with subsequent CD81 which has important roles in the subsequent entry process. The expression level of SRB1 also correlates with HCV internalization rates. Serum amyloid A (SAA) is an important acute-phase protein widely utilized in clinics. Current research indicates that the close relation between SAA and HDL might affect HCV infectivity. SAA binds SRB1 and is internalized by this receptor, and HCV entry is therefore impaired by the interaction between SAA and the virus (Table 13.1) (Cai et al. 2007). Antibodies targeting SRB1 are demonstrated to impede virus entry and spread both in vitro and in vivo (Table 13.1) (Catanese et al. 2007; Lacek et al. 2012). ITX5061 is a preclinical small molecule anti-HCV compound which hinders viral infection by blocking the uptake of HDL through SRB1. Besides, this compound is also found to possess a synergistic effect when combined with DAAs in cell culture, indicating its prospective potential in future combination therapy (Zhu et al. 2012). ITX5061 has just finished a phase Ib pharmacology toxicological trial study and is now being evaluated in a phase II clinical trial in HCV patients (Table 13.1). In gentype1 patients, ITX5061 treatment before and after transplant and daily for 1 week thereafter induces a sustained reduction in HCV RNA levels compared to the control group.

CD81 is the first identified entry factor of HCV infection with systemic research. It is critical for viral post-binding steps by its interactions with HCV glycoprotein (Pileri et al. 1998). This transmembrane protein has a molecular weight of 26 kDa and is ubiquitously expressed in host cells, consisting of a large and a small extracellular loop (LEL and SEL). The LEL of CD81 is thought to interact with HCV E2 protein and facilitates HCV infection directly. And the interaction could further activate RTKs of EGFR which causes HRas activation to promote viral internalization and interaction with another essential entry factor CLDN1 on hepatocytes (Zona et al. 2013). Small molecule inhibitors such as imidazole-based compounds potently block HCV entry in vitro. They simulate the D-helix of CD81 and abolish the function of this essential receptor selectively during viral entry. Meanwhile, most physiological functions of CD81 are still preserved, indicating that the clinical application of these compounds might be safe (Table 13.1) (VanCompernolle et al. 2003). Monoclonal antibodies (mAbs) specifically targeting the key epitopes of CD81, for instance JS-81 or the recently developed K04, interfere the interactions between E2 and CD81, blocking viral entry during post-binding steps and suppress establishment of HCV infection in humanized mice model (Table 13.1) (Vanwolleghem et al. 2008; Ji et al. 2015). Furthermore, a soluble CD81 LEL-derived recombinant peptide is also shown to be an effective HCV entry inhibitor, impeding the entry of HCVpp, HCVcc, as well as patient-derived HCV in vivo (Table 13.1) (Molina et al. 2008; Kapadia et al. 2007). Nevertheless, the cellular toxicity issues should not be ignored especially for the utilization of CD81 antibodies or targeted compounds since CD81 is a ubiquitously distributed host factor in all tissues.

CLDNs and OCLNs all belong to the TJ family. The CLDN1 and CD81 complex is thought to be contributive to efficient viral internalization (Farquhar et al. 2012). Study shows that human embryonic kidney 293 cells could get cell susceptibility of HCVpp entry after the expression of CD81, SRB1, and CLDN1. As CLDN1 is highly expressed in HCV-targeted hepatocytes, antiviral agents specifically targeting this host factor would get ideal and promising effects and compensate for the development of prophylactic intervention. It is composed of four transmembrane domains which form two extracellular loops of EL1 and EL2. CLDN1 EL1 is highly conserved and makes direct interaction with viral E1 glycoprotein to promote HCV entry. A human CLDN1-derived peptide (CL58) is identified to inhibit HCV infection during post-binding step while the cellular TJ integrity remains unchanged (Table 13.1) (Si et al. 2012). CLDN1 mAbs and pAbs are also demonstrated to have effective antiviral activities on HCV infection, the

mechanism of which might involve the neutralization of E2-CD81-CLDN1 complex with low cellular toxicity both in primary human hepatocytes (PHHs) and humanized mice models (Table 13.1) (Fofana et al. 2010; Mailly et al. 2015). The lead antibody has been successfully humanized for further clinical investigation (Colpitts et al. 2018), as it might be able to prevent graft reinfection in HCV-infected transplant recipients, and supplement current anti-HCV therapy. Administration of CLDN1 antibodies manifests no side effects on liver or other organs in various animal models, confirming their application safety. However, a recent study finds that HCV can utilize CLDN6 instead of CLDN1 in the host cells, making it possible that the virus could escape from CLDN1 Abs or specifically targeted drug, and this situation should be taken into account in future CLDN usage of HCV inhibitors (Haid et al. 2014). OCLN is also essential for efficient HCV entry. Human CD81 and OCLN expression in mouse hepatocytes provides the originally non-susceptible cells with HCV permissivity, while knockdown of OCLN blocks viral entry during a late post-binding step (Dorner et al. 2013). It also has four transmembrane domains forming two extracellular loops of EL1 and EL2. The EL2 of OCLN is a key element to promote HCV entry by interaction with GTPase dynamin II which plays a role in mediating viral endocytosis. Although OCLN does not have direct interactions with viral envelope proteins like CLDN1, it acts during a similar period as CLDN1. Recent study finds that miR-122 overexpression in host cells perturbs HCV entry probably by downregulating the expression and distribution of OCLN (Sendi et al. 2015). Monoclonal antibodies targeting either OCLN EL1 or EL2 completely prevent HCV infection into host cells and a mouse model without apparent side effects (Shimizu et al. 2018). And it seems that mAb targeting EL2 has a more potent effect than the one targeting EL1, demonstrating the unique function of EL2 in HCV entry.

EGFR and EphA2, two well-studied RTKs are screened out through a functional siRNA kinase library to be essential host co-factors of HCV entry (Lupberger et al. 2011). EGFR plays a key role in the regulation of CD81-CLDN1 association, and recruitment of clathrin, therefore enhancing viral internalization for efficient HCV entry. The two factors distribute highly in human hepatocytes, and inhibitors specifically targeting these two kinases such as erlotinib (an EGFR inhibitor) and dasatinib (an EphA2 inhibitor), two marketing anti-cancer drugs, significantly inhibit HCV entry in both hepatoma cells and PHHs (Lupberger et al. 2011). The antiviral activity of erlotinib is also demonstrated in human-liver chimeric mice, perturbing viral entry which is mediated by SRB1-dependent high-density lipoproteins (Table 13.1) (Lupberger et al. 2011). Besides, these two clinically approved RTKs inhibitors interfere the formation of CD81-CLDN1 complex and impede cell-to-cell transmission as well, realizing the blockage of HCV entry during post-binding steps. Therefore, the RTKs become promising targets for the development of HCV entry inhibitors with the distinctive advantage of preventing graft reinfection in end-stage chronic HCV patients who have to take the liver transplantation. Nevertheless, further study is still needed to evaluate the efficacy and safety of the clinically licensed RTKs inhibitors to be applied as members of HCV therapeutics.

Study shows that an iron metabolic is frequently observed in clinical case of HCV patients (Kaito 2007). Transferrin receptor 1 (TfR1) is highly expressed in human hepatocytes which functions as an iron uptake receptor, and its trafficking protein (TTP) is reported to play a role in HCV entry. Inhibitors such as ferristatin, or mAb specifically targeting TfR1 inhibit HCV infection during the initial entry step when the treatment is carried out before virus inoculation (Table 13.1) (Martin and Uprichard 2013). Further kinetic assay demonstrates that TfR1 promotes viral entry during a post-binding period, most likely between the viral interaction and association of E2-CD81-CLDN1 and clathrin-mediated endocytosis. However, the functional region of TfR1 during HCV entry has not been identified yet, requiring more intensive study to decipher its exact mechanism before related targeted interventions being developed. Furthermore, TfR1 antibody or inhibitor cannot reduce viral

cell-to-cell transmission entirely, narrowing down its application potential in HCV treatment.

NPC1L1 is a cholesterol transport receptor with 13 transmembrane domains, and widely distributed on the surfaces of human enterocytes and hepatocytes to mediate uptake and modulate homeostasis of cholesterol in the body (Altmann et al. 2004). The expression level of NPC1L1 is related to HCV infection. Antibodies specifically targeting the LEL1 of NPC1L1 significantly impede viral entry during post-binding process, suggesting that the receptor functions in similar ways as other entry factors (Del Campo et al. 2012). NPC1L1 antagonist ezetimibe is a clinically approved drug in the treatment of hypercholesterolemia. It is found to block viral entry and cell-to-cell transmission in vitro most likely by perturbing NPC1L1 internalization (Sainz et al. 2012). In vivo, ezetimibe suppresses HCV genotype 1 infections in human-liver chimeric mice (Table 13.1) (Sainz et al. 2012). Further studies are needed to determine the therapeutic effect in clinical application.

13.2.3 Clathrin-Mediated Endocytosis and Subsequent Membrane Fusion

Immediately after the post-binding interaction with the essential host factors, the virion particles along with several entry factors, such as CD81 and CLDN1, are internalized into cytoplasm through a highly coordinated way, probably the clathrin-mediated endocytosis**.** The CD81-CLDN1 complex trafficking effectively facilitates coexisting viral internalization and fusion, and the early and late endosomes are formed after internalization to prepare for the following virion-cell membrane fusion. Specific inhibitors such as chlorpromazine or siRNAs specifically targeting the heavy chain of clathrin effectively inhibit the formation of clathrin-coated pit, thus blocking HCVpp entry and HCVcc infection in vitro (Table 13.1). Arbidol, a clinically approved anti-influenza drug in China and Russia, is a derivative of indole which is believed to inhibit HCV cell entry through several approaches. It interferes with clathrin-mediated endocytosis by hindering the release of clathrin-coated pit and impairs membrane scission induced by dynamin-2 (Table 13.1) (Blaising et al. 2013b). A flavonolignan compound silibinin interferes with membrane scission mediated by dynamin2, thereby suppressing clathrin-mediated endocytosis of HCV (Blaising et al. 2013a).

Subsequent to endocytosis, the virion–host cell membrane fusion takes place immediately to release viral genome into the cytosol for the following viral protein translation and RNA replication. It is a critical entry step for envelope viruses like HCV. The envelope proteins (noncovalent heterodimer of HCV E1 and E2 glycoproteins) are activated by the interactions between virus and host entry factors especially the CD81-E2 association to become susceptible to low pH environment, triggering the fusogenic conformational changes of the related peptides under the acidic environment and proper temperature of the endosomal lumen (Qian et al. 2016). Indeed, the mechanism concerning how E1-E2 heterodimer mediates membrane fusion still needs to be further elucidated, limiting rational designs targeting the HCV fusion process. Nevertheless, E2-derived peptides are found to block viral infection during a post-binding process (Liu et al. 2010). HCV fusion needs the facilitation of lipid components, such as cholesterol and sphingomyelin (SM). Studies suggest that HCV fusion is promoted under the treatment of cholesterol. Moreover, according to the related research that HCVcc which has the lowest particle density, exhibits the highest fusogenicity among all types of HCV virions.

HCV fusion inhibitors are classified specifically according to their action modes into three types. The inhibitors of first type interfere with the acidification environment which is an essential triggering element of virion-cell membrane fusion. The acquainted-known acidification inhibitors consist of bafilomycin A and concanamycin A which suppress the function of vesicular ATPases to lead the inhibition of acidification (Table 13.1) (Meertens et al. 2006). Other similar inhibitors include chloroquine and ammonium chloride, interfering with endosome

acidification and thus impeding membrane fusion process in a dose-dependent manner (Table 13.1) (Blanchard et al. 2006). The membrane inhibitors of second type target viral or host lipid compositions, which play important roles in the entire proceeding of viral fusion. The above endocytosis inhibitor arbidol is also capable of blocking viral membrane fusion, through a dual-binding mode that links viral glycoproteins in their aromatic residues with phospholipids, therefore hindering the essential conformational change formation of viral fusion peptides necessary in the process of virion–host cell fusion (Table 13.1) (Boriskin et al. 2008). Recently, phenothiazine-derived compounds which are featured with nitrogen and sulfur tricyclic structures such as benzhydrylpiperazines, aminoquinolines, and curcumin have been demonstrated to be effective entry inhibitors of HCV infection (Table 13.1)**.** These small molecule compounds inhibit virion–host cell membrane fusion either by integrating into the cholesterol-rich membranes of target cells to enhance the membrane fluidity, or binding in the vicinity of a putative fusion domain, destabilizing the pre-fusion state of the virus (Chamoun-Emanuelli et al. 2013; Uddin and Downard 2018). Polyunsaturated liposomes could decrease cellular cholesterol levels in HCV-infected cells, and thus blocking viral fusion (Pollock et al. 2010). Synthetic phospholipids analog rigid amphipathic fusion inhibitors (RAFIs) such as representative AUY11 are able to interact with viral envelope lipid components and make the viral fusion proteins hard to be activated, resulting in the inhibition of the additive negative curvature during the primary viral fusion stage and reduction of the membrane fluidity (Table 13.1) (St Vincent et al. 2010; Colpitts et al. 2013). Small molecule LJ001 is an entry inhibitor to suppress viral fusion through the specific incorporation into viral membranes, preventing the virion from the pre-fusion state (Table 13.1) (Wolf et al. 2010). Silymarin is composed of multiple flavonolignans and flavonoid taxifolins. The treatment of silymarin blocks HCV infection both in vitro and in animal models through a similar effecting mechanism of arbidol (Table 13.1) (Wagoner et al. 2010). The third group of fusion inhibitors include several compounds that interact with viral components critical in fusion process with possible or unclear mechanisms. A chloroquine analog called ferroquine is found to interact with viral E1 glycoprotein, and amphipathic DNA polymers of phosphorothioate oligonucleotides (PS-ONs) most likely interfere with viral fusion step, all of which have potent antiviral activity against HCV infection both in vitro and in vivo (Vausselin et al. 2013; Matsumura et al. 2009). HCV infectivity inhibitor-1 (HCV-II1) is believed to cage viral envelope proteins in a pre-fusion conformation, thus inhibiting HCV fusion (Bush et al. 2014). A triazine inhibitor is reported to interact with E2 and inhibit HCV entry during pre-fusion process at post-binding step (Baldick et al. 2010). Fluoxazolevir is an aryloxazole-based fusion inhibitor of HCV. It inhibits HCV infection in humanized chimeric mice by binding HCV E1 to prevent viral fusion with host cells (Ma et al. 2020) (Table 13.1).

Some natural, plant-derived or synthesized compounds are also found to be potent entry inhibitors of HCV infection. Phenolic compound flavonoid ladanein inhibits viral infection during a post-attachment entry step, and is effective against all major HCV genotypes. Combined administration of ladanein with a post-entry antiviral agent has a synergistic effect in the inhibition of HCV infection (Haid et al. 2012). Terpenoids saikosaponin is an efficient entry inhibitor by neutralizing virus particles, preventing viral attachment to host cells, and inhibiting viral fusion, probably through the mechanism of acting on HCV E2. Moreover, saikosaponin blocks viral infection by several genotypic strains, and especially inhibits daclatasvir-resistant mutant strains in combination with daclatasvir (Lin et al. 2015). Natural oleanane-type triterpenes are found to have an inhibitory effect on HCV entry. Subsequent modifications further enhance these compounds' potency in suppressing HCV entry. Mechanism study reveals that the functional triterpenes exhibit the antiviral activity by interrupting the interaction between HCV E2 glycoprotein and CD81 via binding to E2, thus hampering the virus

and host cell recognition (Yu et al. 2013). Tannic acid, derived from a plant, is a polymer of gallic acid and glucose molecules. This polyphenol has a potent inhibitory effect on HCV entry in in vitro study, suppressing the docking of HCV virions on host cell surfaces, and also blocks viral cell-to-cell transmission in infectious cell cultures. Small molecule compound PF-429242 is an inhibitor of SKI-1/SIP and could inhibit HCV infection at the early steps of virus life cycle (Blanchet et al. 2015). The action modes of the compound involve two different routes. One is SREBP/SKI-1/S1P dependent, relating LDLR and NPC1L1, while the other is SREBP independent. KgF25 from *khaya grandifoliola* C.DC has antiviral activities against HCV both in entry and post-entry steps. The inhibitory effect of KgF25 on viral entry is pan-genotypic, and is through direct inactivation of free viral particles (El-Tantawy and Temraz 2020). Thiophen urea derivatives possess antiviral activity against major HCV genotypes during viral entry step, and the lead compound is found to interact with HCV E2 glycoprotein to exert its inhibitory effect (Ryu et al. 2021) (Table 13.1). However, the exact antiviral mechanisms of these compounds and their potential applications in clinical practice require further investigation and evaluation.

Besides, targeting virion components is also a prophylactic strategy to block HCV infection from the very beginning. Polyclonal immunoglobulins against HCV have been found to suppress HCV infection in human-liver chimeric mice (Meuleman et al. 2011) and are under evaluation in a clinical trial of preventing graft reinfection in HCV-positive recipients (NCT01804829). ApoE is detected on the surfaces of HCV particles, and is reported to directly interact with HCV E2 to enhance the viral infectivity. Therefore, antibodies targeting this virion-associated component could disturb HCV binding, and peptides derived from human apoE which contain both a receptor binding fragment and a lipid binding fragment also specifically block the entry of HCV particles (Liu et al. 2012).

Furthermore, a series of marketing drugs which are used in clinics have also been shown to inhibit HCV infection during the initial entry step. Anti-histamine compound chlorcyclizine HCl (CCZ) is a member of over-the-counter drugs to alleviate allergy symptoms. Studies show that CCZ impedes HCV infection at a late stage of viral entry, probably during fusion process by targeting HCV glycoprotein E1 to form interaction with E1 putative fusion peptide, and is currently being evaluated in a phase Ib clinical trial (Hu et al. 2020). Flunarizine, a piperazine-derived drug approved for treating migraine, inhibits HCV fusion by targeting E2 and a potential fusion peptide in E1 (Perin et al. 2016). Sorafenib is an inhibitor of multiple kinases which has been clinically used to treat patients who are suffered from hepatocellular carcinoma. Sorafenib is found to inhibit HCV entry as well as efficient viral production by affecting the CLDN1 expression and localization (Descamps et al. 2015). Aspirin, a clinically applied remedy for anti-platelet and analgesic treatment, suppresses HCV entry by decreasing the expression level of CLDN1 (Table 13.1) (Yin and Zhang 2016). These marketing drugs are comparatively safe and available agents with affordable medical expenditure, saving massive time for drug development, thus repurposing of these drugs for novel antiviral candidates is a prospective way to optimize current anti-HCV therapy.

13.3 Conclusions and Perspectives

The molecular mechanisms of HCV entry have been deciphered clearly in recent studies, which contributes greatly for the identification of antiviral targets throughout this process and the development of related entry inhibitors during different periods of the initial viral life cycle. On the account of the unique features of viral entry to initiate HCV infection, as well as to contribute to viral spread and maintenance, entry inhibitors are provided with great advantages and promising perspectives to be utilized as components in future HCV cocktail therapy. Besides, many entry inhibitors, including some natural plant-derived compounds might have low productive costs, making it possible for all needed patients to

receive proper anti-HCV therapy. Despite numerous entry inhibitors have been identified in recent years, the majority of them are still under evaluation in cell cultures or animal models. So far, only a small fraction of the drugs have progressed into clinical stages, of which ITX5061 is the best-known and most advanced entry inhibitor, targeting HCV essential host factor SRB1 to block viral entry process. ITX5061 is now being evaluated in a phase II clinical trial and seems to be an ideal option for future HCV cocktail therapy. Even if we do not consider the final outcome of ITX5061 to be a mature anti-HCV drug, the study process of this compound sets a good example for entry inhibitors development. Furthermore, repurposing novel therapeutic direction of currently marketing drugs with favorable antiviral potencies against HCV is an effective and economical way to screen for novel HCV entry inhibitors. The data in Table 13.1 include the effecting targets and study stages of current anti-HCV agents during viral entry steps.

Differed from the currently clinically applied DAAs which target viral nonstructural proteins, most entry inhibitors interfere with conservative host factors essential for viral entry with relatively high genetic barriers, providing them with unique advantages of avoiding the emergence of viral escapes. However, the cytotoxicity issues of these drugs need careful evaluations. Since HCV of different genotypes are considered to enter host cells in a similar way, most entry inhibitors inhibit viral infection independent of virus genotypes. Besides, entry inhibitors tend to be capable of preventing cell-to-cell transmission if their antiviral mechanisms involve the inhibition of the entry elements necessary in both cell-free infection and cell-to-cell spread, and thus helping end-stage HCV patients resist from graft reinfection after liver transplantation. Numerous novel entry inhibitors have been identified recently and are under intensive investigations in cell cultures or animal models experiments. Some of them have even been evaluated in clinical HCV-positive patients to figure out the exact in vivo efficacy. The most qualified and satisfying entry inhibitors should comply with the goodness of complementing current anti-HCV interventions, bringing more effective, economical and safer antiviral agents with better tolerance for HCV patients, especially the refractory patients.

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Human Papillomavirus (HPV) Entry Inhibitors

14

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Abstract

Chronic or persistent human papillomavirus (HPV) infection is essential for the development of many types of carcinomas, such as cervical carcinoma. Developing new diagnostic and prognostic biomarkers and designing more effective targeted therapeutics and treatment strategies remains urgent. Numerous efforts have been made to design new drugs and vaccines to treat HPV infections. Due to the special HPV infection pathway, entry inhibitors to block viral entry into target cells have been extensively and deeply studied. This chapter reviews the basic characteristics of HPV infection and the various types of HPV entry inhibitors, which were found to have high safety, potent antiviral effects, and broad-spectrum activity against multiple HPV subtypes. Together with the use of prophylactic vaccines, the development and application of these entry inhibitors will reduce the incidence of HPV infection and associated cancers in the future.

Keywords

HPV · Entry inhibitor · Cervical cancer · Viral infection · Viral surface protein

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14.1 HPV Structure and Encoded Proteins

Human papillomavirus (HPV) is characterized as a non-enveloped, double-stranded DNA virus whose genome encodes six early regulatory proteins (E1, E2, E4, E5, E6, and E7) and two late structural proteins (L1 and L2) (zur Hausen 2002). Based on genome sequences, more than 200 officially recognized HPV subtypes have been identified and classified into five genera (Doorbar et al. 2015). The mature HPV virion exhibits icosahedral symmetry, with a diameter of approximately 60 nm. Its major capsid protein L1, which has a molecular weight of ~55 kDa, has the ability to self-assemble into virus-like particles (VLPs). Five L1 proteins form a stable star-shaped pentamer, and 72 L1 pentamers form a regular VLP in a $T = 7$ icosahedral lattice (Buck and Trus 2012). In some cases, 12 L1 pentamers can form a small VLP in a $T = 1$ icosahedral lattice, with a diameter of approximately 30 nm (Chen et al. 2000). The minor capsid protein L2 has a molecular weight of 64–78 kDa and lacks the capacity to form VLPs. L2 can enhance the assembly of L1 into VLPs and facilitate encapsidation of the ~8 kbp viral genome during assembly of mature virions.

The structure of HPV virions and capsid pentamers has been extensively studied, revealing their assembly details and the specific functions of viral capsid proteins. The 72 pentamers interact with each other by invading arms and disulfide

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bonds to form knobby virions (Buck and Trus 2012). The L1 protein is made up of an eight-stranded β-jellyroll structure named the "nucleoplasmin-like" structure. In HPV-16, Cys175 in the invaded L1 protein binds to Cys428 in the invading L1 protein to form a disulfide bond, joining the neighboring capsomers. These cysteine residues are highly conserved among different HPV subtypes (Buck et al. 2005; Modis et al. 2002). The L1 protein plays a key role in viral binding to the host cell surface, so it is the major target for entry inhibitor design.

There are empty holes located inside and beneath the L1 pentamer, which are believed to be L2 binding sites (Buck et al. 2008). The amino (N)- and carboxyl (C)-termini of neighboring L2 molecules are packed together, indicating L2 function during the assembly and entry phases of the papillomavirus life cycle. The copy number of L2 in the HPV capsid is indistinct, while the upper estimation is one L2 monomer per L1 pentamer (Bissett et al. 2016). L2 binds to L1 through its C-terminal conserved proline-rich motif (PxxP), which might facilitate the bending and looping of L2 inside the L1 pentameric cavity (Fahey et al. 2009). L1-L2 VLP complexes could be formed only when these two proteins were co-expressed, implying that L2 binds to the L1 pentamer prior to capsid assembly (Mallon et al. 1987). Additionally, L2 is required to interact with the viral genome and cell surface receptors, making it an important research object for HPV entry inhibitors (Wang and Roden 2013). Moreover, L2 was also found to play a role in cytoskeleton adhesion, viral conformational changes, subcellular trafficking, and degradation of endosomal membranes (Fahey et al. 2009).

The six early regulatory proteins have important regulatory functions during HPV lifecycles. (1) E1, which is involved in the regulation of viral replication and transcription, is the largest and most conserved protein in the HPV genome (Borgogna et al. 2012). It has an N-terminal domain for replication regulation, a central DNA-binding domain and a C-terminal hexametric helicase domain (Bergvall et al. 2013). The presence of E1 is mandatory for genome amplification in the viral life cycle. (2) The E2 protein is composed of an N-terminal transactivation domain and a C-terminal DNA dimerization domain. The E2 protein can help the E1 helicase load onto the replication origin, enhance replication complex assembly, and stabilize HPV replicons (McBride 2013). (3) The E4 protein has variable size among different HPV subtypes, and it is involved in the virion production process and is regarded as a biomarker for active HPV infection (Wang et al. 2011). (4) The E5 protein is a small transmembrane protein that plays important roles in carcinogenesis and immune evasion processes in HPV-infected cells. It can downregulate the expression of antigen presentation-related cell surface proteins (Campos-Leon et al. 2017). (5) The E6 protein has approximately 150 residues that form two zinc-like finger domains and is regarded as a major oncoprotein that inactivates the p53 gene in host cells (Filippova et al. 2007). (6) The E7 protein is approximately 100 residues in length and shares sequence similarity with adenovirus E1A proteins in the N-terminal region. Its major function is to maintain the viral genome in host cells and to facilitate cellular proliferation, angiogenesis, and malignant progression (McLaughlin-Drubin et al. 2005).

14.2 HPV Classification and Subtypes

HPV can be classified according to different criteria. Since the L1 ORF is highly conserved in the HPV genome, it is used for the identification and taxonomic classification of HPV subtypes. HPV virions with <60% sequence similarity in the L1 region are considered to belong to different genera, while those within 60–70% similarity belong to different species (Bzhalava et al. 2015). To date, there are five known HPV genera, α-papillomavirus (65 subtypes), β-papillomavirus (51 subtypes), γ-papillomavirus (79 subtypes), μ-papillomavirus (3 subtypes), and ν-papillomavirus (3 subtypes).

Different HPV subtypes cause different clinical diseases, including warts, intraepithelial

neoplasia, and even cancers. Many HPV types, such as the majority of HPVs in the β and γ genera, can cause only asymptomatic infection in immunologically capable individuals. Some low-risk subtypes (lrHPVs) (e.g., HPV-6 and HPV-11) can cause epithelial diseases, such as condyloma acuminatum, while some high-risk subtypes (hrHPVs) can cause tumors (e.g., HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) (Rautava and Syrjanen 2012). Persistent hrHPV infection is a major risk factor for precancerous lesion formation and carcinogenesis in related sites, leading to stagnation of cell differentiation and out-of-control cell cycle regulation and causing development of most cancers and precancerous lesions in the cervix, penis, vulva, vagina, anus, head and neck, and other sites. The key differences between lrHPV and hrHPV are believed to be the expression and function of their E6/E7 proteins (Doorbar 2006; Pett et al. 2004).

HPV generally infects two types of tissues in the human body, the skin and mucosa. On this basis, HPV can be classified into two categories: the cutaneotropic and mucosotropic subtypes. Cutaneotropic HPVs, including HPV-1, 4, 5, 8, 41, 48, 60, 63, 65, etc., usually cause cutaneous lesions and plantar warts in immunocompromised patients. Mucosotropic HPVs, including HPV-6, 11, 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69, 73, 82, etc., are always associated with benign and malignant lesions of the anogenital tract (Vashisht et al. 2019).

14.3 HPV Life Cycle

The study of the HPV life cycle is important for the development of viral entry inhibitors. HPV infection is a complex multi-step process. The major target cells for HPV infection are keratinocytes in the basement membrane (BM), the only mitotic active cell type in the epithelium tissue. At present, it is generally believed that HPV needs to infect basal cells through epithelial cell injury (Wilkinson et al. 2002). Once in contact with the BM, the L1 protein first interacts with heparan sulfate proteoglycan (HSPG) (which may include growth factor receptors or α6 integrins) on the host cell, and then a series of conformational changes are triggered by multiple proteases and chaperones (e.g., furin), enabling the L1 and L2 proteins to bind to different receptors (Richards et al. 2006). For example, cyclophilin B and proprotein convertase promote L2 protein cleavage, increasing L2 N-terminus exposure, then virions can bind to L2-specific receptors, such as annexin A2/S100A10 heterotetramer (A2t) (Raff et al. 2013).

Subsequently, virions are internalized through a non-traditional endocytosis mechanism (named micropinocytosis) in either a clathrin- or caveolae-dependent manner. Before viral DNA enters the nucleus for replication, the virion has been shown to be transported via endosomes, the trans Golgi network (TGN), the Golgi complex, and the endoplasmic reticulum (ER) (Aksoy et al. 2017). The viral DNA can interact with microfilaments via the motor protein dynein, followed by transport of this complex to the nucleus. Once HPV DNA is integrated into the host genome, autonomous replication is responsible for the development of persistent infection. HPV does not enter the blood to cause systemic viremia or destroy epithelial cells (Senapati et al. 2016).

The transmission of HPV infection usually occurs by direct contact with the skin or mucous membrane of the infected individual or sometimes by contact with contaminated objects. HPV can evade the host immune response and establish long-lasting infection. Its life cycle occurs entirely in differentiated keratinocytes, which are eventually shed from the epithelial surface by terminal differentiation. During HPV infection, there is always no viremia, cell lysis, cell death, inflammation, or inflammatory cytokine expression. In this way, the virus can evade the immune surveillance of the host. Moreover, hrHPV can disrupt innate immune signaling pathways in host cells. The viral E7 protein inhibits the initiation of the cellular signaling cascade by binding to STING and inhibiting downstream production of type I interferon (IFN). E5, E6, and E7 proteins block the type I IFN and proinflammatory signal transduction pathways to inhibit Janus kinase/signal

transduction and transcriptional activator (JAK/STAT) and nuclear factor κB (NF-κB) signaling. E6 and E7 inhibit the formation of host inflammatory complexes by reducing the secretory expression of the proinflammatory cytokines IL-1 and IL-1B (Della Fera et al. 2021).

14.4 HPV-Related Diseases

HPV infections are predominant in underdeveloped regions of the world, such as south-eastern Asia, Latin America, and sub-Saharan Africa, with an incidence rate of approximately 19–30% (de Martel et al. 2017). HPV usually causes unapparent infection. Even if there is no clinical appearance, the virus can still be detected using medical techniques. HPV usually does not have cytolytic activity, cause cell death, produce viremia, or induce the local inflammatory process. As a result, the natural humoral immune response against HPV infection is usually very weak and does not produce neutralizing antibodies (NAbs) (Tommasino 2014).

The diseases caused by HPV are closely related to the sites of infection (Vashisht et al. 2019). For example, condyloma acuminatum (CA) is a sexually transmitted disease caused by HPV infection in the cervix, vagina, vulva, urethra, perianal region, anal canal, and other parts. It is related to a variety of HPV subtypes, but 90% of CA is caused by the HPV-6 and 11 subtypes. For skin, major clinical manifestations include common warts (HPV-1, 2, 4, 40, etc.), plantar warts (HPV-1, 2, 4, etc.), flat warts (HPV-3, 10, etc.), filiform warts (HPV-2), pigmented warts (HPV-4, 60, and 65), periungual warts (HPV-1, 2, and 4), Butcher's warts (HPV-7), pityriasis-like plaques (HPV-5, 8, etc.), squamous cell carcinomas of sun-exposed skin (HPV-5, 8, etc.), etc. For the anogenital tract, major clinical manifestations include CA (HPV-6, 11, 42, 81, etc.), giant condyloma acuminatum (HPV-6 and 11), Bowenoid papulosis (HPV-16, 55, etc.), erythroplasia of Queyrat (EQ) (HPV-8 and 16), vaginal, vulvar, penile, and anal intraepithelial neoplasia (HPV-16, 18, etc.), etc. For the oral cavity, major clinical manifestations include oral squamous papilloma (HPV-2, 6, 7, 11, 16, 18, 32, and 57) and focal epithelial hyperplasia (HPV-13, 32, etc.). For the respiratory tract, eye, digestive tract, and breast, the major clinical manifestations are laryngeal/glottal papillomatosis (HPV-6, 11, etc.), conjunctival papillomatosis (HPV-6, 11, etc.), oesophageal papilloma (HPV-16 and 18), and breast papillomatosis (HPV 18, etc.), respectively.

Persistent HPV infection may lead to various cancers, such as cervical cancer (HPV-16, 18, etc.), vulvar and vaginal cancer (HPV-16, 18, 33, etc.), anal cancer (HPV-16, 18, etc.), penile cancer (HPV-16, 18, etc.), head and neck cancer (HPV-16, 18, etc.), oesophageal cancer (HPV-16 and 18), lung cancer (HPV-16, 18, etc.), and breast cancer (HPV-16, 18, and 33) (Vashisht et al. 2019). Cervical cancer is the most common gynecological malignancy. In 2020, cervical cancer had the fourth highest number of new cases and the fourth highest mortality among cancers in women worldwide (World Health Organization 2020). The HPV-16 and 18 subtypes are the most prevalent types causing cervical cancer worldwide. Skin cancer is closely associated with infection by hrHPV subtypes, and rates of infection with detectable HPV DNA in the normal skin of healthy adults can be up to 80% (Antonsson et al. 2000). HPV-16 DNA was detected in 19% of non-melanoma skin cancer samples from squamous cell cancer or basal cell cancer (Pierceall et al. 1991). In addition to the above diseases, HPV is also associated with oropharyngeal cancer, juvenile laryngeal papilloma, lung cancer, oesophageal cancer, and prostate cancer (Westra and Lewis 2017; zur Hausen 2002). With the development and application of HPV vaccines and viral inhibitors, the incidence of HPV-related diseases will be significantly reduced in the future.

14.5 HPV Vaccines

Animal experiments have proven the possible effect of prophylactic HPV vaccines, such as vaccines made of purified bovine papillomavirus virus for cattle (Jarrett et al. 1990) and formalin-

inactivated virus for dogs (Bell et al. 1994). However, as papillomaviruses cannot grow in culture, it was difficult to find a proper source of antigens for developing human HPV vaccines. Later, it was found that the recombinantly expressed L1 protein was able to produce VLPs, and this kind of VLP could induce the production of protective NAbs (Breitburd et al. 1995). Further studies examined the neutralizing activity of polyclonal antisera and found that the protection was HPV-type specific, so vaccines need to be multivalent (Frazer 2014).

To date, there are three major prophylactic HPV vaccines available in many countries: 2-, 4-, and 9-valent vaccines. They consist of purified HPV L1 proteins that self-assemble to form HPV type-specific VLPs. The 2-valent vaccine is designed against HPV-16 and HPV-18; the 4-valent vaccine against HPV-6, HPV-11, HPV-16, and HPV-18; and the 9-valent vaccine against HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58 (World Health Organization 2017). Several clinical trials have evaluated the efficacy and safety of HPV-targeted vaccines, among which six large phase III clinical trials are regarded as landmarks for HPV vaccine development, 2 for the 2-valent vaccine (Hildesheim et al. 2014; Paavonen et al. 2007), 3 for the 4-valent vaccine (Garland et al. 2007; Giuliano et al. 2011; Group FIS 2007), and 1 for the 9-valent vaccine (Joura et al. 2015). These clinical results showed that the HPV vaccines could prevent infection and disease related to the corresponding HPV subtypes and generate an antibody response to those subtypes. However, these vaccines did not prevent infection and disease related to other HPV subtypes.

In addition, the demand for clearance of established HPV infection has prompted research on therapeutic vaccines, including live vector, nucleic acid, peptide-based, protein and cellular vaccines, with several vaccine candidates currently in clinical trials (Liu et al. 2019b). However, there are currently no approved therapeutic HPV vaccines for clinical usage. Therefore, specific anti-HPV agents, especially broad-spectrum antiviral drugs, are still needed to treat HPV infections.

14.6 Chemical Antivirals that Inhibit HPV Replication

Traditional chemical antivirals play important roles in the treatment of viral infections, mainly targeting the enzymes encoded by the viral genome. However, HPV hijacks the host replication system during its life cycle, providing few targets inside virus-infected cells for traditional drug design. Certain acyclic nucleoside phosphonates (ANPs), such as cidofovir, were found to have anti-HPV potential by inhibiting DNA polymerase and slowing DNA replication (Johnson and Gangemi 1999). Cidofovir is more likely to be activated in HPV-infected cells than in uninfected cells, and the replication process of the viral genome is more susceptible to chain-terminating factors than that of the human genome. A phase II clinical trial showed that those who adopted topical cidofovir reported a 60.8% response rate, compared with 20% in the control group (Van Pachterbeke et al. 2009), providing an alternative treatment for patients with concerns regarding post-operative complications. Recently, the cidofovir derivative ODE-Bn-PMEG was designed and found to be effective in inhibiting HPV-11, 16, and 18 replication (Beadle et al. 2016), making it a promising candidate for the local treatment of HPV infection.

In addition to ANPs, HPV E1/E2-targeting antivirals, such as indandiones, have also been found to be effective against HPV-6 and 11 (White et al. 2003). Further modification of indandiones may allow them to inhibit additional HPV subtypes. E1-specific inhibitors, such as biphenysulfonacetic acid, may affect ATP-binding ability and reduce viral genome replication (White et al. 2005). Some small-molecule inhibitors were screened against E6 and E6AP, which may provide new insights into drug development for HPV (Baleja et al. 2006). Furthermore, HPV-associated host proteins may also serve as effective drug targets to design host-dependent viral inhibitors. For example, class I histone deacetylases (HDACs) are HPV E7-associated proteins and are responsible for

viral long-term episome maintenance (Longworth and Laimins 2004). Cyclin-dependent kinase (Cdk) 2 is stimulated by E7, promotes cellular proliferation and accelerates viral genome amplification (Hebner and Laimins 2006). The cellular transcription factor Sp1 can bind to the viral genome and promote HPV transcription (Gloss and Bernard 1990). Inhibitors of these HPV-related host proteins were found to effectively inhibit HPV infection and have potential in the clinical treatment of HPV-related diseases (Liu et al. 2019b).

14.7 HPV Entry Inhibitors

The replication cycle of HPV has three major stages: the virus enters host cells, the viral genome replicates in host cells, and finally, the packaged progeny virus is released from the host cells. The first entry stage is the initial step of the viral replication cycle, making it the ideal target to design specific prevention and treatment antiviral agents, that is, "keep the enemy out of the country." Based on the characteristics of the HPV structure and cell receptors, variable entry inhibitors with unique mechanisms of action have played important roles in the treatment of HPV infections. These inhibitors can be generally classified into compounds, peptides, proteins, and polysaccharide inhibitors, which are described below.

14.7.1 Small Compounds

Caffeic acid is a small molecule compound widely found in a variety of agricultural products, and it has a wide range of antiviral activities, such as inhibition of human immunodeficiency virus (HIV)-1 and herpes simplex virus (HSV)-2-proliferation in vitro. A recent study found that caffeic acid could block HPV entry into target cells. It acts at the early stage when the virus attaches to the target cell, possibly by binding to the L1 protein to prevent the interaction of the virus and cell receptor. It can effectively inhibit different subtypes of HPV infection with an IC50 (half-maximal inhibitory concentration) range of 12.1–16.5 μg/ml. It is expected to be developed into a new type of HPV infection inhibitor (Ding et al. 2018).

It has been reported that A2t heterotetramer facilitates infectious entry of HPV into epithelial cells through the interaction between the S100A10 subunit of A2t and the viral L2 capsid protein (Woodham et al. 2012). Therefore, A2t is a promising target to design viral entry inhibitors against HPV. For example, two inhibitors of A2t (A2ti) that specifically disrupt A2t heterotetramer formation were identified and investigated for their ability to prevent HPV-16 infection in vitro, named A2ti-1 and A2ti-2 (Woodham et al. 2015). A2ti-1 and A2ti-2 inhibited the interaction between S100A10 and its binding partner annexin A2 with IC50 values of 24 μM and 230 μM, respectively. Both A2ti compounds were found to be non-toxic at the maximum concentrations tested and did not affect cell growth after 72 h. According to HPV internalization and infection assays, A2ti-1 significantly inhibited HPV-16 pseudovirus (PsV) infection of HeLa cells in a dose-dependent manner, with ~100% inhibition activity at 100 μM, while A2ti-2 had weaker activity against HPV-16 PsV infection, with <50% reduction at 100 μM. Based on isothermal titration calorimetry (ITC) and fluorescence assays, A2ti was found to target the S100A10 dimer of A2t and block HPV-16 entry into target cells. This result highlights the importance of A2t in HPV infection and confirms its potential as a drug target to design HPV entry inhibitors.

14.7.2 Peptides

Lactoferricin (Lfcin) is a peptide generated by proteolytic cleavage of the N-terminal part of lactoferrin by pepsin. Lfcin was found to have antimicrobial and anticancer properties, such as inhibition of infection by HSV-1 and HSV-2

(Andersen et al. 2003), HIV (Berkhout et al. 2002), and echovirus 6 (Pietrantoni et al. 2006). These broad-spectrum defense properties were found to be related to its ability to form amphipathic structures with clear hydrophobic and positively charged faces. Among various species, bovine Lfcin (17–42 aa) and human Lfcin (1–49 aa) have been widely and deeply studied, and disulfide bridges are formed in both peptides. It has been reported that Lfcin can inhibit the entry of HPV PsV and block its binding to HaCaT cells (Drobni et al. 2004), probably due to blocking of cell surface heparan sulfate (HS) used by the virus for initial attachment. A follow-up study found that Lfcin and its derived peptides have strong inhibitory activity against HPV-5 and HPV-16 and that this activity depends on the structure, net charge, and amino acid sequence of the peptide (Buck et al. 2006b; Mistry et al. 2007). Bovine Lfcin 17–31 aa is the most potent inhibitor of both HPV-5 and HPV-16 PsV infection. Lfcin 1–49 aa from human has weaker antiviral activity, while bovine Lfcin 17–42 aa could inhibit only HPV-5 PsV infection in one cell line. Both bovine and human Lfcin could block the binding of HPV-16 VLPs to host cells by interacting with both the virions and the receptor HS, indicating that their mechanism of action occurs via viral entry inhibition. Further studies are needed to analyze the in vivo effects of these Lfcin peptides in the treatment of HPV-related diseases.

Defensins are effector peptides of the human innate immune system, with two categories: α- and β-defensins. It has been reported that α-defensins inhibit HPV infection, and microscopic studies revealed that they could block virion escape from endocytic vesicles (Buck et al. 2006a). HD5 is one of the six α-defensins, and it is expressed and secreted in the genitourinary tract of both women and men. In healthy women, the concentration of HD5 in vaginal lavage fluid is approximately 16.5 μM (Wiens and Smith 2015). Recently, HD5 was found to exhibit potent antiviral activity against HPV at physiological concentrations (Wiens and Smith 2015). Through direct binding to HPV-16 PsV, HD5 inhibits the furin-mediated cleavage of viral L2 protein after cyclophilin B-mediated unfolding of L2, blocking the exposure of the RG-1 epitope in L2. Once this conserved and critical step in HPV entry is disrupted, the viral genome cannot escape the endosomal pathway (Wiens and Smith 2015). Moreover, HD5 does not interfere with the interaction between an anti-RG-1 antibody and the epitope on the L2 protein, indicating that HD5 should most likely directly bind to HPV L1 instead of L2. These results proved the potential of HD5 for development as a drug against mucosal and cutaneous HPV types.

Tetraspanins regulate the composition of cell membrane components and control intracellular transport, and there are 33 members in humans. They are vulnerable to pathogens entering target cells, making them putative antiviral targets. For HPV, tetraspanin CD151 is able to interact with viral entry factors to facilitate viral uptake via a clathrin-, caveolin-, and dynamin-independent endocytic pathway (Scheffer et al. 2013), and another tetraspanin, CD63, is required for post-endocytic trafficking (Gräßel et al. 2016). It has been reported that after cellular depletion of CD9, CD63 and CD151, HPV-16 infection in HeLa cells was reduced by 50–80%. The C-terminal peptides of CD63 and CD151 exhibit a moderate to potent inhibitory effect (IC50 <10 μM) on HPV entry, while the C-terminal tail of CD9 has no effect at all (Fast et al. 2018). This result proved the importance of the CD151 and CD63 C-termini for HPV entry into host cells and highlights the potential of tetraspanin-derived peptides to be used to design entry inhibitors for HPV infections.

The L1 and L2 capsid proteins of HPV play a crucial role in HPV entry and infection. Thus, the positively charged peptides derived from the L1 and L2 proteins can be used to inhibit HPV infections. It has been reported that 6 positively charged peptides derived from L1 and L2 proteins have significant activity against HPV-31 PsV infection, with IC50 values in the range of 10–40 μg/mL (Bousarghin et al. 2004). Peptides that did not bind to heparin receptors had no antiviral effect. Moreover, this blocking effect was not type specific since a similar reduction in transfection was observed with peptides from

other HPV types, and all these peptides had no obvious cytotoxicity up to the highest concentrations tested. This finding confirms that the cell entry process is an effective target for antiviral drug design and that L1- and L2-derived peptides could block the binding of HPV virions to their receptor.

The N-terminal region (residues 13–78) of the HPV L2 protein is highly conserved among HPV subtypes and is closely associated with HPV infections. Using fine mapping analysis, it was found that a 36 aa peptide sequence of the L2 N-terminus (L2N) is critical for HPV infection and that expression of L2N with the transmembrane sequence on the target cell surface could block HPV infection (Yan et al. 2019). The L2N-derived lipopeptide with chemical lipidation at the C-terminus efficiently inhibited HPV infection. A stearoylated lipopeptide spanning 13–46 aa of L2N exhibited the most potent anti-HPV activity, with an IC50 of ~0.2 nM. The mechanism of action may be due to the rapid degradation of virion particles. Since L2N is highly conserved among HPV subtypes and is indispensable in the process of HPV infection, its derived lipopeptide has therapeutic potential to be developed as an effective anti-HPV agent (Yan et al. 2019).

14.7.3 Neutralizing Antibodies

Viral NAbs can bind the surface epitopes of virions to block virion binding to target host cells, thus inhibiting the subsequent cell entry process. Therefore, antiserum or purified antibodies against HPV could be developed as viral entry inhibitors. Various neutralizing monoclonal antibodies (mAbs) have been isolated to study the neutralization mechanism and their specific binding sites on HPV virions. Two mAbs, 5B6 and #9, were isolated and found to have different binding sites on the papillomavirus capsid (Booy et al. 1998). mAb #9 could bind to L1 molecules of both pentavalent and hexavalent capsomeres, inhibiting viral binding to the host cell surface, while mAb 5B6 binds to two L1 molecules of the adjacent hexavalent capsomeres to neutralize the virus by preventing capsid uncoating. Similarly, two other NAbs, H11.B2 for HPV-11 and H16.V5 for HPV-16, bind to the center region of the capsomere or the apex region of the capsomere, respectively (Zhang et al. 2016), both leading to a highly efficient neutralization effect against HPV PsV infection.

In another study, 3 HPV-16-specific antibodies, mAbs H16.1A, H16.14J, and H263.A2, were purified and found to effectively block HPV infection by interacting with epitopes similar to H16.V5 epitope (Guan et al. 2015). Recently, it was reported that three potent NAbs, 5D3, 17D5, and 15F7, could bind to the HPV-6 surface and inhibit viral infection (Liu et al. 2019a). Cryo-electron microscopy (EM) structures revealed that 5D3, 17D5, and 15F7 interact with the center-distal ring, center, and center-proximal ring of the HPV pentamer surface, respectively. Therefore, 5D3 and 17D5 could block HPV PsV attachment to the extracellular matrix (ECM) and the cell surface, while 15F7 allowed PsV attachment but inhibited PsV entry into the cell (Liu et al. 2019a). Moreover, this study also revealed that non-neutralizing antibodies had little activity against HPV PsV entry. The different antibody-mediated neutralization mechanisms provide an important basis for the development of specific drugs against HPV entry.

In addition to the major capsid protein L1, the minor capsid protein L2 of HPV is also involved in the viral entry process, serving as an effective target for NAbs. A mAb named RG-1 can bind the N-terminal region of L2 (17–36 aa) and block infection by both HPV-16 and HPV-18 (Gambhira et al. 2007). Treatment with RG-1 protected mice from HPV infection. Antiserum against HPV L2 comprising 17–36 aa could inhibit infection by multiple HPV PsVs, including those of HPV-5, 6, 16, 18, 31, 45, 52, 58, etc. Once the L2 17–36 aa peptide-specific antibody was removed, the anti-HPV activity of the remaining antiserum was largely reduced. These results confirm the mechanism of action for HPV vaccines and provide potential for the development of therapeutic vaccines and antibody-based antiviral drugs for HPV treatment.

14.7.4 Non-Antibody Proteins

Lactoferrin (LF) is an iron-binding glycoprotein consisting of a single polypeptide chain forming N-lobe and C-lobe domains. LF has a molecular weight of ~80 kDa and exhibits a high degree of homology between different species. LF is at high concentrations in colostrum (up to 7 mg/mL) and exists in the human endocervix and vaginal mucus. LF is an important component of the non-specific immune system, which displays antimicrobial properties against multiple viruses, including rotavirus, herpesviruses, poliovirus, respiratory syncytial virus, hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV (Hara et al. 2002; van der Strate et al. 2001). LF binds directly to multiple viruses or viral receptors, preventing the attachment of viruses to target host cells. Based on the use of CFDA-SE-labeled HPV VLPs that only fluoresce after internalization, LF was found to also inhibit internalization of HPV-16 particles, indicating the anti-HPV entry activity of LF (Drobni et al. 2004). Bovine LF could inhibit HPV-16 binding to the HaCaT cell surface in a dose-dependent manner, with an IC50 of 35 μg/mL, which is more potent activity than that of human LF, with an IC50 of 90 μg/mL. Subsequent studies further confirmed that LF could inhibit HPV-5 and HPV-16 infection in two cell lines, indicating the potential for LF to be used as an entry inhibitor against HPV.

Bovine β-lactoglobulin (β-LG) is a readily available protein whose structure and function have been studied extensively. It contains 162 residues with a molecular weight of 18.4 kDa. On the surface of β-LG, there are 18 positively charged residues, including 15 lysine and 3 arginine residues, making it easy to modify with chemical compounds to enhance the electrical properties of the protein. In the 1990s, 3-hydroxyphthalic anhydride (3HP)-modified bovine β-lactoglobulin (3HP-β-LG) was found to have potent inhibitory activity against HIV, HSV-1, HSV-2, and some Chlamydia strains (Kokuba et al. 1998; Neurath et al. 1995, 1996). A few years later, 3HP-β-LG was further found to exhibit highly potent antiviral activity against infection of HeLa or HaCaT cells by HPV-6, HPV-16, and HPV-18 subtypes. The anti-HPV activities were correlated with the percentage of modified lysine and arginine residues in 3HP-β-LG (Lu et al. 2013). When almost all the lysine and arginine residues on the protein surface were modified, the anti-HPV-16 activity of 3HP-β-LG reached an IC50 of 27 nM. Subsequent studies revealed that 3HP-β-LG did not inactivate HPV PsV but rather blocked its entry into the target cell via its interaction with the virus, not the cells (Hua et al. 2019). 3HP-β-LG binds to the HPV L1 protein through an interaction between the negatively charged region in 3HP-β-LG and the positively charged region in the HPV L1 protein, relying on both electrostatic binding and matching conformations of the binding sites in both proteins. 3HP-modified chicken ovalbumin (3HP-OVA) showed no anti-HPV activity, which was probably due to the mismatch of the two protein conformations. When topically applied, 3HP-β-LG could not be detected inside host cells or in blood circulation, demonstrating that 3HP-β-LG inhibits viral infection outside the cell. These results indicate that 3HP-β-LG has the potential to be developed as an effective, safe, and inexpensive antiviral agent for the prevention of HPV infection.

14.7.5 Polysaccharides

In early events of HPV infection, HSPG mediates the initial binding of virions to the cell surface (Giroglou et al. 2001). Of the HSPGs, syndecan-1 is the major epithelial form and is strongly upregulated in wound edge keratinocytes, as well as serves as a primary receptor protein in natural HPV infection of keratinocytes (Shafti-Keramat et al. 2003). Heparin-like polysaccharides, such as 3,6-*O*-sulfated chitosan (36S), were reported to prevent the binding of HPV to the cell surface (Gao et al. 2018). 36S could effectively inhibit multiple genital HPV genotypes in different cell lines with low cytotoxicity, with IC50 values against HPV-16, HPV-18, HPV-6, HPV-45, HPV-5, and HPV-31 of 2.4,

3.7, 19.4, 38.1, 73.9, and 82.6 μg/mL, respectively. The main mechanism of action may be that the direct binding of 36S to viral capsid protein blocks the absorption of virions on the target cell surface. Moreover, 36S was able to enter HeLa cells and downregulate the cellular PI3K/Akt/mTOR pathway to enhance autophagy (Gao et al. 2018). Thus, the marine-derived sulfated chitosan 36S has high potential to be developed as a novel anti-HPV agent.

HSPGs consist of a core protein and glycosaminoglycan chains of unbranched sulfated polysaccharides, known as HSs (Joyce et al. 1999). As its name implies, heparin is structurally related to HS or HSPGs, so heparin and other sulfated polysaccharides prevent the binding of HPV to the cell surface by mimicking HS. For example, the K5 capsular polysaccharide from *Escherichia coli* has structure similar to that of HS. Sulfated K5 derivatives [K5-OS(H), K5-N, OS(H), and K5-N,OS(L)] exhibited significant inhibitory activity against HPV-16, HPV-18, and HPV-6 PsV infections, with IC50 values between 0.1 and 0.9 μg/mL (Lembo et al. 2008). The results of a cell viability assay with keratinocytes treated with each compound at concentrations from 1 to 100 μg/mL revealed no evidence of cytotoxicity. K5 derivatives not only blocked the initial adhesion of HPV virions to the cell surface but also exhibited a post-adhesion inhibitory effect on HPV infections. In addition, dextran sulfate, polystyrene sulfonate, and cellulose sulfate all showed direct microbicidal activity with IC50s between 10 and 100 μg/mL (Christensen et al. 2001). These results provide insights into the design of safe and broad-spectrum microbicides against genital HPV infections.

Carrageenan is a class of sulfated polysaccharide extracted from marine red algae (seaweed) and has a structure similar to that of HS. In 2006, it was reported that carrageenan had potent infection inhibitory activity against a broad range of sexually transmitted HPV subtypes, with IC50s in the low ng/ml range (Buck et al. 2006b). Carrageenan could prevent the binding of HPV virions to the receptor HS on host cells and was three orders of magnitude more potent than heparin, which has been regarded as a highly effective HPV inhibitor. Moreover, carrageenan can also block HPV infection through a second, post-attachment HS-independent effect. A subsequent study included the development of a mouse model of cervicovaginal infection with HPV-16 and confirmed that carrageenan could inhibit HPV-16 infection in vivo (Roberts et al. 2007). A few years later, a subsequent study further revealed carrageenan's effectiveness against several native high-risk HPV types (16, 18, 31, and 45) and showed that carrageenan was effective against HPV-18 and HPV-31 but not HPV-16 or HPV-45 (Cruz and Meyers 2013). In addition, it was found that the combination of carrageenan with the broad-spectrum microbicide griffithsin (GRFT) could effectively inhibit HPV infection (Levendosky et al. 2015). Instead of blocking the initial attachment of the virus to the cell, GRFT interacts with the HPV secondary receptor α6 integrin to decrease its availability on the cell surface. These studies provided a better understanding of the development of carrageenan-based HPV entry inhibitors.

DC-SIGN, a C-type lectin receptor expressed by immature dendritic cells and macrophages, recognizes mannose clusters on the viral surface to mediate viral infection. It was found that an amphoteric cationic polyamidoamine (PAA) named AGMA1 could effectively inhibit several HSPG -dependent viruses, including HPV-16, while the amphoteric anionic PAA named ISA23 proved inactive. However, biocompatible linear PAAs carrying different amounts of mannosyl-triazolyl pendants, such as Man-AGMA6.5 and Man-AGMA14.5, maintained anti-HPV-16 activity (Mauro et al. 2016). Moreover, AGMA1 or ISA23 derivatives carrying different glycomimetic substituents could all inhibit HPV infection with significant activity, proving their potential for development as broad-spectrum, dual-action anti-HPV drugs (Mauro et al. 2016).

14.8 Clinical Effect of HPV Entry Inhibitors

14.8.1 Carrageenan Gel

Carrageenan, which has been identified as a potent HPV infection inhibitor, is widely used as a thickener in many cosmetic and food products. Safety and acceptability trials of lubricants containing carrageenan supported clinical efficacy testing for a carrageenan-based lubricant gel in reducing genital HPV incidence (Kilmarx et al. 2006). Recently, a randomized, double-blind, placebo-controlled, phase 2B trial was conducted to evaluate the efficacy of a carrageenan-based lubricant gel in reducing the risk of genital HPV infection in women (Magnan et al. 2019). Between 2013 and 2017, a total of 280 women aged 18 years and older were randomly assigned to the carrageenan gel ($n = 141$) or the placebo gel ($n = 139$) group. All gels were self-applied every other day for the first month and used before and after each intercourse during follow-up. The primary outcome was the incidence of a new infection by an HPV type that was not present at baseline. The median follow-up time was 9.2 months. The results showed that 42% of participants in the carrageenan group and 57% in the placebo group became infected by at least one new HPV type, suggesting that using a carrageenan-based lubricant gel can reduce the risk of genital HPV infections in women.

14.8.2 3HP-β-LG Gel

3HP-β-LG is highly effective at inhibiting the entry of both hrHPV and lrHPV subtypes. The surface of 3HP-β-LG is rich in negative charge, and 3HP-β-LG can effectively bind to the HPV capsid protein to block the binding of virions to their cell receptor (Hua et al. 2019; Lu et al. 2013). Therefore, 3HP-β-LG may also block the repeated infection of progeny HPVs on cervical basal layer cells, leading to reduced HPV viral load and preventing the occurrence of cervical lesions and cervical cancer. Moreover, it has been proven that 3HP-β-LG does not enter the host cell or blood circulation and does not induce the production of specific antibodies upon topical application (Hua et al. 2019). These studies supported the clinical usage of 3HP-β-LG to inhibit HPV infection in women.

The dosage form of 3HP-β-LG in clinical trials is a biological dressing, which are 3 g each and contain 0.01% (w/w) 3HP-β-LG. In a study, 77 women aged 25–65 years old infected by hrHPV (without a high level of cervical lesions) were randomly divided into a treatment group ($n = 38$) and a blank control group ($n = 39$). For the treatment group, the 3HP-β-LG dressing was administered intravaginally every other day for 3 months, avoiding the menstrual period. After the trial, no serious adverse events, including burning, tingling, or other serious symptoms, were reported by participants in this study (Guo et al. 2016a). The vaginal microenvironment in the treatment group returned to normal. According to efficacy evaluation, 60.5% of HPV-positive women in the treatment group became HPV-negative, compared with 13.5% of women in the blank control group ($P < 0.001$) (Guo et al. 2016b). These data suggest that the 3HP-β-LG dressing is a safe and effective topical biological agent for the treatment of cervical HPV infection.

In the clinical use of 3HP-β-LG dressings, they also showed significant clinical effects and few adverse reactions, compared with other traditional medicines or medical devices. In one study, 150 patients with cervical hrHPV infection were randomly divided into three groups and treated with a 3HP-β-LG dressing, Baofukang suppository (one Chinese patent medicine), or recombinant human interferon α2b gel (Ying et al. 2016). The results showed that the 3HP-β-LG dressing could significantly reduce the HPV DNA load of patients much better than the other two groups. In another study, 148 patients with hrHPV infection complicated with cervical intraepithelial neoplasia (CIN) level I were treated with a 3HP-β-LG dressing or were untreated (Zhang et al. 2018).

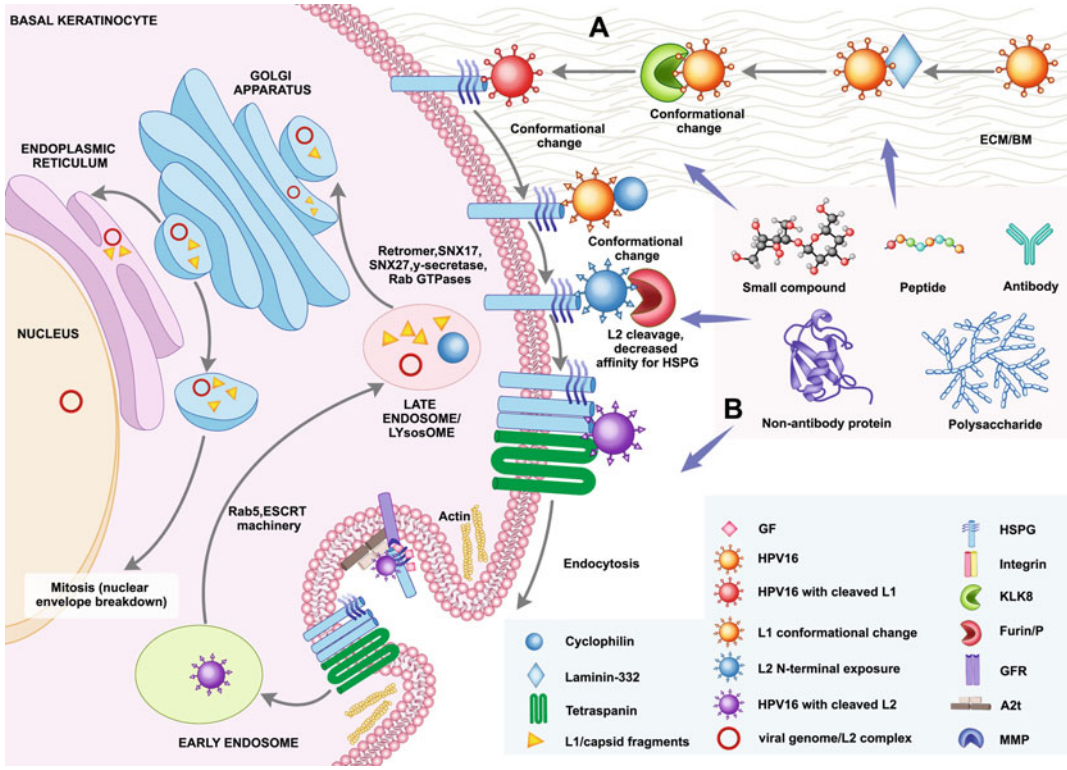

Fig. 14.1 The entry process of HPV and related entry inhibitors. **(a)**In the ECM or BM region, laminin-332 binds with HPV-16, and kallikrein-8 (KLK8) cleaves its L1 capsid protein. Then, HPV binds to HSPGs on the target cell membrane, and cyclophilins may facilitate L2 N-terminal exposure. Furin/proprotein convertases (PCs) then cleave the N-terminus of L2, leading to the internalization of HPV virions via a macropinocytosis-like mechanism involving actin. Inside the cell, the virion traffics through several organelles and releases its genome into the nucleus (Aksoy et al. 2017). **(b)**Various HPV entry inhibitors, including small compounds, peptides, antibodies, non-antibody proteins, and polysaccharides, block viral entry in different ways

The results showed that the eradication rate of HPV infection in the 3HP-β-LG group was 80.25%, which was significantly higher than the 46.27% rate in the control group. Moreover, the negative conversion rate of patients with HPV complicated with the cervical cytology atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion (ASCUS/LSIL) in the 3HP-β-LG group was 80.00%, which was significantly higher than the 33.33% rate in the control group. These results showed that vaginal administration of 3HP-β-LG could improve HPV clearance and was also effective against the cervical cytology ASCUS/LSIL.

14.9 Conclusion

HPV is a non-enveloped, double-stranded DNA virus, and its major capsid protein L1 and minor capsid protein L2 are responsible for the assembly of mature virions and viral entry into target host cells. More than 200 different HPV subtypes can be divided into five genera (α, β, γ, μ, and ν), lrHPV and hrHPV subtypes, or cutaneotropic and mucosotropic subtypes. HPV virus infection is a complex multi-step process, and multiple cell receptors and proteases are required for the viral entry step. HPV infection usually causes unapparent infections, but persistent HPV infection can lead to various warts and cancers, such as cervical

cancer. To prevent these diseases, HPV vaccines and viral inhibitors have been widely studied. There are three major prophylactic HPV vaccines available in many countries, the 2-, 4-, and 9-valent vaccines, which all consist of purified HPV L1 VLPs. Since HPV has very few targets inside infected cells for traditional drug design, entry inhibitors are the ideal method to design specific prevention and treatment antiviral agents against HPV. Caffeic acid and A2t heterotetramer inhibitors are small compounds that block HPV entry. The peptides derived from Lfcin, defensins, tetraspanins, and the HPV L1 and L2 proteins have all been shown to be effective in inhibiting HPV entry into host cells. Multiple NAbs against the HPV L1 and L2 proteins, together with bovine or human LF and 3HP-β-LG, are protein antiviral agents for the prevention of HPV infection. Moreover, polysaccharides also have high potential to be developed as novel anti-HPV agents, including heparin-like polysaccharides, sulfated polysaccharides, carrageenan, etc. To date, several clinical trials have been conducted to evaluate the safety and clinical efficacy of these HPV entry inhibitors, such as carrageenan- and 3HP-β-LG-containing gels. These results suggested that HPV inhibitors exhibit high safety for clinical use and are effective in reducing the risk of HPV infection and related diseases. With the development and application of an increasing number of HPV vaccines and inhibitors, the incidence of HPV-related diseases will be significantly reduced in the future (Fig. 14.1).

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