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Antiviral Drug Discovery and Development

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Preface

During the last 40 years, we have witnessed an unprecedented progress in the design and development of antiviral drugs. Since their introduction in the early 1960s, compounds targeting infections caused by herpesviruses remained as the only drugs approved for antiviral treatment. The emergence of the human immunodeficiency virus (HIV) and its further expansion throughout the world causing disease and death promoted research that aimed to develop an HIV cure or vaccine. As a result, today we have more than 30 antiretroviral drugs available to control HIV infection. These treatments have turned HIV infection into a treatable but chronic disease, although further efforts will be needed to achieve an effective cure. Lessons learnt from the development of antiretroviral therapies have allowed the eradication of hepatitis C virus (HCV) infection through the introduction of new interferon-free treatments based on the use of inhibitors of key processes in the HCV replicative cycle. Despite these positive advances, effective therapies are available only for a few viral families and emerging and re-emerging pandemics are still a major threat to human health, as sadly seen these days with COVID-19. Medicinal chemistry efforts towards the discovery of new antiviral drugs will be important to respond to major challenges imposed by pandemic viral infections but also by the continuously changing landscape of drug resistance that may arise in response to widely used antiviral drugs.

In this book, we have covered the field of antiviral drug design and development by first describing the state of the art for viruses of major relevance, starting from herpesviruses, and following by retroviruses, hepatitis B and C viruses, dengue virus, influenza virus, and ending with the current efforts to find an effective antiviral therapy against coronaviruses. The second part of the volume concentrates on specific types of compounds useful to combat different types of infections, such as peptide-based or covalent antiviral agents, and in the description of novel approaches towards the discovery of antiviral drugs targeting host proteins or intracellular mechanisms.

We believe that this book provides researchers, graduate students, and clinic practitioners with a cutting-edge and comprehensive summary of

research on antiviral drug design and development and an updated description of current drugs available to fight major viral diseases. We express our most sincere gratitude to all the authors and reviewers for their great contributions.

Jinan, Shandong, China — Xinyong Liu
Jinan, Shandong, China — Peng Zhan
Madrid, Spain — Luis Menéndez-Arias
Odense M, Denmark — Vasanthanathan Poongavanam

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Antiviral Drugs Against Herpesviruses

1

Jocelyne Piret and Guy Boivin

Abstract

The discovery of the nucleoside analogue, acyclovir, represented a milestone in the management of infections caused by herpes simplex virus and varicella-zoster virus. Ganciclovir, another nucleoside analogue, was then used for the management of systemic and organ-specific human cytomegalovirus diseases. The pyrophosphate analogue, foscarnet, and the nucleotide analogue, cidofovir, have been approved subsequently and constitute the second-line antiviral drugs. However, the viral DNA polymerase is the ultimate target of all these antiviral agents with a possible emergence of cross-resistance between these drugs. Recently, letermovir that targets the viral terminase complex was approved for the prophylaxis of human cytomegalovirus infections in hematopoietic stem cell transplant recipients. Other viral targets such as the protein kinase and the helicase-primase complex are also evaluated for the development of novel potent inhibitors against herpesviruses.

Keywords

Herpesviruses · Antiviral agents · Drug resistance

Abbreviations

ACV	Acyclovir
ATP	Adenosine triphosphate
AIDS	Acquired immunodeficiency syndrome
BAC	Bacterial artificial chromosome
BCV	Brincidofovir
BVDU	Brivudin
CDK	Cyclin-dependent kinase complex
CDV	Cidofovir
dNTP	Deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EC_{50}	Effective concentration that reduces virus-induced cytopathic effects by 50%
FCV	Famciclovir
FOS	Foscarnet
GCV	Ganciclovir
HAT	Histone acetyltransferase
HCMV	Human cytomegalovirus
HHV-6A	Human herpes virus 6A
HHV-6B	Human herpes virus 6B
HHV-7	Human herpes virus 7
HHV-8	Human herpes virus 8

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HIV	Human immunodeficiency virus
HPI	Helicase-primase inhibitor
HSCT	Hematopoietic stem cell transplant
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
LMV	Letermovir
MBV	Maribavir
NBS	Nucleoside-binding site
NEC	Nuclear egress complex
ORF	Open reading frame
PCV	Penciclovir
pol	Polymerase
rb	Retinoblastoma
RNase	Ribonuclease
SOT	Solid organ transplant
TK	Thymidine kinase
TPA	Tetradecanoyl phorbol ester
UL	Unique long
VACV	Valacyclovir
VGCV	Valganciclovir
VZV	Varicella-zoster virus

1.1 Introduction

Herpesviridae consists in a large family of DNA viruses. *Herpesviridae* family includes nine different human viruses that are classified in three subfamilies, the *Alphaherpesvirinae* (herpes simplex viruses 1 and 2 [HSV-1 and HSV-2] and varicella-zoster virus [VZV]), the *Betaherpesvirinae* (human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 [HHV-6A, HHV-6B, and HHV-7]), and the *Gammaherpesvirinae* (Epstein-Barr virus [EBV] and HHV-8]). Herpesviruses are characterized by their abilities to enter in a latent state into the host and to reactivate periodically. These viruses are ubiquitous and cause different clinical manifestations, the severity of which is dependent on the immune status of the infected individuals. The clinical manifestations induced by HSV-1 and HSV-2 consist of orolabial and genital infections as well as keratitis, encephalitis, and neonatal infections. Herpes simplex virus may also cause severe infections in immunocompromised patients, particularly those presenting an altered cell-mediated immunity. Individuals infected with human immunodeficiency virus (HIV) and recipients of solid organ transplants (SOT) can develop extensive herpetic lesions which can persist for longer periods of time and have a potential to disseminate. The frequency of recurrent infections can be higher in these patients, and the appearance of the lesions may be atypical. Mucocutaneous lesions tend to persist over time, and the virus can disseminate to cause visceral infections such as esophagitis, hepatitis, pneumonitis, and meningoencephalitis. Varicella-zoster virus is the etiological agent of varicella and herpes zoster which can result in prolonged pain, known as postherpetic neuralgia. Infections caused by VZV may be more prolonged and severe with unusual clinical presentations in immunocompromised patients. Human cytomegalovirus causes mononucleosis-like syndromes in immunocompetent individuals. However, infections induced by HCMV can be severe and life-threatening in immunocompromised patients. They manifest as systemic and organ-specific diseases (e.g., pneumonitis, gastroenteritis, hepatitis, pancreatitis, myocarditis, retinitis, encephalitis, and peripheral neuropathy). In this patient population, infection is either due to reactivation of a latent virus, a primary infection, or a reinfection from infected tissue or organ that occurs after transplantation. Primary infections due to HHV-6A are usually asymptomatic, whereas those caused by HHV-6B, and more rarely HHV-7, are associated with exanthema subitum in children. Both HHV-6A and HHV-6B can also induce severe diseases (pneumonitis, hepatitis, and encephalitis) in immunocompromised patients. EBV is responsible for classical infectious mononucleosis in immunocompetent individuals. More severe clinical manifestations such as nasopharyngeal carcinoma, Burkitt's lymphoma, non-Hodgkin B-cell lymphomas, and posttransplant lymphoproliferative diseases can occur in immunocompromised patients. HHV-8 is mainly associated with Kaposi's sarcoma, a neoplasm which is frequently observed in patients infected with HIV and a major health problem in central African countries.

1.2 Herpesviruses Replicative Life Cycle

The replication of the viral DNA, its cleavage into unit length genomes, their packaging into procapsids, and the maturation of capsids filled with DNA occur in host cell nucleus (Fig. 1.1). The nucleocapsids are then transported out of the nucleus by egress into the cytoplasm. During this step, the nucleocapsids are enveloped and de-enveloped as they cross the nuclear membrane. The virion assembly continues to progress in distinct compartments of the cytoplasm. Finally, the fully assembled viral particles are released from infected cells through an exocytic pathway or after cell lysis. The virus-encoded enzymes involved in DNA synthesis (DNA polymerase (pol), protein kinase, helicase-primase complex), encapsidation process (terminase complex), and nuclear egress (protein kinase) were evaluated as targets for the development of potent inhibitors against herpesviruses.

1.3 The Viral DNA Polymerase

The viral DNA pol is the target of most antiviral agents approved for the prevention and treatment of infections caused by herpesviruses [1]. The DNA pol of herpesviruses are multifunctional enzymes [2] which possess a 5′-3′ polymerase activity that extends DNA chain from a primer strand [3], an intrinsic 3′-5′ exonuclease activity that removes mismatched nucleotides from the elongated primer [4], and a ribonuclease (RNase) H activity [5].

The DNA pol of herpesviruses and bacteriophage RB69 belong to the family B DNA pol. Their common structure looks like a right hand with palm, thumb, and fingers domains (Fig. 1.2a) that are, respectively, involved in the catalytic reaction, the DNA molecule binding, and the incoming nucleotide binding [7]. The 5′-3′ polymerizing activity of these enzymes consists in the addition of new nucleotides to the 3′-OH extremity of a primer strand. These enzymes present in three different modes called the apoenzyme state in the absence of DNA binding [8], the editing mode corresponding to a binary complex with the DNA molecule [9] and the replicating mode consisting of a ternary complex with the DNA molecule and incoming nucleotide [10] that have been resolved from structure analyses of RB69 DNA pol. During the reaction of polymerization, the DNA molecule binds to the apoenzyme, and the thumb closes down around the DNA chain, while the fingers domain adopts an open conformation (Fig. 1.2b). The incoming nucleotide binds to the enzyme-DNA binary complex and induces a conformational change in the fingers domain which rotates toward the palm domain. The nucleotide is trapped, and the enzyme conformation is closed (Fig. 1.2b). The nucleotide is transferred to the 3′-OH end of the primer strand (pre-translocated state). The pyrophosphate is released and the DNA is translocated (post-translocated state). The fingers domain moves away from the palm domain, and the enzyme adopts an open conformation so that the next nucleotide can be incorporated. This resets the polymerase for a new round of catalysis. When the viral DNA molecule is completely synthetized, it leaves the thumb and palm domains.

1.3.1 Antiviral Agents Targeting the Viral DNA Polymerase

First-line antiviral agents indicated for the treatment of infections caused by HSV and VZV include acyclovir (ACV) and penciclovir (PCV) (Fig. 1.3). Acyclovir ([9-(2-hydroxyethoxymethyl)guanine, Zovirax®]) is a guanosine analogue which must be triphosphorylated to be active. In infected cells, the first phosphorylation is performed by the viral thymidine kinase (TK) encoded by the *UL23* (HSV) and *ORF36* (VZV) gene. Thereafter, two additional phosphorylations are successively made by host cellular guanosine monophosphate kinase and nucleoside diphosphate kinase [11, 12]. The viral DNA pol encoded by *UL30* (HSV) or *ORF28* (VZV) gene can use ACV triphosphate (ACV-TP) as a substrate as it

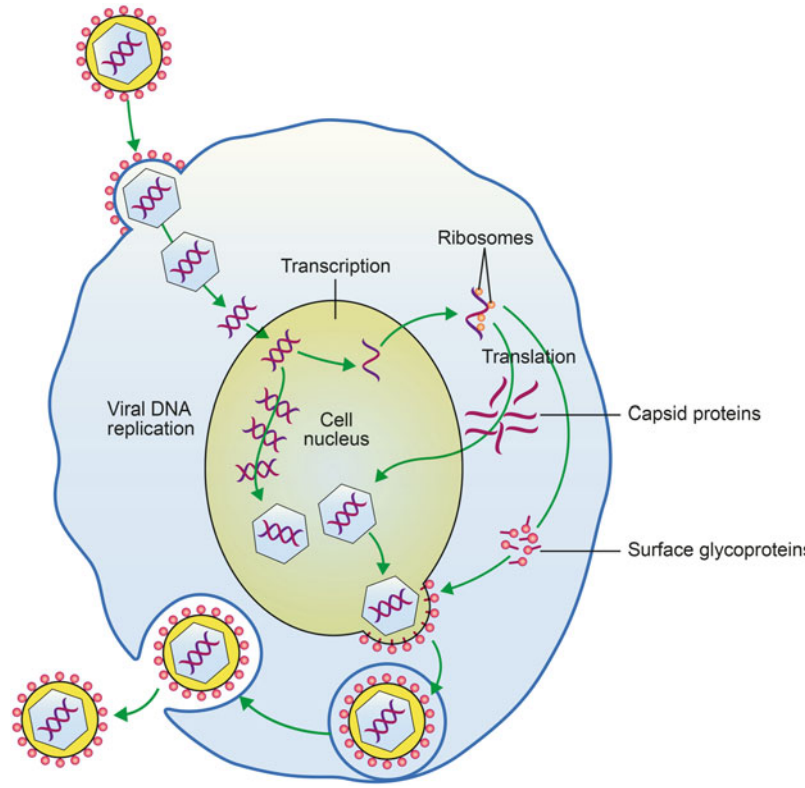

Fig. 1.1 Replicative cycle of herpesviruses

can be incorporated at the 3′-end of elongating DNA [13]. The binding of the next deoxynucleoside triphosphate (dNTP) to the primer-template results in the formation of a dead-end complex [14]. As ACV-TP does not have a hydroxyl group in 3′ position, it also acts as an obligate DNA chain terminator [14]. Furthermore, it is proposed that ACV monophosphate residue could bind to the 3′-terminus of elongating DNA and cannot be excised by the 3′-5′ exonuclease activity of the DNA pol [15], thereby preventing further chain elongation. The concentrations of ACV that reduce HSV-1, HSV-2, and VZV cytopathic effects by 50% (EC_{50}s) vary between 0.01 to 2.7 μM, 0.01 to 4.4 μM, and 0.17 to 26 μM, respectively [16]. In the clinic, ACV demonstrates a favorable safety profile [16, 17]. Its use is limited by its relatively poor oral bioavailability (i.e., 15–35%) which led to the development of its L-valyl ester prodrug, valacyclovir (VACV, Valtrex®, Zelitrex®) (Fig. 1.3) [18, 19]. After oral administration of VACV, the drug is transferred by the human intestinal peptide transporter in the small intestine and is then rapidly converted to ACV by ester hydrolysis [20]. This increases the absolute bioavailability of ACV to 54% [21].

Penciclovir (9-[4-hydroxy-3-hydroxymethyl-but-1-yl]guanine, Denavir®, Vectavir®) is an acyclic guanine derivative which is not commercially available as an oral agent. Its spectrum of activity and its mechanism of action are similar to those of ACV [22]. However, as PCV possesses a 3′ hydroxyl group on its acyclic side chain, PCV triphosphate allows limited chain elongation and is thus a short-chain terminator. The EC_{50} values of PCV against HSV and VZV are dependent on the cell types but are usually twofold higher than those of ACV. When given orally, PCV is very poorly absorbed which prompted the development of the oral prodrug famciclovir (FCV, Famvir®) (Fig. 1.3) which is a diacetyl ester of 6-deoxypenciclovir [23]. After oral

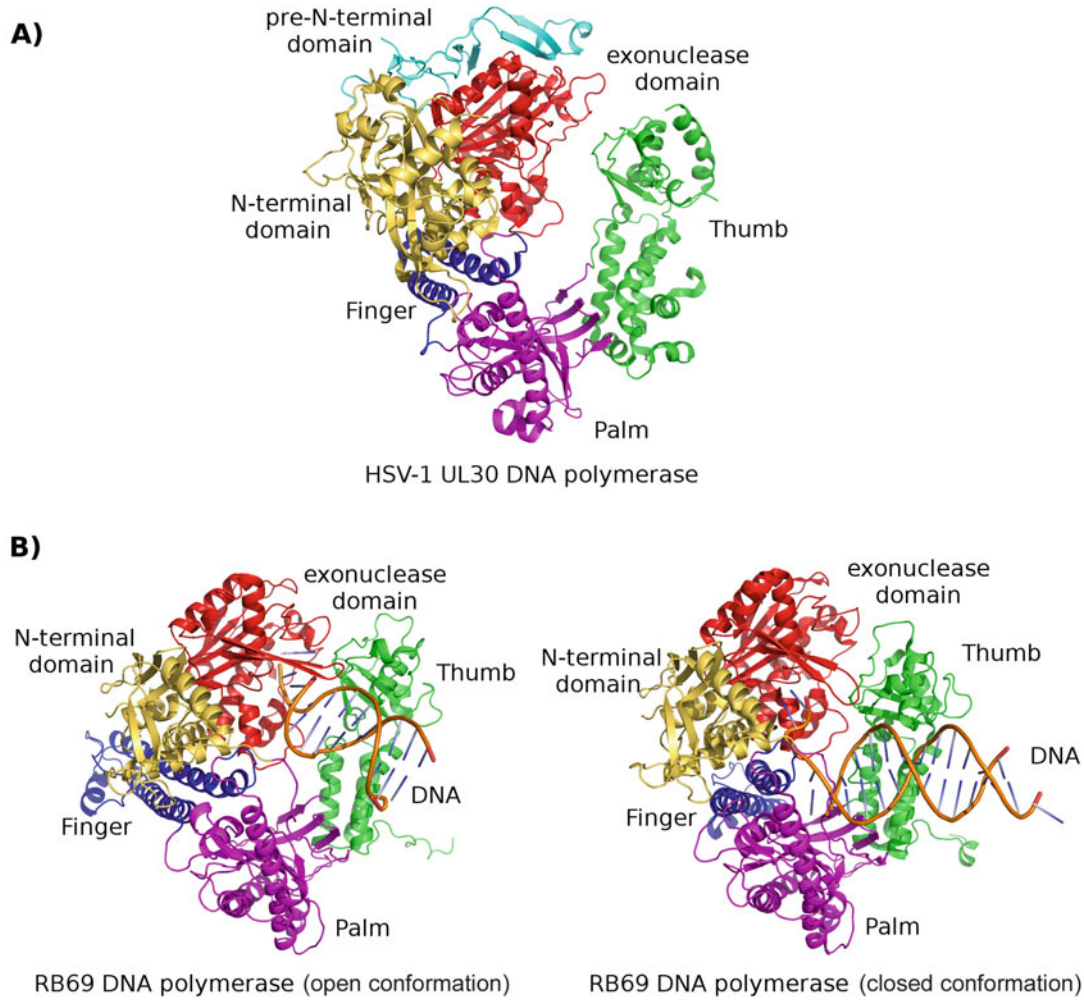

Fig. 1.2 Cartoon representation of B family DNA polymerases. (**a**) pUL30 DNA polymerase of HSV-1 in apo form (PDB 2GV9). Pre-NH_2-terminal domain (cyan), NH_2-terminal domain (light yellow), exonuclease domain (red), palm domain (magenta), fingers domain (blue), and thumb domain (green). (**b**) gp43 DNA polymerase of RB69 bacteriophage in open (PDB 1CLQ, left) and closed (PDB 3LDS, right) conformations. The color of the different domains is similar as in (**a**), except that there is no pre-N-terminal domain. The DNA molecules bound to the enzymes are also represented. This figure was prepared by using PyMol software (Schrödinger, Inc.). Reprinted from Zarrouk et al. [6], Copyright 2017, with permissions of Elsevier

administration of FCV, the absolute bioavailability of PCV is 77% [23]. FCV is rapidly absorbed when administered orally and efficiently converted to PCV. The first acetate group of FCV is cleaved by esterases found in the intestinal wall [24]. Thereafter, an aldehyde oxidase catalyzed the conversion of 6-deoxypenciclovir to PCV during first passage through the liver [25].

Oral ACV, VACV, and FCV are indicated for short-term therapy of primary and recurrent HSV infections (particularly genital herpes), long-term suppressive therapy of recurrent genital herpes, and treatment of herpes zoster as well as for prophylaxis of infections caused by herpesviruses in recipients of SOT and hematopoietic stem cell transplant (HSCT). The intravenous formulation of ACV is used for the management of severe

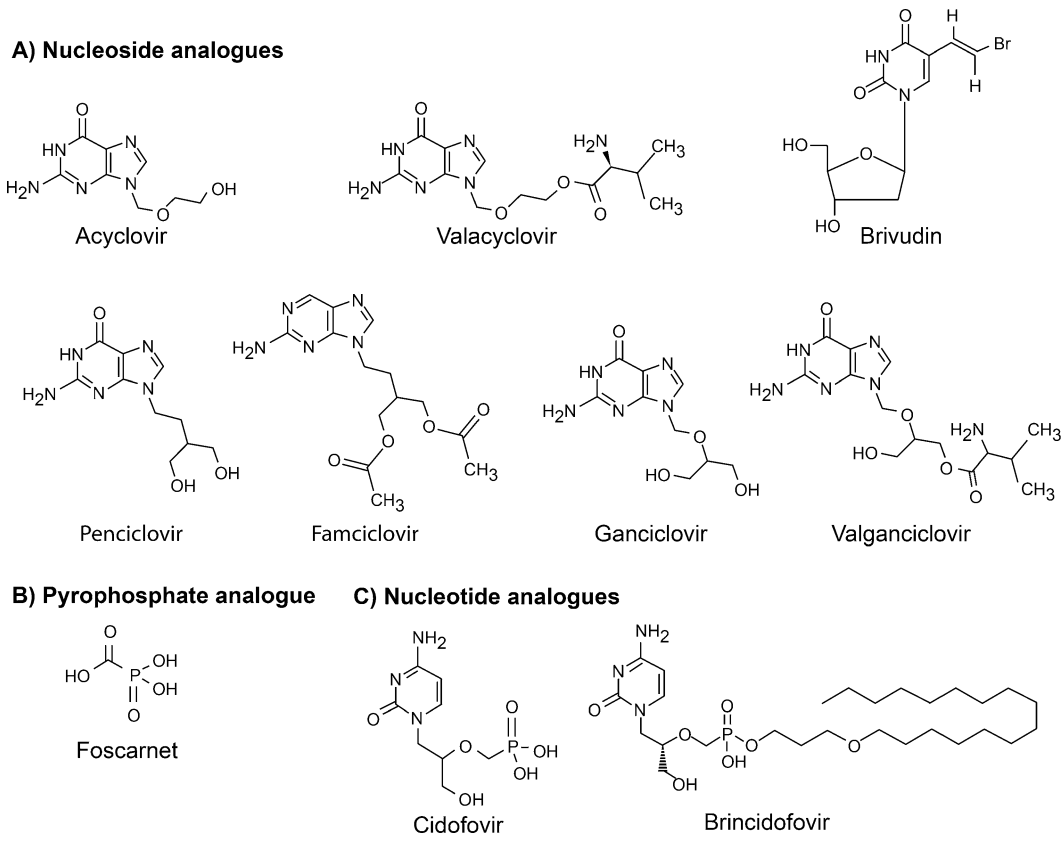

Fig. 1.3 Antiviral agents targeting the viral DNA polymerase

HSV (including encephalitis and neonatal herpes) and VZV infections. Topical formulations of ACV and PCV are available for the treatment of herpes labialis and keratitis.

In some European countries, an oral formulation of brivudin (BVDU, [*E*]-5-[2-bromovinyl]-2′-deoxyuridine, Zostex®, Zonavir®, Zerpex®, Zovudex®) is approved for the treatment of herpes zoster in immunocompetent adults (Fig. 1.3). BVDU demonstrates a specific antiviral activity against HSV-1 and VZV but not HSV-2 [26, 27]. The EC_{50} value of BVDU against VZV is 0.0072 μM [28]. The thymidine analogue BVDU is phosphorylated to mono- and diphosphate forms by the viral TK and then to its triphosphate form by cellular kinase. Brivudin triphosphate can compete with deoxythymidine triphosphate for incorporation into replicating DNA or can be incorporated as an alternate substrate, thus leading to the formation of a dead-end complex [26]. Approximately 90% of an oral dose of BVDU is absorbed, and 70% is converted to (E)-5-(2-bromovinyl)uracil on first pass through the liver [29]. Brivudin should not be coadministered with 5-fluorouracil. Indeed, (E)-5-(2-bromovinyl)uracil was shown to inhibit dihydropyrimidine dehydrogenase that is involved in the catabolism of pyrimidines as well as of 5-fluorouracil which significantly increases its half-life [30]. The administration of BVDU at a dose of 125 mg once daily for 7 days was shown to be effective against the development of zoster lesions and to prevent postherpetic neuralgia [31, 32].

Ganciclovir (GCV, 9-[1,3-dihydroxy-2-propoxymethyl]guanine, Cytovene®) constitutes the first-line antiviral drug for the prevention and treatment of HCMV infections and diseases (Fig. 1.3). Ganciclovir is a deoxyguanosine analogue that requires a first phosphorylation by the pUL97 kinase encoded by the *UL97* gene and two subsequent phosphorylations by cellular kinases

to be converted in its active form. Ganciclovir triphosphate competes with deoxyguanosine triphosphate for incorporation into replicating DNA where it slows down the polymerization reaction by the DNA pol encoded by the *UL54* gene and eventually stops chain elongation [33]. The EC_{50} value of GCV against HCMV in fibroblastic cells is approximately 3.5 ± 2.3 μM [34]. Ganciclovir can be given orally or intravenously. Ganciclovir has a poor bioavailability (~6%) following oral administration. Valganciclovir (VGCV, Valcyte®) (Fig. 1.3), its L-valyl ester prodrug, was thus developed and exhibits an approximately tenfold increase in oral bioavailability [35]. Oral VGCV and intravenous GCV are indicated in the treatment of established HCMV diseases in immunocompromised patients and in the prevention of symptomatic episodes, especially in transplant recipients. The main side effect associated with the administration of GCV consists in myelosuppression which limits its use especially for HSCT recipients. Ganciclovir can be also administered as an intravitreal implant (Vitrasert) for the treatment of HCMV retinitis.

Second-line antiviral drugs for the treatment of HCMV infections and diseases include foscarnet (FOS, phosphonoformic acid, Foscavir®) and cidofovir (CDV, [*S*]-1-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine, Vistide®) (Fig. 1.3). Due to their side effects and the absence of oral formulation, they are usually administered to patients failing or not tolerating therapy with nucleoside analogues. Foscarnet is a pyrophosphate analogue which does not require to be phosphorylated by viral or cellular kinases. It thus directly inhibits the viral DNA pol activity. Foscarnet binds to the pyrophosphate binding site and prevents the cleavage of pyrophosphate upon incorporation of incoming dNTP leading to termination of DNA chain elongation [36]. It is suggested that FOS has a higher binding affinity for the viral DNA pol in its closed conformation [37]. The EC_{50} values of FOS against HSV and HCMV strains range from 30 to 90 μM and 50 to 800 μM, respectively. The intravenous formulation of FOS is used for the treatment of HCMV retinitis in individuals with the acquired immunodeficiency syndrome (AIDS) and infections caused by GCV-resistant HCMV in immunocompromised patients. Foscarnet may also be administered as an alternate therapy for nucleoside analogue-resistant HSV and VZV infections. The most frequent toxicities associated with the use of FOS are electrolyte abnormalities and renal impairment requiring administration of fluid (hydration) and monitoring of serum creatinine levels. Cidofovir is an acyclic nucleoside phosphonate which requires only two phosphorylations by cellular kinases to exert its antiviral activity. Cidofovir diphosphate acts as a non-obligate DNA chain terminator [38]. Cidofovir exerts a potent antiviral activity against all the human herpesviruses [39]. Typical EC_{50} values of CDV against HCMV range from 0.2 to 0.4 μM. The intravenous formulation of CDV is used for the treatment of HCMV retinitis in AIDS patients and can be occasionally administered in transplant recipients with drug-resistant HCMV infections. Topical and intravenous formulations of CDV may be used off-label in the treatment of infections caused by HSV isolates resistant to ACV and/or FOS [1]. Nephrotoxicity is the main side effect of CDV and requires adequate hydration of the patients and administration of probenecid to prevent kidney failure.

An orally bioavailable ether lipid ester prodrug of CDV (hexadecyloxypropyl-cidofovir), brincidofovir (BCV) (Fig. 1.3), was developed to reduce the dose-limiting renal toxicity of the parental drug [40]. Indeed, BCV is not a substrate of anion transporters present in renal proximal tubules, and the risk of nephrotoxicity is substantially reduced compared to CDV. Upon entry into infected cells, BCV is converted into CDV which inhibits the viral DNA pol activity [41]. The antiviral activity of BCV against all human herpesviruses was increased by two- to threefold compared to CDV [42]. Brincidofovir was also reported to be effective against HCMV and HSV isolates resistant to nucleoside analogues [39] and could be a safer alternative than CDV for the treatment of drug-resistant infections in immunocompromised patients. Brincidofovir has a high oral bioavailability and a long intracellular half-life that allow reducing the number of

administration to twice a week. In a phase II study, recipients of HSCT who received oral doses of 100 mg BCV twice weekly until week 13 after transplantation had a significant reduction in the incidence of HCMV infections [43]. Diarrhea was a dose-limiting adverse event in the group who received a dose of 200 mg twice weekly. However, recipients of HSCT who received oral dose of 100 mg BCV twice weekly for 14 weeks after transplantation did not exhibit a reduction in HCMV infections through week 24 in a phase III prophylaxis study [44]. Furthermore, the administration of BCV was associated with gastrointestinal toxicity. A retrospective study showed that BCV at doses not exceeding 200 mg/week for adults and 4 mg/kg/week for pediatric administered for 14 days was effective as an HSV and VZV prophylaxis in HSCT recipients [45]. In an attempt to reduce the gastrointestinal side effects of the drug, an intravenous formulation of BCV has been developed and is currently evaluated for its safety and for the determination of pharmacokinetic parameters.

No antiviral agents have been approved for the treatment of HHV-6A, HHV-6B, HHV-7, EBV, and HHV-8 infections. Several DNA pol inhibitors such as GCV, FOS, CDV, and BCV were shown to be effective against HHV-6A, HHV-6B, and HHV-7 in vitro as well as in case reports (see reviews in [46, 47]). In a post hoc analysis of the phase III prophylaxis study, a decrease incidence of HHV-6B in plasma was detected through day 42 in HSCT recipients who received oral BCV compared to placebo [48]. HHV-8 is sensitive to GCV, FOS, and CDV but not to ACV in tetradecanoyl phorbol acetate (TPA)-induced BCBL-1 cells latently infected with the virus [49]. EBV was shown to be susceptible to ACV, GCV, FOS, and CDV in vitro [50].

1.3.2 Resistance of Herpesviruses to Viral DNA Polymerase Inhibitors

In HSV clinical isolates, resistance to ACV is associated with mutations in the viral TK in 95% of the cases and to mutations in the viral DNA pol in the remaining cases [51–53]. The TK of HSV-1 and HSV-2 are encoded by the *UL23* gene and are composed of 376 and 375 amino acids, respectively [54]. The TK polypeptides of *Herpesviridae* contain six highly conserved domains (Fig. 1.4) [55]. These conserved regions play major roles in the enzyme activity and include the ATP-binding site, the nucleoside-binding site, and the cysteine at codon 336 (HSV-1) or 337 (HSV-2). This cysteine residue is important to maintain the three-dimensional conformation of the active site [56]. Additions or deletions in homopolymer runs of G's and C's represent hot spots for ACV resistance in *UL23* gene and lead to premature stop codon [57–59]. Single amino acid substitutions in conserved and nonconserved regions of the TK polypeptide are also detected in ACV-resistant clinical isolates [51–53]. Globally, each mechanism (additions/deletions of nucleotides or amino acid substitutions) occurs in approximately 50% of clinical isolates resistant to ACV [57]. However, the proportion of additions/deletions has been reported to increase to 62% [60] or even 80% [61] of *UL23* gene mutations in more recent studies. Mutations conferring resistance to PCV are located in the *UL23* gene and inevitably mediate cross-resistance with ACV [22, 62, 63].

The DNA pol of HSV-1 and HSV-2 are encoded by the *UL30* gene and are composed of 1235 and 1240 amino acids, respectively [2]. The DNA pol of *Herpesviridae* are members of the α-like DNA pol family [64] and share regions of homology numbered I to VII (Fig. 1.4). Their numbering indicates the degree of conservation among these enzymes, with region I being the most conserved. The DNA pol of *Herpesviridae* also contain a δ-region C, which is shared by eukaryotic DNA pol δ family members [65]. Moreover, a 3′-5′ exonuclease domain (composed of Exo I, Exo II, and Exo III conserved motifs) is associated with the NH_2-terminal region of these enzymes. In HSV clinical isolates, mutations conferring resistance to ACV are located in conserved regions of the DNA pol, especially in regions II, III, VI, and VII, most of

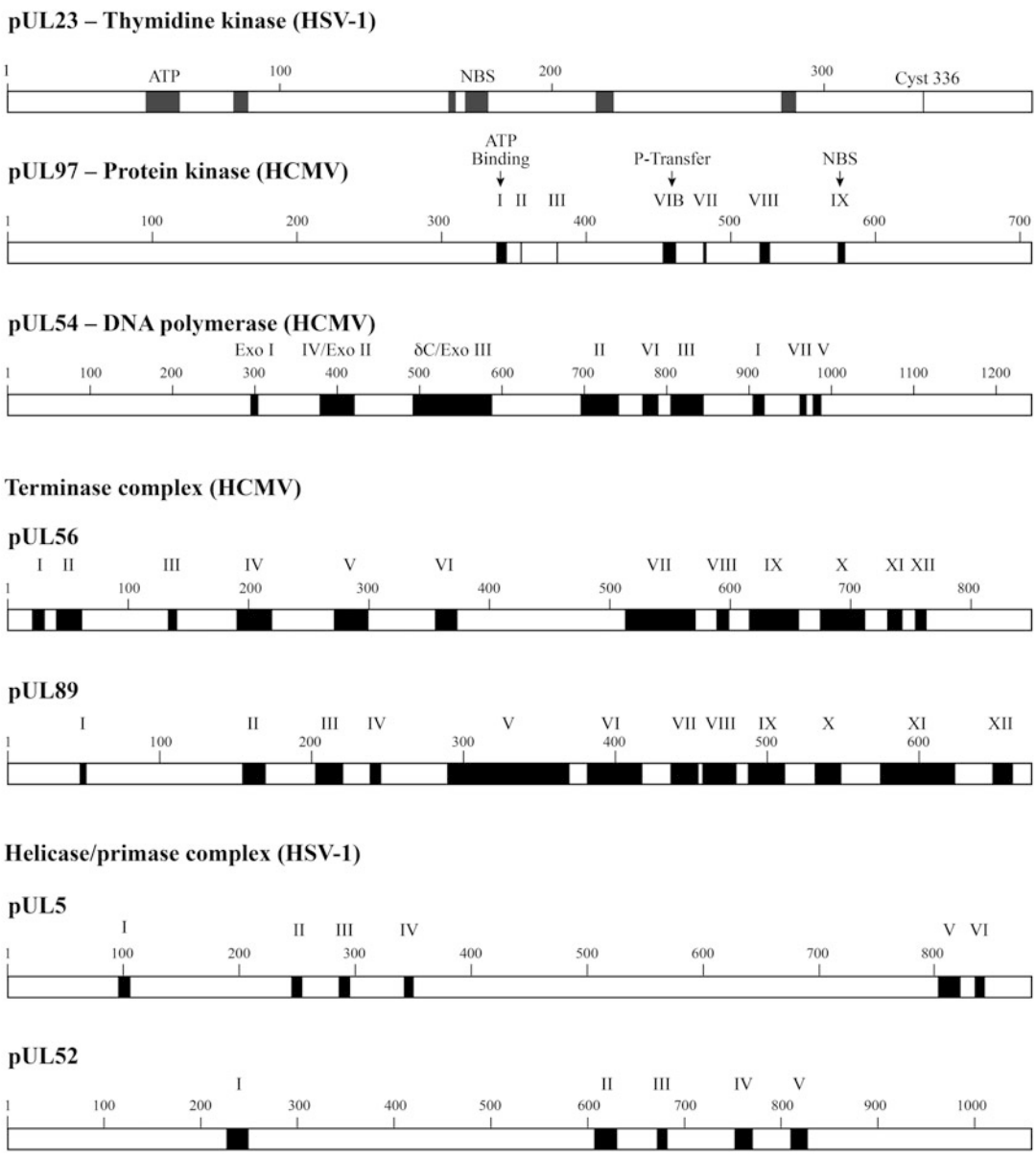

Fig. 1.4 Schematic representations of the viral proteins targeted by anti-herpetic agents. The pUL23 thymidine kinase of HSV-1 was taken as representative of pUL23 of HSV-2, ORF36 of VZV, and pU69 of HHV-6. pUL97 corresponds to the protein kinase of HCMV. The pUL54 DNA polymerase of HCMV was chosen as representative of pUL30 of HSV-1 and HSV-2, ORF28 of VZV, and pU38 of HHV-6. pUL56 and pUL89 are the two major subunits of the terminase complex of HCMV. The pUL5 helicase and pUL52 primase correspond to the two major subunits of the helicase-primase complex of HSV-1 and were taken as representatives of ORF55 and ORF6 of VZV, respectively. Graduations represent the number of amino acids in the different proteins. Black boxes show the conserved motifs in the different proteins and are numbered (roman digits) according to their level of homology for pUL97 and pUL54. In pUL23 and pUL97, ATP and NBS correspond to the ATP-binding site and the nucleoside-binding site, respectively. In pUL97, P transfer corresponds to the phosphate transfer domain (P-loop). In pUL54, Exo I, Exo II, and Exo III represents the three exonuclease motifs, whereas δ̃C is the conserved δ̃-region C

them being found in regions II and III [66, 67]. Only a few mutations have been reported within the other conserved domains or in nonconserved regions [67]. Most clinical isolates resistant to FOS contain single amino acid substitutions in conserved regions II, III, and VI as well as between regions I and VII of the DNA pol [67, 68]. Mutations located in conserved regions II and VI frequently mediate resistance to both ACV and FOS. Some mutations in regions II (S724N) and VI (L778M) of the HSV-1 DNA pol were reported to confer cross-resistance to ACV, FOS, and CDV [69].

In VZV clinical isolates, resistance to ACV is mainly associated with mutations in the viral TK and, to a lesser extent, with mutations in the viral DNA pol [51, 53]. The TK of VZV is encoded by the *ORF36* gene and is composed of 341 amino acids. The GC content is lower in the genome of VZV (46%) compared to that of HSV (68%). Therefore, the *ORF36* gene contains only a few homopolymer stretches [70]. The homopolymer run of six cytosines located at codon position 493–498 within this gene emerged as a hot spot for the insertion or deletion of nucleotides leading to ACV resistance [70–73]. Clinical isolates resistant to ACV often contain deletions of nucleotides that result in frameshift reading leading to a stop codon at position 231 [72]. In addition, amino acid substitutions conferring resistance to ACV are widely dispersed in the viral TK [71, 72, 74–78] and occurred more frequently in the ATP-binding and nucleoside-binding sites [72].

A few reports have described VZV clinical isolates resistant to ACV and/or FOS harboring mutations in the viral DNA pol [76, 79, 80]. The DNA pol of VZV is encoded by the *ORF28* gene and is composed of 1194 amino acids. The amino acid substitutions are mostly detected in the catalytic site and in the other conserved regions of the DNA pol and may confer cross-resistance to ACV and FOS.

The phenotypes of HSV and VZV clinical isolates resistant to ACV are associated with one of the following mechanisms: (1) TK-deficient which is a complete deficiency in viral TK activity; (2) TK low producer which corresponds to a decreased production of viral TK; (3) TK altered which represents a viral TK protein with altered substrate specificity, i.e., the enzyme phosphorylates thymidine, the natural substrate, but is not able to phosphorylate ACV; and finally (4) DNA pol altered which corresponds to a viral DNA pol with altered substrate specificity [57, 81–86]. It is estimated that 95% of HSV or VZV clinical isolates resistant to ACV exhibits TK-negative or TK-low producer phenotypes, whereas the remaining consists of TK-altered and DNA pol-altered mutants [57, 84].

More than 90% of drug-resistant HCMV clinical isolates selected from initial GCV therapy contain one or more mutations in the pUL97 protein kinase, and the remaining harbors mutations in the viral DNA pol [87, 88]. The viral pUL97 kinase is encoded by the *UL97* gene and is composed of 707 amino acids. The protein kinases are conserved among members of the herpesvirus family. The catalytic domain of protein kinases contains eleven major conserved regions numbered I to XI (Fig. 1.4), with region I having the highest level of homology [89]. The ATP-binding site, the phosphate transfer domain (P-loop), and the substrate-recognition site (catalytic loop) correspond to codon ranges located at positions 337–345 (region I), 481–483 (region VII), and 574–579 (region IX), respectively. Amino acid substitutions in the pUL97 kinase that were the most frequently identified in GCV-resistant clinical isolates include A594V, L595S, M460V/I, H520Q, C592G, and C603W. Other amino acid changes that occur at codons 460 and between codons 590 and 607 of the pUL97 kinase are less frequently encountered. Based on recombinant phenotyping, mutations in *UL97* gene are considered as mediating a low-grade drug resistance when they induce a two- to fivefold increase in GCV EC_{50} values over that of the wild-type strain, whereas mutations are considered as conferring a moderate level of drug resistance when they are associated with five- to tenfold increase in GCV EC_{50} values [90]. There is no major defect in the replicative capacity of recombinant viruses resistant to GCV harboring substitutions or small

deletions of amino acids in the pUL97 kinase compared to the wild-type counterpart.

The viral DNA pol of HCMV is encoded by the *UL54* gene and is composed of 1242 amino acids. HCMV clinical isolates resistant to GCV with an altered DNA pol activity possess numerous amino acid changes widely distributed in the different conserved domains of the enzyme but mostly occur at codons 395–545 and 809–987. Mutations that emerge under GCV therapy in *UL54* gene can be associated with cross-resistance to CDV and, less frequently, to FOS. Cross-resistance to GCV and CDV is mediated by amino acid substitutions located in the exonuclease domain (codons 301, 408–413, and 501–545) and in conserved region V (codons 981–987) of the DNA pol. However, clusters of amino acid changes associated with resistance to FOS alone or to both FOS and GCV were detected in conserved regions II, VI, and III. Importantly, some mutations may also confer cross-resistance to all three antivirals. In contrast to pUL97 mutants, recombinant viruses harboring amino acid substitutions conferring drug resistance in the DNA pol usually demonstrate an altered growth in cell culture compared to their wild-type counterpart. The effect of individual mutations in *UL97* and *UL54* genes are multiplied when they are combined in a single recombinant virus leading to increase in GCV EC_{50} values by more than 15-fold and are considered as conferring high-level drug resistance [91, 92].

A first HCMV strain resistant to BCV was generated under in vitro selective pressure with the drug and harbored mutation D542E in the *UL54* gene [93]. This mutation was shown to be associated with BCV and CDV (tenfold reduced susceptibility) but not to GCV or FOS by recombinant phenotyping. In vitro selection experiments under BCV pressure also elicited amino acid substitutions N408K and V812L in the viral DNA pol as well as D413Y, E303D, and E303G in the exonuclease domain [94]. These mutations confer GCV and CDV resistance with 2.4- to 17-fold higher BCV EC_{50} values. No genotypic resistance to the drug was reported during a phase II trial of BCV prophylaxis [95]. Administration of BCV after FOS in a kidney transplant recipient led to the emergence of F412L mutation in the *UL54* gene that confers cross-resistance to GCV and CDV [96].

HCMV and HHV-6 coinfections have been reported in immunocompromised patients [97]. Drug resistance mutations could thus emerge in HHV-6 isolates following prolonged prophylaxis/treatment of HCMV disease with GCV. Mutations conferring resistance of HHV-6 to GCV were detected in the *U69* and/or *U38* genes coding for the protein kinase and the DNA pol, respectively. The pU69 kinase and pU38 DNA pol of HHV-6 are composed of 563 and 1012 amino acids, respectively. An HHV-6 mutant selected by GCV pressure in vitro had two mutations, namely, M318V in the pU69 kinase and A961V in the pU38 DNA pol [98]. The M318V mutation (equivalent to the mutation M460V in the HCMV pUL97 kinase) located in the sub-domain VIb was confirmed as mediating resistance to GCV by using recombinant baculovirus phenotyping [99, 100]. Mutation A961V, which is not located in a conserved region (after region V), was proposed to confer cross-resistance to GCV, FOS, and CDV by a flow cytometry-based susceptibility assay [98]. However, resistance to FOS and GCV was not confirmed when evaluating FOS- and GCV triphosphate-induced inhibition of mutant A961V and wild-type recombinant DNA pol in a filter-based assay [101]. A series of mutations (A447D, C448G, L450S, A462D, and C463Y) located in the functional sub-domain XI of pU69 kinase of HHV-6 have been extrapolated by homology with already described GCV resistance mutations (A591D, C592G, L595S, A606D, and C607Y) in the pUL97 kinase of HCMV. Recombinant baculovirus phenotyping confirmed the role of these mutations in drug resistance [99, 100]. A series of mutations H507Y, C525S (in δ-region C), and F292S (outside conserved regions) were selected under FOS pressure in vitro [102]. Their roles in drug resistance were confirmed by testing FOS-induced inhibition of mutant and wild-type DNA pol enzymatic activity. Mutation R798I, selected under CDV pressure in vitro, confers resistance to both GCV and CDV [103]. This mutation is located in region VII and is closed

to the highly conserved motif KKRY which interacts with the primer-template DNA. The mutation M318V has been identified in peripheral blood mononuclear cells of AIDS patients infected with HHV-6 who was treated with GCV and presented a clinically resistant HCMV infection [98].

1.4 The Viral Terminase Complex

The replication of the genome of HCMV generates DNA concatemers made of head-to-tail viral genomes. The viral terminase complex cleaves these concatemeric viral DNA into unit length linear genomes that are then packaged into preformed capsids. The heterotrimeric complex of the HCMV terminase is composed of pUL56, pUL89 (Fig. 1.4), and pUL51 proteins [104]. The pUL56 subunit binds to the packaging signal (also called *pac* site) on the concatemeric viral DNA [105] as well as to the pUL104 protein that forms the portal through which the viral genome is packaged into the capsid [106]. The pUL56 subunit possesses an ATPase activity [107, 108] that probably provides the energy required for the translocation of the viral genome into the capsid and for the cleavage of the genome by the pUL89 subunit [109]. When pUL56 and pUL89 proteins are associated, the ATPase activity is increased by up to 30% [107]. The mutual interactions between the three subunits pUL56, pUL89, and pUL51, which represents the smallest component [104], regulate the stability, subcellular localization, and assembly of the functional HCMV terminase complex [110].

1.4.1 Antiviral Agents Targeting the Viral Terminase Complex

To date, derivatives that were developed as potent inhibitors of the viral terminase complex belong to three chemical classes. The first series of compounds targeting the HCMV terminase complex consists in benzimidazole D-riboside derivatives such as BDCRB (β-D-ribofuranoside-2-bromo-5,6-dichlorobenzimidazole) and its more metabolically stable analogue GW275175X (2-bromo-5,6-dichloro-1-beta-D-ribofuranosyl-1H-benzimidazole) (Fig. 1.5) [111, 112].

The second class of compounds that act on the viral terminase complex is phenylenediamine sulfonamide derivatives. Among them, BAY 38-4766 (3-hydroxy-2,2-dimethyl-N-[4([[5-(dimethylamino)-1-naphthyl]sulfonyl] amino)-phenyl]propanamide) also called tomeglovir (Fig. 1.5) showed a broader spectrum of antiviral activity compared to BDCRB [113]. GW275175X demonstrated good safety, tolerability, and pharmacokinetics profiles during a phase I single escalating dose study. Single oral doses of up to 2000 mg of BAY 38-4766 were safe and well tolerated in healthy male volunteers [114]. However, there was no further evaluation of both GW275175X and BAY 38-4766 beyond phase I clinical trials.

The third chemical class of viral terminase inhibitors is the quinazolines. Their lead compound is AIC246 (3,4-dihydro-quinazoline-4-yl-acetic acid) also known as MK-8228 or letermovir (LMV) (Fig. 1.5) [115]. The antiviral activity of LMV is highly specific to HCMV. It targets the pUL56 subunit of HCMV terminase complex and inhibits the cleavage of viral DNA concatemers and their packaging in preformed capsids [116]. The LMV EC_{50} values range in the low nanomolar concentrations, and its selectivity index is greater than 15,000 [115, 117]. Letermovir is not effective against other herpesviruses or nonhuman CMV strains [117]. Letermovir is approximately 1000-fold more potent than GCV against HCMV and exhibits an activity against viral isolates resistant to currently available drugs [117]. With the exception of UL51, homologues of proteins that composed the HCMV terminase complex have been identified in bacteriophages and herpesviruses. As no counterparts of these proteins were found in mammalian cells, it is expected that LMV would demonstrate a good safety profile. Studies in cell culture systems showed that combinations of LMV with currently available antiviral agents resulted in additive effects (with GCV and CDV) and additive/minor antagonistic effects (with FOS) against

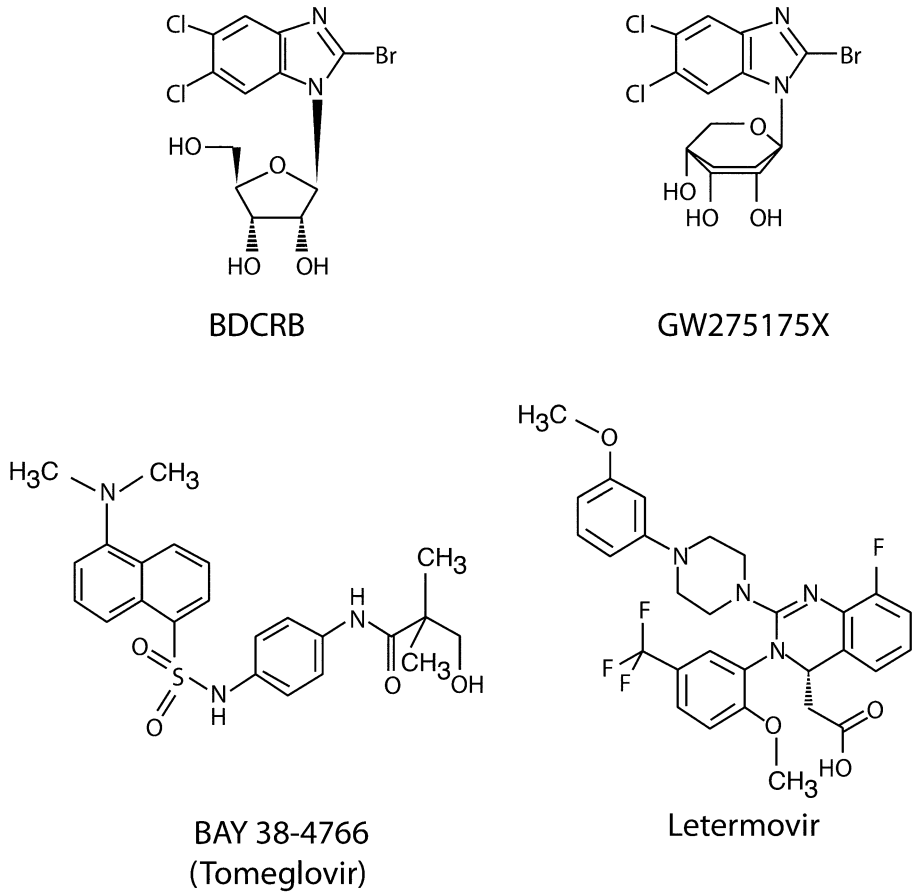

Fig. 1.5 Antiviral agents targeting the viral terminase complex

HCMV [118]. When LMV was combined with artesunate, a moderate synergistic effect against HCMV has also been reported in vitro [119]. These studies suggest that combinations of LMV with other antiviral drugs or artesunate should be further evaluated for the treatment of HCMV infections.

The US Food and Drug Administration (as of November 2017) and the European Commission (as of January 2018) approved the use of LMV under the trade name of Prevymis™ for the prophylaxis of HCMV infection in adult recipients of an allogeneic HSCT seropositive for HCMV. Letermovir is available as an oral and an intravenous formulation. The administration of the intravenous hydroxypropyl β-cyclodextrin formulation of LMV is indicated immediately after transplantation as well as in patients who present difficulties for the ingestion and absorption of oral drugs due to gastrointestinal complications [120]. The oral bioavailability of LMV is moderate (35%). Letermovir is well tolerated and demonstrates a safety profile which may allow to initiate prophylaxis during the pre-engraftment period in HSCT recipients.

Letermovir has been approved for primary HCMV prophylaxis in HSCT recipients. However, its efficacy for the treatment of HCMV disease or secondary prophylaxis has been only evaluated off-label in case series. A lung transplant recipient with disseminated multidrug-resistant HCMV disease who received low doses of LMV (from 120 mg to 240 mg once daily) successfully recovered [121]. A heart transplant recipient who received oral daily doses of 480 mg LMV as secondary prophylaxis for GCV-resistant

HCMV syndrome had a favorable outcome [122]. A first case series described four SOT recipients who received oral daily dose of 720 mg LMV (which was increased to 960 mg daily due to lack of effect in one patient) as salvage therapy for drug-resistant HCMV retinitis. All patients showed clinical improvement. However, the viral load was not reduced for 3 of them, and they were switched to alternative treatments [123]. In another case series, five lung transplant and one HSCT recipients with refractory or resistant HCMV infection were treated with LMV [124]. The viral load was reduced in three patients with asymptomatic viremia treated with oral doses of 240 mg or 480 mg LMV, partially decreased in one HSCT recipient with HCMV colitis/pneumonitis treated with IV doses of 480 mg LMV with a good clinical outcome, whereas it increased in one lung transplant recipient with HCMV syndrome treated with IV doses of 240 mg LMV (which was suggested to have been suboptimal). However, the optimal use of LMV in the treatment of HCMV infections including those that are refractory or resistant to currently available antiviral agents require to perform clinical studies involving a large number of patients and evaluating the safety, dosage, and rate of emergence of drug resistance. The efficacy of LMV in the treatment of patients experiencing refractory or resistant HCMV infection or disease with concurrent organ dysfunction is evaluated in an ongoing phase II clinical trial (ClinicalTrials.gov Identifier: NCT03728426). Patients with refractory or resistant HCMV infection will be treated with IV or oral daily doses of 480 mg LMV (240 mg if given with cyclosporine) for up to 12 weeks (if clinically indicated, treatment for secondary prophylaxis could be extended for an additional 12 weeks).

1.4.2 Resistance of HCMV to Viral Terminase Inhibitors

In cell culture studies, mutations conferring resistance to LMV were mainly detected in the *UL56* gene and more rarely in the *UL89* and *UL51* genes encoding the three subunits of the HCMV terminase complex. pUL56 is encoded by the *UL56* gene of HCMV and is composed of 850 amino acids. pUL56 is conserved among herpesviruses, and 12 conserved regions (numbered I to XII) (Fig. 1.4) were identified based on homology studies between proteins of different herpesviruses [125]. pUL56 also contains two variable regions between conserved regions VI and VII and after conserved region XII. Mutations conferring resistance to LMV are mediated by amino acid substitutions in the pUL56 subunit and are located at codon 25 and between codons 229 and 369. The impacts of these mutations on viral growth fitness were minimal to low compared to wild-type counterparts.

pUL89 is encoded by the *UL89* gene of HCMV and is composed of 674 amino acids. pUL89 is conserved among herpesviruses, and 12 conserved regions (numbered I to XII) (Fig. 1.4) were identified between HCMV and other herpesvirus counterparts [126]. The T4 bacteriophage contains four amino acid motifs (i.e., adenine binding site, Walker A or motif I [-γ-phosphate sensor], Walker B or motif II [ATPase motor], and motif III [ATPase coupling helicase]) [127] that are located in conserved regions II, III, and V (for the last two motifs), respectively. Mutations conferring resistance to LMV in the *UL89* gene were selected by exposure of an error-prone exonuclease HCMV mutant to LMV, GW275175X, and tomeglovir [128, 129]. All the amino acid changes identified (N320H, N329S, D344E, and T350M) are located in conserved region V of the pUL89 subunit and were associated with low-grade resistance to LMV. The cytopathic effects and viral growth of recombinant viruses harboring LMV-resistant mutations in the *UL89* gene were similar to those of wild-type counterparts.

The third component of the viral terminase complex, pUL51, is encoded by the *UL51* gene of HCMV [129]. pUL51 is a 157-amino-acid protein which has no equivalent in bacteriophages. pUL51 is homologous to pUL33 in HSV-1 with a sequence similarity limited at the C-terminal part (amino acids 73 to 149 of pUL51), whereas the N-terminal part of the protein is not conserved among herpesviruses

[104]. A bacterial artificial chromosome (BAC) clone of HCMV strain AD169 was exposed to LMV and allowed the selection of P91S substitution which confers low-grade resistance to the drug. The mutant recombinant virus had a similar viral growth compared to the wild-type strain.

HCMV clinical isolates resistant to LMV harbor mutations that were all located in the *UL56* gene. These mutations consist in amino acid substitutions and include V236M [130–133], E237G [131], C325W [131, 133, 134], C325F [123, 135], C325Y [123, 130, 134, 136–138], and R369T [131] that confer levels of resistance to LMV of >19-, 13-, 9300-, >3000-, >3000-, and 52-fold, respectively. The mutation C325Y was shown to preexist in the *UL56* gene in HCMV-infected tissues of patients who have never received LMV or other viral terminase inhibitors [139]. No mutations were detected in *UL89* and *UL51* genes in HCMV strains isolated from patients under LMV.

1.5 The Viral pUL97 Kinase

The pUL97 is a serine/threonine protein kinase (Fig. 1.4) that phosphorylates itself and a series of viral and host proteins that are involved in different steps of the viral replication cycle [140]. In the HCMV virion, pUL97 is associated with pp65 tegument protein [141]. During the infection, pUL97 is expressed early and mainly localizes in the nucleus and is detected later in the cytoplasm [140]. The viral pUL97 kinase is involved in the phosphorylation of several viral proteins such as the major immediate early promoter [142], the viral polymerase accessory protein pUL44 [143, 144], the viral pp65 tegument protein [145], and the nuclear mRNA export factor pUL69 [146]. The viral pUL97 kinase exhibits functional homologies with the cellular cyclin-dependent kinase complexes (CDKs). Indeed, pUL97 kinase is able to phosphorylate the retinoblastoma tumor suppressor protein (Rb) on sites that are normally phosphorylated by CDKs. Phosphorylated Rb cannot repress host genes that are required for the progression of the cell cycle to the S phase and that are crucial for HCMV DNA synthesis [147]. The viral pUL97 kinase is also recruited to the nuclear rim by the nuclear egress complex (NEC) which is composed of the pUL50 and pUL53 subunits. The pUL97 kinase then phosphorylates the lamin A/C [148] and the NEC leading to disruption of the nuclear lamina and egress of viral particles into the cytoplasm [149]. Thus, the viral pUL97 kinase supports the viral DNA synthesis by modulating the cell cycle, regulates the expression of viral genes, promotes virion morphogenesis, and induces nuclear lamina disassembly to facilitate the nuclear egress of nascent viral particles [150]. The autophosphorylation of pUL97 is suggested to be involved in the regulation of its enzymatic activities [151].

1.5.1 Antiviral Agents Targeting the Viral pUL97 Kinase

The benzimidazole L-ribosides were discovered while searching for more stable derivatives of BDCRB. The lead compound 1263W94 (1H-β-L-ribofuranoside-2-isopropylamino-5,6-dichlorobenzimidazole) also known as SHP620 or maribavir (MBV) (Fig. 1.6) exerts antiviral activity against HCMV and EBV [152]. Maribavir acts by inhibiting the pUL97 kinase activity [152, 153]. Maribavir is the active form and does not require intracellular phosphorylation. In cell culture, the EC_{50} value of MBV against HCMV is approximately 0.3 μM which is tenfold lower than that of GCV. An antiviral activity against HCMV strains resistant to GCV was also reported [154].

The essential role of this enzyme in the viral replicative cycle was demonstrated by the use of laboratory-engineered pUL97-deficient HCMV mutant which exhibits a severe growth defect compared to the wild-type parental strain [155]. The kinase active site contains an invariant lysine at position 355. Its deletion (K355del) or amino acid changes at this position such as K355M or K355Q abolish the kinase activity [151, 156]. Infection of cells with pUL97-defective HCMV mutants results in an inefficient viral DNA synthesis and the formation of nuclear

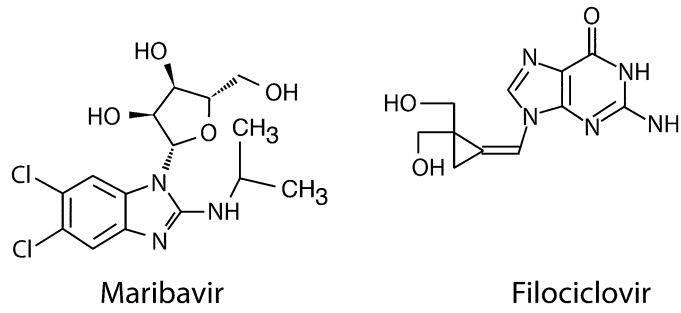

Fig. 1.6 Antiviral agents targeting the viral protein kinase and both the viral protein kinase and DNA polymerase

aggregates containing sequestered structural proteins, especially the pp65 tegument protein, resulting in an altered virion morphogenesis. The defects in the viral replicative cycle observed after inhibition of pUL97 kinase activity by MBV are similar to those caused by pUL97-defective HCMV mutants. Thus, MBV interferes with the virion morphogenesis and the nuclear egress of viral particles in infected cells [157]. Maribavir is a competitive inhibitor of adenosine triphosphate (ATP) binding to the pUL97 kinase as shown by enzyme kinetic experiments [158]. Docking of the MBV molecule to the pUL97 kinase structure obtained by three-dimensional modeling confirmed that the drug competes with the binding of ATP [159]. The benzimidazole ring appears to be close to residues 353 and 409–411 that are located between the P-loop and the catalytic loop [160].

The pUL97 kinase of HCMV is involved in the phosphorylation of GCV, but it does not seem to phosphorylate natural deoxynucleosides [140]. As MBV is an inhibitor of pUL97 kinase activity, a combination of GCV and MBV results in an antagonistic effect against HCMV infection [161]. Synergistic effects against wild-type and drug-resistant HCMV strains were induced by combinations of MBV with FOS, CDV, and LMV, whereas it was strongly synergistic when using combinations of MBV with rapamycin [162, 163]. Additional investigations are needed to further evaluate the benefit of combining MBV with other antiviral agents or rapamycin. Bone marrow progenitors and different human leukemia cell lines exposed to MBV exhibit low toxicity [153, 164]. In contrast to GCV, the lack of toxicity of MBV for the bone marrow may be compatible with pre-engraftment prophylaxis in HSCT recipients.

Preclinical studies showed that MBV has a favorable safety profile, good oral bioavailability, and lower toxicity than currently available antiviral agents [165]. After failing in a phase III study [166], higher doses of MBV were investigated in two phase II, dose-ranging, efficacy studies. Firstly, the safety, tolerability, and efficacy of preemptive 400 mg, 800 mg, or 1200 mg doses of MBV and 900 mg dose of VGCV administered twice daily for up to 12 weeks were compared in HSCT and SOT recipients. In all dose groups, treatment with MBV had similar efficacy compared to VGCV for clearing HCMV viremia within 6 weeks (77% *versus* 65%) with a comparable effect between MBV doses (78%, 83%, and 72% of patients in the 400 mg, 800 mg, and 1200 mg dose groups, respectively) [167]. Secondly, the safety, tolerability, and efficacy of 400 mg, 800 mg, or 1200 mg escalating doses of MBV administered twice daily for up to 24 weeks were evaluated for the treatment of refractory or drug-resistant HCMV disease in transplant recipients. In all MBV dose groups, the levels of viral DNA were not detectable in the plasma of 67% of patients within 6 weeks of treatment with similar effects observed between the different MBV doses (70%, 63%, and 67% of patients in the 400 mg, 800 mg, and 1200 mg dose groups, respectively) [168]. Two phase III studies with MBV are ongoing. The first one compares the efficacy and safety of 400 mg MBV and 900 mg VGCV administered twice daily for the treatment of HCMV infections in HSCT recipients (ClinicalTrials.gov Identifier: NCT02927067).

The second phase III study compares the efficacy and safety of 400 mg MBV given twice daily with investigator-assigned drug for the treatment of refractory or drug-resistant HCMV infections in transplant recipients (ClinicalTrials.gov Identifier: NCT02931539).

1.5.2 Resistance of HCMV to pUL97 Kinase Inhibitors

In cell culture systems, MBV resistance mutations were mainly detected in the *UL97* gene. Compensatory mutations were also found in the *UL27* gene. The majority of amino acid changes conferring resistance to MBV do not generally affect susceptibility to GCV and are located at the vicinity of the ATP-binding site of the pUL97 kinase. Two amino acid changes are located in the ATP-binding loop (L337M and F342S); the substitution F342S confers resistance to both MBV and GCV. A few amino acid substitutions (D456N, V466G, C480R, and P521L) or deletion (Y617del) is found at distance from the ATP-binding site and confers cross-resistance to MBV and GCV.

Four conserved regions numbered I to IV were identified in pUL27 following the analysis of intra- and interspecies conservation of the protein [169]. Laboratory-engineered pUL27-deficient HCMV mutant exhibits a modest half log decrease in viral titers in vitro and no apparent alteration of viral growth in vivo [170]. After exposure of HCMV strains to MBV, amino acid substitutions were selected in pUL27 and shown to confer low levels of drug resistance. Frameshift mutations leading to truncated proteins were also described. During HCMV replication, it is suggested that pUL27 delays the progression of the cell cycle toward the G_1/S phase. The mechanism involves the induction of proteasome-dependent degradation of Tip60, a cellular histone acetyltransferase (HAT), leading to an increased expression of p21, a cellular CDK inhibitor [171]. In contrast, pUL97 phosphorylates and inactivates Rb to favor the progression of the cell cycle toward the S phase and promote viral DNA synthesis. When pUL97 kinase activity is inhibited by MBV, Rb cannot be inactivated, and S phase genes that are needed for viral DNA synthesis remain silent. Thus, the loss of pUL27 activity could compensate for the inhibition of pUL97 functions by MBV explaining the mechanism of emergence of drug resistance mutations in the *UL27* gene [172]. Furthermore, mutations were shown to spontaneously emerge in the *UL27* gene of pUL97-deficient HCMV strains that are not exposed to MBV [173]. This confirms that the apparition of mutations in pUL27 could compensate for the loss of pUL97 kinase activity.

In HCMV clinical isolates, two mutations mediating resistance to MBV were identified in the *UL97* gene. Amino acid changes T409M [168, 174, 175] and H411Y [168, 175] confer levels of resistance of 81- and 12-fold, respectively. No mutations conferring resistance to MBV were detected in the *UL27* gene in clinical isolates.

1.5.3 Antiviral Agents Targeting Both the pUL97 Kinase and the DNA Polymerase

Filociclovir ([Z]-9-[[2,2-bis-(hydroxymethyl)-cyclopropylidene]methyl]guanine) also known as MBX-400 or cyclopropavir (Fig. 1.6) belongs to the methylenecyclopropane nucleoside analogues which is a new chemical class. Filociclovir exerts a potent activity against HCMV, EBV, HHV-6A, HHV-6B, and HHV-8 in vitro [176, 177]. The EC_{50} value of filociclovir against HCMV is 1.0 ± 0.6 μM. Most GCV-resistant HCMV clinical isolates remain susceptible to this drug. Filociclovir inhibits HCMV replication by a dual mechanism of action that involves the inhibition of the activities of both the viral DNA pol and pUL97 kinase [176, 178]. Recombinant viruses with large deletions in pUL97 kinase or with the K355M mutation are resistant to filociclovir suggesting that the compound requires pUL97 kinase for its antiviral activity [176]. In enzymatic studies, filociclovir was shown to be a better substrate for the pUL97 kinase than GCV [179]. The high

affinity of filociclovir for the pUL97 kinase could explain the inhibition of the enzyme activity in HCMV-infected cells [178]. Filociclovir also appears to exert its antiviral activity through the inhibition of DNA synthesis [176]. Filociclovir is phosphorylated in filociclovir monophosphate by pUL97 kinase and then further phosphorylated by the cellular guanosine monophosphate kinase in its triphosphorylated form which could inhibit the viral DNA pol activity [178]. The accumulation of filociclovir triphosphate in HCMV-infected cells was shown to be higher than that of GCV triphosphate [180]. Filociclovir triphosphate is a more potent inhibitor of UL54 DNA pol activity than GCV triphosphate [181]. Filociclovir triphosphate competes with deoxyguanosine for incorporation into elongating DNA. Filociclovir triphosphate is also a substrate for the DNA pol and acts as a non-obligate chain terminator of DNA synthesis with termination occurring after incorporation of the next nucleotide. Single oral dose of filociclovir ranging from 35 to 1350 mg was safe and well tolerated in healthy volunteers in a phase Ia study (ClinicalTrials.gov Identifier: NCT01433835). The most frequently reported side reactions were gastrointestinal adverse effects. A phase Ib trial evaluating escalating doses of filociclovir (100 mg, 350 mg, 750 mg, and 1000 mg) given once daily for 7 days in healthy volunteers demonstrated that adequate drug exposure could be achieved from doses as low as 100 mg [182].

1.5.4 Resistance of HCMV to Filociclovir

Mutations conferring resistance to filociclovir have been detected in the pUL97 kinase. Two of the most frequently encountered GCV-resistant mutations, C592G and A594V, also decrease the susceptibility to filociclovir [183]. Mutations M460I, H520Q, and C603R, which confer resistance to GCV, were selected under pressure with filociclovir in vitro and induce high levels of resistance to the drug. A mutant harboring a deletion of nucleotide 498 in *UL97* gene that results in a truncated protein lacking the kinase domain was also selected under pressure with filociclovir in vitro [184]. This mutant confirms that pUL97 kinase is involved in the mechanism of action of the drug. Deep sequencing analysis of laboratory strains resistant to filociclovir also revealed mutations in *UL27* gene which is consistent with an inhibition of pUL97 kinase activity [178]. Mutations located in the catalytic domains of pUL54 DNA pol that mainly confer resistance to FOS and variable cross-resistance to GCV and CDV (Q578H, E756K, V781I, A809V, T813S, A834P, M844T, and G841A) were shown to decrease the susceptibility to filociclovir [185]. In contrast, mutations located in the exonuclease domain and in conserved region V that are commonly associated with cross-resistance to GCV and CDV confer increased susceptibility to filociclovir. It is suggested that the structural conformation of the methylcyclopropane moiety of filociclovir could prevent its excision by the exonuclease. Mutations E756D and M844V in the *UL54* gene were selected under pressure with filociclovir in vitro and confer resistance to the drug.

1.6 The Helicase-Primase Complex

The helicase-primase is a heterotrimeric complex composed of the helicase, primase, and cofactor subunits (Fig. 1.4). The helicase-primase unwinds double-stranded DNA and produces primers for DNA synthesis by the viral DNA pol. The helicase and primase subunits were shown to be, respectively, involved in the 5′-3′ helicase/single-stranded DNA-dependent ATPase activities and the primase functions. The cofactor subunit has no known catalytic activity but is involved in multiple protein-protein interactions. The cofactor subunit could interact with other components of the replication machinery and also possibly coordinates the replication fork progress. The helicase-primase complex is well conserved among members of the *Herpesviridae* family. The helicase, primase, and cofactor subunits are encoded by the *UL5*, *UL52*, and *UL8* genes for HSV and by the *ORF55*, *ORF6*, and *ORF52* genes for VZV. Due to its essential role in the

viral replication, the helicase-primase complex constitutes an excellent target for the development of potent antiviral agents.

1.6.1 Antiviral Agents Targeting the Helicase-Primase Complex

Three classes of compounds have been shown to target the helicase-primase complex. The first class consists in 2-amino-thiazole thiazolylphenyl derivatives such as BILS 179 BS (*N*-[2-[4-(2-amino-1,3-thiazol-4-yl)anilino]-2-oxoethyl]-*N*-[(1*S*)-1-phenylethyl]pyridine-4-carboxamide) (Fig. 1.7). BILS 179 BS was shown to be active against HSV-1 and HSV-2 including strains resistant to ACV but not against other herpesviruses [186]. BILS 179 BS acts by inhibiting the activities of viral helicase, single-stranded DNA-dependent ATPase, and primase.

The second class of helicase-primase inhibitors (HPI) is thiazole urea derivatives such as BAY 57-1293 (N-methyl-N-(4-methyl-5-sulfamoylthiazol-2-yl)-2-(4-(pyridin-2-yl)phenyl)acetamide) or AIC316 or pritelivir (Fig. 1.7). Pritelivir is highly active against HSV-1 and HSV-2, including ACV-resistant strains, but not against VZV [187]. Pritelivir is about tenfold more potent than ACV in vitro. Pritelivir acts by inhibiting the single-stranded DNA-dependent ATPase activity of the helicase-primase complex. A combination of pritelivir with nucleoside analogues showed synergistic effects in cell culture systems [188]. Three phase I clinical studies showed that the drug was generally well tolerated and demonstrated a high and long-lasting exposure in humans (up to 80 h). Oral pritelivir (5 mg, 25 mg, or 75 mg daily or 400 mg weekly) reduced the rates of genital HSV-2 shedding and days with lesions in a phase II trial [189]. A phase II study compared the efficacy of daily oral doses of pritelivir (100 mg) with VACV (500 mg) for the treatment of patients with frequently recurring genital HSV-2 and showed that both drugs reduced the percentage of swabs positive for HSV detection on 28 days [190]. However, the clinical trial was terminated due to adverse events (such as dry skin, crusty skin lesions, alopecia, and anemia) in a nonclinical study performed at the same period. Further studies are needed to evaluate the efficacy and toxicity of this drug. AiCuris has been granted breakthrough therapy designation by the US Food and Drug Administration for oral pritelivir for the treatment of HSV infections in immunocompromised patients in June 2020.

The third class of HPI includes oxadiazolephenyl derivatives such as ASP2151 (*N*-[2,6-dimethylphenyl]-*N*-[2-[4-(1,2,4-oxadiazol-3-yl)anilino]-2-oxoethyl]-1,1-dioxothiane-4-carboxamide) or amenamevir (Fig. 1.7). This compound is highly active against HSV-1, HSV-2, and VZV in vitro [191]. Amenamevir was also active against an ACV-resistant VZV strain. The EC_{50} values of amenamevir against HSV-1 and HSV-2 isolated from genital lesions were 0.043 μM and 0.069 μM, respectively [192]. The EC_{50} values of amenamevir against VZV strains in human embryonic fibroblasts were <0.1 μM. Amenamevir inhibits the activities of the helicase, single-stranded DNA-dependent ATPase, and primase in a recombinant helicase-primase complex assay system. In contrast to ACV, amenamevir directly inhibits the viral helicase-primase activity and is not affected by supply in deoxyribonucleosides induced by the replication of VZV and HSV [193]. A combination of amenamevir with ACV and other nucleoside analogues demonstrated synergistic/additive effects against HSV and VZV infections [194, 195]. The oral bioavailability of amenamevir was 86% [196]. Amenamevir was safe and well tolerated. The most common side effects are the excretion of N-acetyl-β-glucosaminidase and α1-microglobulin in urine. However, there are no safety concerns for the kidney in patients with normal or impaired renal function. In a phase II study, three daily (100 mg, 200 mg, or 400 mg) or single daily (1200 mg) dosing of amenamevir for 7 days or VACV (500 mg twice daily) for 3 days was effective in the treatment of recurrent genital herpes [197]. The same amenamevir dosages were also compared with VACV (1000 mg three times daily for 7 days) against herpes zoster. The proportion of patients with cessation of new

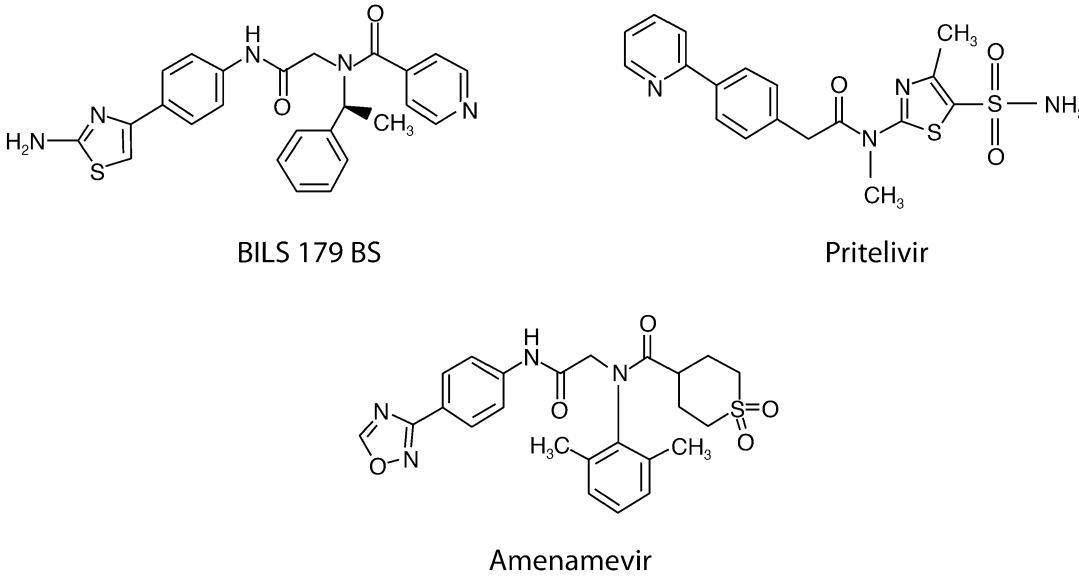

Fig. 1.7 Antiviral agents targeting the viral helicase-primase complex

zoster lesions by day 4 were 90.9%, 85.5%, 87.7%, and 87.3% in 400 mg, 200 mg, and 100 mg amenamevir and VACV groups, respectively [198]. In a phase III study, amenamevir (400 mg once daily for 7 days) was evaluated for the treatment of herpes zoster and was effective at reducing the formation of new lesions and preventing postherpetic neuralgia in immunocompetent Japanese patients [199]. Amenamevir was also reported to potently suppress the development of acute herpetic pain and postherpetic neuralgia in a murine model of zosteriform-like skin lesions induced by HSV-1 [200]. In 2017, amenamevir (Amenalief®) was approved for the treatment of herpes zoster in Japan. The usual oral dose is 400 mg amenamevir once daily for 7 days.

1.6.2 Resistance of Herpesviruses to Helicase-Primase Inhibitors

Mutations conferring resistance to pritelivir were selected in vitro in the *UL5* and *UL52* genes encoding the helicase and primase subunits of HSV-1 [201]. Most mutations associated with resistance to pritelivir were located in the *UL5* gene. The pUL5 subunit of HSV-1 has 882 amino acids and contains six putative conserved helicase motifs (Fig. 1.4). Amino acid substitution N342K is located in the functional helicase motif IV [202]. Mutations G352V, M355T, and K356N (or K355N in HSV-2) are located downstream of helicase motif IV [187, 203]. Other amino acid substitutions at the same position, K356Q and G352R, are associated with increased and decreased virus growth in cell culture [201]. Of note, mutations K356N, G352V, and G352C located in the *UL5* gene of HSV-1 were also selected under pressure with BILS 22 BS (an HPI in the same class as BILS 179 BS) and confer resistance to the drug [204]. The pUL52 subunit has 1058 amino acids and contains seven conserved primase motifs, including a zinc finger motif (Fig. 1.4). Amino acid substitution A899T (A905T in HSV-2) selected under pressure with pritelivir was identified in the *UL52* gene of HSV-1 [205]. Mutation K356T was shown to emerge spontaneously at high frequency in HSV-1 laboratory isolates [206]. The highly resistant mutation K366N (more than 100-fold resistant to the drug) was also found at high frequency in 2 of 10 clinical isolates of HSV-1 not exposed to the drug [207]. Mismatch primer-based PCR revealed that mutations K356N,

K356T in *UL5* gene, and A899T in *UL52* gene preexisted in HSV-1 clinical isolates and were not induced during exposition to the drug [208]. No evidence of mutations conferring resistance to pritelivir was observed after 4 weeks of daily therapy for treatment of frequently recurrent genital HSV-2 in immunocompetent individuals [209].

After in vitro selection in the presence of high concentrations of amenamevir, amino acid substitutions were detected in *ORF55* and *ORF6* genes encoding the helicase and primase subunits of VZV. The mutation N336K (analogous to N342K selected under pritelivir in HSV-1) was identified in motif IV of the helicase subunit. Two other mutations, R446H and N939D, were detected in *ORF55* and *ORF6* genes, respectively. This mutant showed a marked defect in viral replication. R367H combined with S364G mutations in the *UL52* gene of HSV-1 increased the level of resistance to amenamevir compared to S364G alone [210]. Mutations K355N and K451R in *UL5* gene of HSV-2 also resulted in high levels of resistance to amenamevir (>1000-fold).

1.7 Conclusions

Until recently, all antiviral agents approved for the prevention and treatment of infections caused by HSV, VZV, and HCMV targeted the viral DNA polymerase. In 2017, letermovir, an inhibitor of the terminase complex of HCMV, was approved under the trade name of Prevymis®. This new drug is indicated for the prophylaxis of HCMV infections in adult recipients of an allogeneic HSCT seropositive for HCMV. Flexible nucleoside analogues of acyclovir based on acyclic sugar synthesis were reported to overcome drug resistance and renewed interest in nucleoside analogue development [211]. Nevertheless, the development of new anti-herpetic compounds acting on different viral or cellular targets and presenting a good safety profile is still an urgent need. Furthermore, no antiviral agents are still approved for the treatment of infections caused by HHV-6A, HHV-6B, HHV-7, HHV-8, and EBV. Several steps of the herpesvirus replicative cycle constitute interesting targets for the development of potent inhibitors. A few compounds targeting the protein kinase, the protein kinase/DNA pol, and the helicase-primase complex are being evaluated in clinical trials. Furthermore, the portal protein involved in the encapsidation of DNA emerged as a novel target for the development of herpesvirus inhibitors [212, 213]. More recently, new medicinal chemistry strategies such as virtual screening of compound databases by molecular docking to target proteins [214], structure activity relationship [215], machine learning analysis [216], and fragment-based drug discovery approach to generate optimized hits by using fluorescence-based binding assays [217] and X-ray crystallography [218] have been developed to identify novel compounds with potent activity against herpesviruses.

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An Update on Antiretroviral Therapy

2

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Abstract

Human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) still claim many lives across the world. However, research efforts during the last 40 years have led to the approval of over 30 antiretroviral drugs and the introduction of combination therapies that have turned HIV infection into a chronic but manageable disease. In this chapter, we provide an update on current available drugs and treatments, as well as future prospects towards reducing pill burden and developing long-acting drugs and novel antiretroviral therapies. In addition, we summarize efforts to cure HIV, including pharmaceutical strategies focused on the elimination of the virus.

Keywords

HIV · Antiretroviral drug · Drug resistance · Toxicity · Combination therapy · Eradication

Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALLINI	Allosteric integrase inhibitor
AZT	3′-azido-3′-deoxythymidine
bNAb	Broadly neutralizing antibody
CA	Capsid protein
CRISPR	Clustered regularly interspaced short palindromic repeats
dsDNA	Double-stranded DNA
EFdA	4′-ethynyl-2-fluoro-2′-deoxyadenosine
FDA	Food and Drug Administration
HAART	Highly active antiretroviral therapy
HAM/TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis
HBV	Hepatitis B virus
HDAC	Histone deacetylases
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T cell lymphotropic virus
IC_{50}	50% maximal inhibitory concentration
LEDGF	Lens epithelium-derived growth factor
LRA	Latency-reversing agents
NC	Nucleocapsid protein
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
RT	Reverse transcriptase
SHIV	Simian/human immunodeficiency virus

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TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TLR	Toll-like receptors
WHO	World Health Organization

2.1 Introduction

Retroviruses are enveloped viruses with an RNA genome. They owe their name to their replication cycle, because retroviruses possess an enzyme known as reverse transcriptase (RT) that converts the RNA genome into DNA, the reverse flow of information as defined in the central dogma of molecular biology [1]. Human retroviruses include human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2, respectively) [2] and four types of human T cell lymphotropic viruses (HTLV) [3, 4]. All of them infect T cells. Another retrovirus belonging to the genus *Spumavirus*, known as human foamy virus, has also been isolated from lymphoblastoid cells obtained from a human nasopharyngeal carcinoma from a Kenyan patient, although the virus was probably acquired from chimpanzees through a zoonotic infection. Human foamy viruses have been isolated from patients with various neoplastic and degenerative diseases, but humans zoonotically infected with simian foamy viruses show no signs of associated disease (for a recent review, see [5]).

HIV is the etiological agent of the acquired immunodeficiency syndrome (AIDS), while HTLV causes a type of cancer known as adult T cell leukaemia/lymphoma and a demyelinating disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HIV and HTLV have a high prevalence in the human population. It is estimated that more than 10 million people are infected with HTLV-1 and HTLV-2, although only a small portion (less than 3%) are expected to develop disease. In contrast, HIV infection is a dangerous disease if not treated. Currently, HIV infects around 38 million people around the world, with 1.7 million new infections and 690,000 deaths in 2019 [6]. Considering the whole world population, the HIV prevalence is estimated at 0.37 per thousand people. However, these numbers are remarkably higher in certain countries, particularly in southern sub-Saharan Africa, where Botswana, Lesotho, Mozambique, Namibia, South Africa, Eswatini, Zambia and Zimbabwe have an estimated HIV prevalence above 10%, in adults aged 15–49 [7].

Improved antiretroviral therapies and expanded access programmes aimed to provide universal coverage for the treatment of HIV-infected patients bring hope for HIV eradication. However, success depends on the strength of health systems across the world. Currently, about 67% of the infected individuals receive antiretroviral therapy. Although these numbers are promising, they are still far from the goals needed to achieve HIV eradication across the world. In addition, antiretroviral drug toxicity and the potential appearance of resistance remain as major threats to current therapies. In this chapter, we provide an update on current treatments and future prospects towards reducing pill burden and developing long-acting drugs, as well as novel antiretroviral therapies. In addition, we provide a general overview of different pharmaceutical approaches to cure HIV infection.

2.2 Currently Approved Antiretroviral Therapies

The goal of HIV therapies is to prevent virus replication and propagation. Antiretroviral drugs block different steps of the virus replication cycle [8] (Fig. 2.1). Entry inhibitors block HIV binding to the cellular receptor CD4 (ibalizumab) or its coreceptor CCR5 (maraviroc). After binding, conformational changes in the HIV complex gp120/gp41 facilitate fusion of the viral envelope and the cell membrane. This step is blocked by fusion inhibitors like enfuvirtide, an approved linear 36-amino-acid synthetic peptide with the N-terminus acetylated and a carboxamide at the C-terminus. It is a peptide mimetic of the HR2 region within HIV-1 gp41, the transmembrane subunit of the viral envelope protein. Enfuvirtide binds to the HR1 region of gp41, blocking the

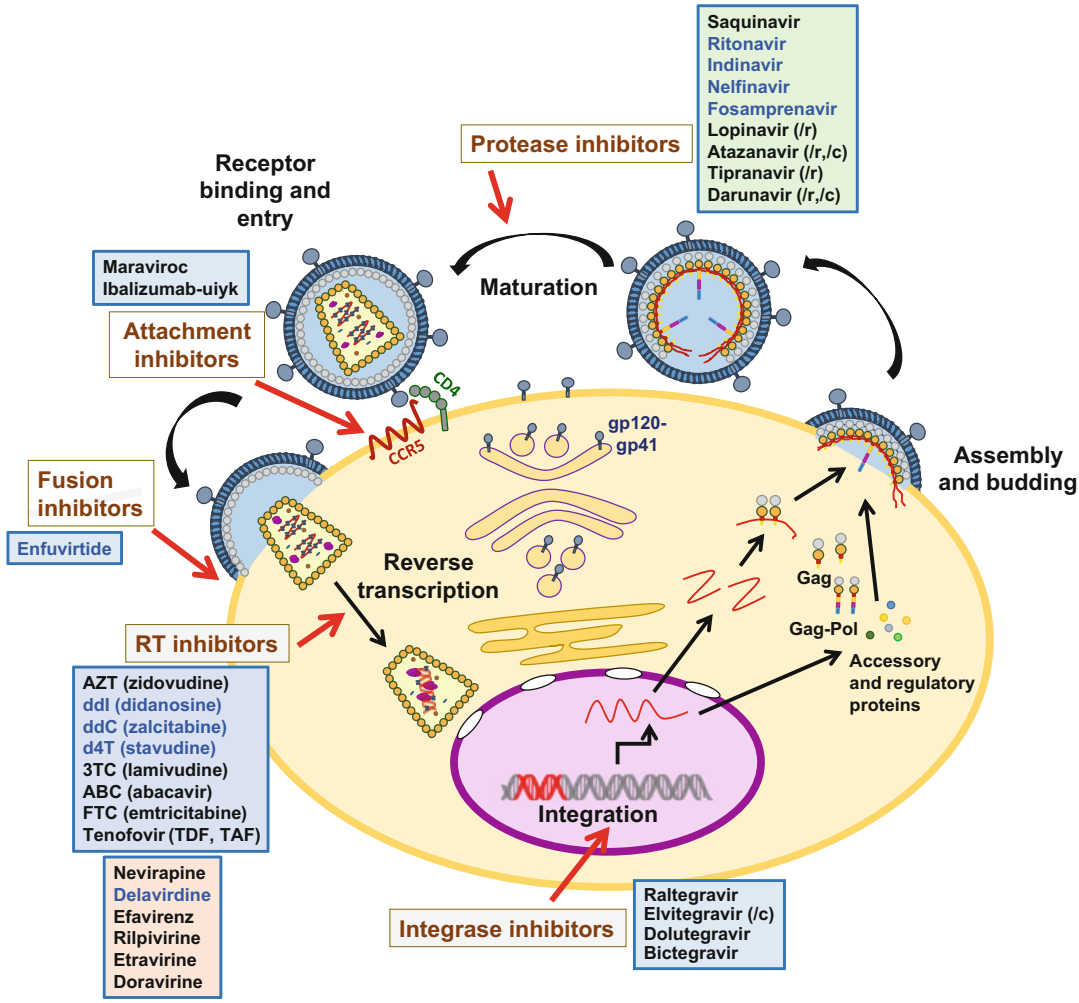

Fig. 2.1 Approved antiretroviral drugs and HIV replication cycle. Less frequently used or discontinued drugs are shown in blue. */c* boosted with cobicistat, */r* boosted with ritonavir, *TAF* tenofovir alafenamide, *TDF* tenofovir disoproxil fumarate. Cobicistat and ritonavir are pharmacokinetic enhancers administered with specific protease inhibitors or the integrase inhibitor, elvitegravir, to optimize their therapeutic concentrations. Their mechanism of action involves the inhibition of cytochrome P450 3A (CYP3A4) enzymes

HR1–HR2 interaction essential for the fusion process.

RT inhibitors block the conversion of the viral single-stranded genomic RNA into double-stranded DNA (dsDNA), necessary for viral replication. The HIV-1 RT is a heterodimer composed of subunits of 66 and 51 kDa, known as p66 and p51, respectively. The 66-kDa subunit contains the active sites of the DNA polymerase and RNase H activities. During DNA polymerization, the RT can use RNA or DNA as template. The RT's DNA polymerase activity is the target of all currently approved RT inhibitors. There are two classes of RT inhibitors in clinical use: nucleoside analogues (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). NRTIs are the backbone of current antiretroviral therapies and together with NNRTIs represent more than half of the drugs approved for clinical use (Fig. 2.2). NRTIs are prodrugs that are converted into triphosphorylated nucleoside analogues by cellular enzymes. The nucleotide analogues are

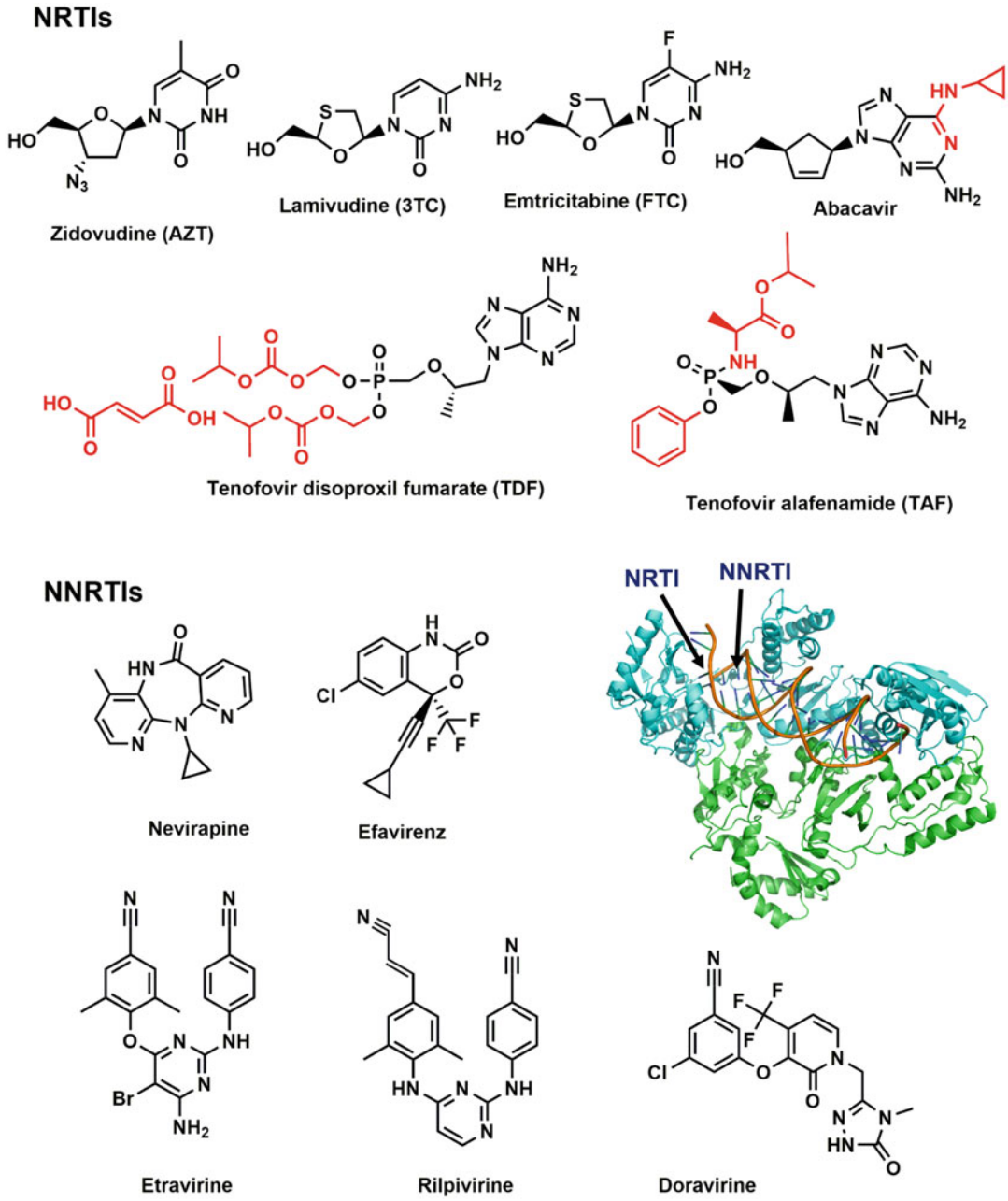

Fig. 2.2 Chemical structures of clinically relevant NRTIs and NNRTIs and HIV-1 RT structure. Atoms modified during the conversion of abacavir to its metabolically relevant derivative carbovir triphosphate and disoproxil fumarate and protide moieties in tenofovir prodrugs are shown in red. A cartoon representation of the structure of HIV-1 RT bound to a template–primer is shown in the insert. Arrows indicate the location of NRTI and NNRTI binding sites. The structure was drawn using Pymol and coordinates from Protein Data Bank file 6UIS

incorporated into the proviral DNA during reverse transcription. However, they lack a 3' OH in the ribose ring, acting as chain terminators and blocking DNA polymerization. Tenofovir

disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) are nucleotide prodrugs, while other drugs shown in Fig. 2.2 (e.g. zidovudine, lamivudine, emtricitabine and abacavir) are nucleoside analogues. NNRTIs are small hydrophobic compounds that bind to an allosteric site located 8–10 Å away from the polymerase active site (for reviews, see [9, 10]). First-generation NNRTIs are represented by nevirapine and efavirenz, while etravirine, rilpivirine and doravirine are second-generation inhibitors (Fig. 2.2).

The proviral DNA (dsDNA formed during reverse transcription) needs to be integrated into the host cell genome. The viral integrase catalyses this process (for a review, see [11]). In HIV-1, the integrase is a 32-kDa protein with three domains: (1) an N-terminal HH-CC zinc-binding domain (a three-helical bundle stabilized by coordination of a Zn^{2+} cation), (2) a catalytic central core and (3) a C-terminal DNA-binding domain containing an SH3 fold. The integrase catalyses two reactions: *3′-processing*, in which nucleotides are removed from one or both 3′ ends of the viral DNA to expose an invariant CA dinucleotide at both 3′ ends of the viral DNA and a *strand transfer* reaction, in which the processed 3′ ends of the viral DNA are covalently ligated to host chromosomal DNA. Clinically approved drugs such as raltegravir, elvitegravir, dolutegravir and bictegravir (Fig. 2.3) act as integrase strand transfer inhibitors. HIV-1 integrase has a tendency to form oligomers, and compounds interfering with integrase oligomerization and binding to host factors are in preclinical development.

Finally, HIV protease inhibitors act late in the viral replicative cycle and block the proteolytic processing of viral polyproteins (i.e. Gag and Gag-Pol), necessary to generate an infectious virion. The HIV-1 protease is a homodimer of subunits of 99 residues each. Saquinavir, ritonavir, lopinavir, atazanavir, darunavir and tipranavir are representative HIV-1 protease inhibitors that bind to the active site of the enzyme preventing polyprotein cleavage (Fig. 2.4). All approved protease inhibitors except tipranavir are transition state mimetics, containing dipeptide isostere scaffolds (for reviews, see [9, 12]).

2.2.1 Combination Antiretroviral Therapies

Zidovudine (AZT, 3′-azido-3′-deoxythymidine) in 1987 was the first antiretroviral drug approved for the treatment of HIV infection (Fig. 2.5). This nucleoside reverse transcriptase inhibitor (NRTI) was followed by other molecules of the same class (e.g. zalcitabine, didanosine and stavudine). However, the use of one or two NRTIs usually led to rapid treatment failure. The approval of saquinavir (an HIV-1 protease inhibitor) in 1996 marked the introduction of combination therapies including three or more drugs directed against at least two different targets. With the advent of highly active antiretroviral therapy (HAART), the number of AIDS-related deaths in the United States and Europe decreased by half in the span of 3 years. In those days, one of the major limitations of HAART regimens was their large pill burden. In 2006, the approval of Atripla, a one-pill combination of tenofovir disoproxil fumarate (TDF), emtricitabine and efavirenz, represented an important advance towards the simplification of antiretroviral drug therapies. The drug combination was taken once daily and improved adherence compared to previous treatments. The efficacy of combination therapies (currently referred to as cART instead of HAART) increased from 43% in the mid-1990s to 78% in 2010, measured as their ability to maintain undetectable viral loads for a minimum of 48 weeks, when administered to patients with less than 100,000 copies/mL at the initiation of treatment [13]. These data, obtained from a large meta-analysis, reflect the impact of Atripla and other novel therapies developed after the introduction of HAART.

Current recommendations in treatment guidelines for the effective suppression of HIV-1 infection include two NRTIs plus a third drug: either a boosted protease inhibitor, an integrase strand transfer inhibitor or an NNRTI [14–16]. The US Food and Drug Administration (FDA) has approved about a dozen single-tablet drug combinations of two or more antiretroviral drugs intended as complete regimens to treat HIV

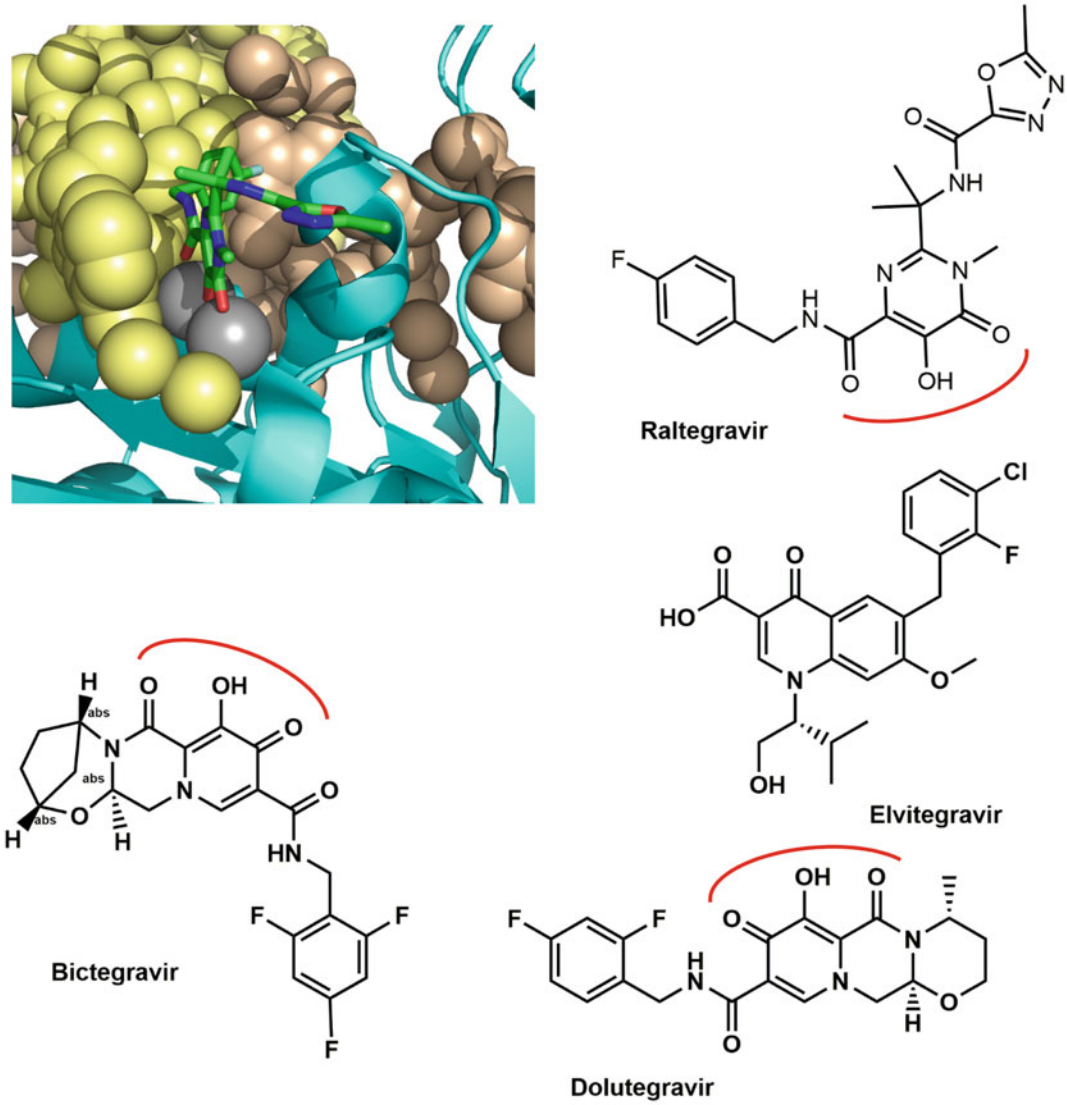

Fig. 2.3 Catalytic site of the integrase with bound raltegravir and chemical structures of approved integrase strand transfer inhibitors. The panel in the upper left side shows a view of raltegravir bound to the catalytic site of PFV integrase (Protein Data Bank file 3OYA). The cartoon (cyan) shows the integrase backbone, and the duplex DNA structure is represented with spheres (yellow and wheat). Mg^{2+} ions are shown as grey spheres. In the chemical structures, chelating oxygens are marked with a red curve

infection (Table 2.1). These medicines are usually administered in simplified dosage regimens—sometimes, only one pill per day—and show high potency with minimal toxicity.

Considering efficacy, tolerability and convenience, nowadays, the preferred three-drug formulations usually contain a two-NRTI combination of tenofovir and lamivudine or emtricitabine, plus an integrase inhibitor that does not require pharmacologic boosting (i.e. raltegravir, dolutegravir or bictegravir). Clinical trials demonstrated that these first-line regimens achieve viral suppression rates close to 90% after 48 weeks [17, 18]. In April 2019,

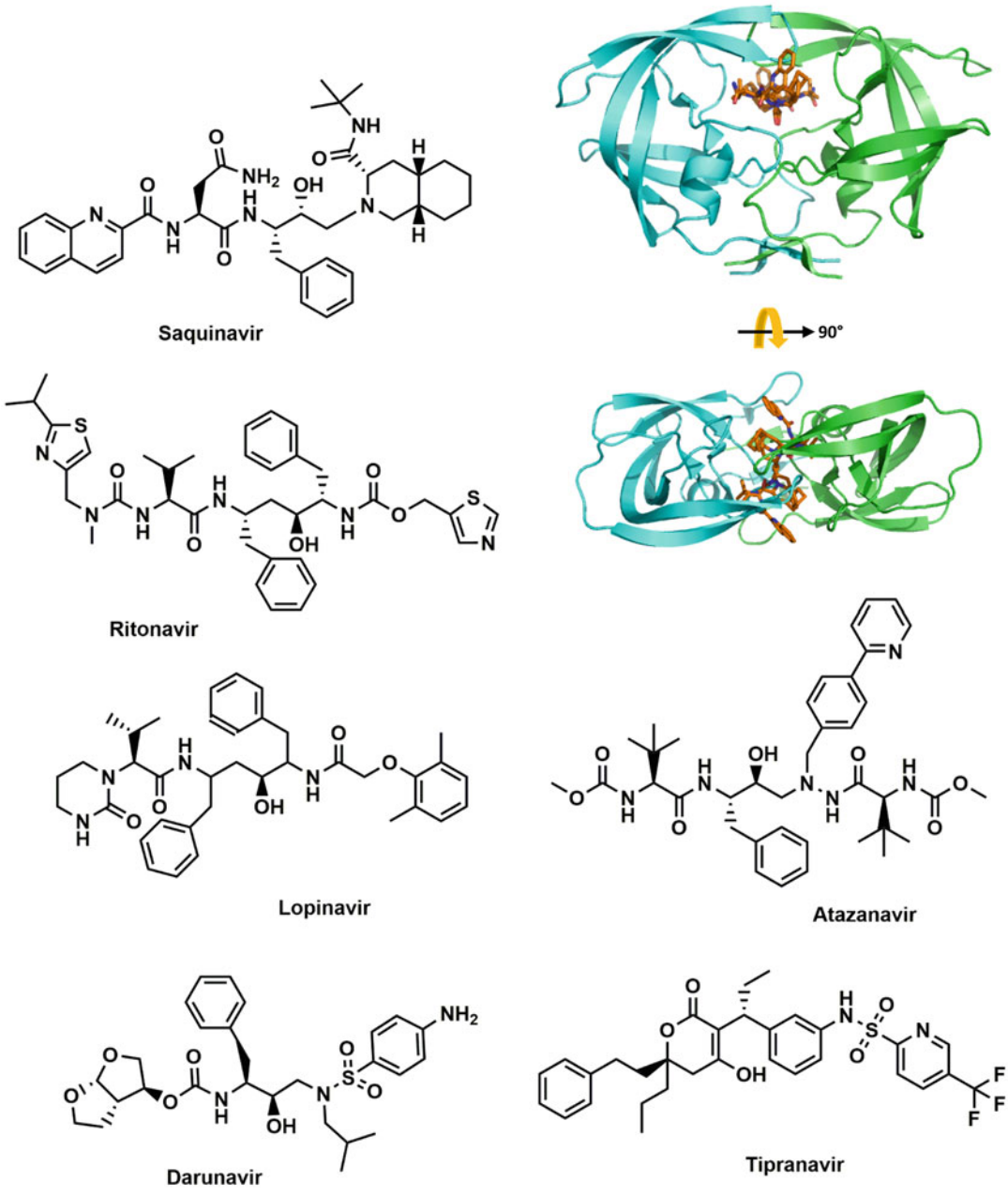

Fig. 2.4 HIV-1 protease and chemical structures of representative protease inhibitors in clinical use. Cartoon representations of the HIV-1 protease bound to saquinavir (red sticks) are shown in the upper right panel. The protease is composed of two polypeptide chains of 99 amino acids each. Homodimer subunits are represented in cyan and green. Side and top views of the enzyme were obtained using the Pymol software and coordinates from Protein Data Bank file 3OXC

Dovato (a combination of dolutegravir and lamivudine) was approved by the FDA and became the first once-daily, single-tablet, two-drug regimen for treatment naïve patients.

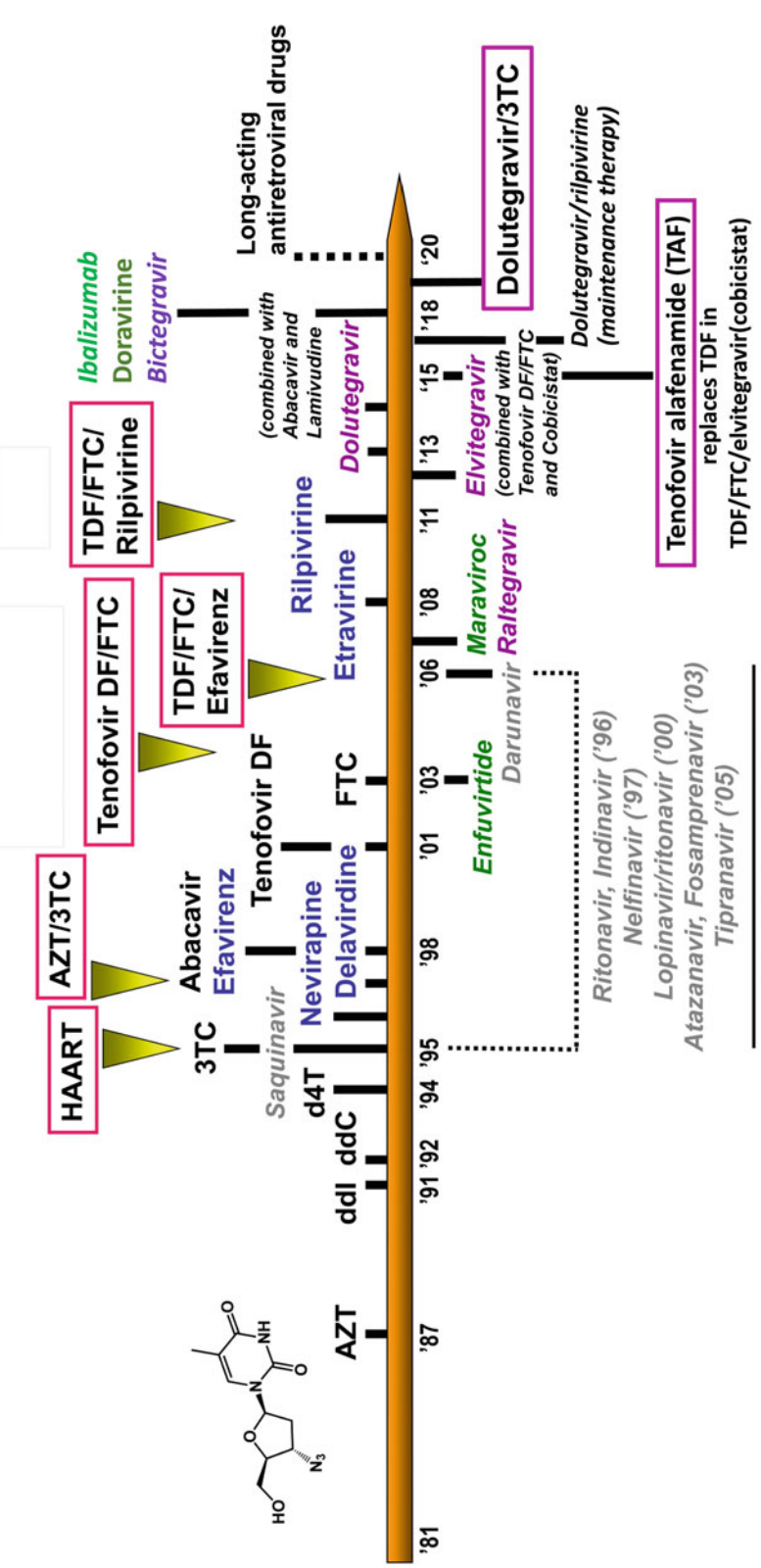

Fig. 2.5 Timeline for approval of antiretroviral drugs. Nucleoside reverse transcriptase inhibitors are shown in black, non-nucleoside reverse transcriptase inhibitors in blue, protease inhibitors in grey, integrase inhibitors in purple and entry inhibitors in green. Abbreviations: 3TC (lamivudine), β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; AZT (zidovudine), β-D-(+)-3′-azido-3′-deoxythymidine; d4T (stavudine), β-D-(+)-2′,3′-didehydro-2′,3′-dideoxythymidine; ddC (zalcitabine), β-D-(+)-2′,3′-dideoxycytidine; ddI (didanosine), β-D-(+)-2′,3′-dideoxyinosine; FTC (emtricitabine), β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; HAART, highly active antiretroviral therapy; DF, disoproxil fumarate

Table 2.1 Approved complete initial therapy regimens of single-tablet drug combinations of two or more antiretroviral drugs[a]

Commercial name	Combination[b]	FDA approval date
Atripla	TDF/emtricitabine/efavirenz	July 12, 2006
Complera	TDF/emtricitabine/rilpivirine	August 10, 2011
Stribild	TDF/emtricitabine/elvitegravir (cobicistat)	August 27, 2012
Triumeq	Abacavir/lamivudine/dolutegravir	August 22, 2014
Genvoya	TAF/emtricitabine/elvitegravir (cobicistat)	November 5, 2015
Odefsey	TAF/emtricitabine/rilpivirine	March 1, 2016
Juluca[c]	Dolutegravir/rilpivirine	November 21, 2017
Biktarvy	TAF/emtricitabine/bictegravir	February 7, 2018
Symfi Lo & Symfi	TDF/lamivudine/efavirenz	February 5/March 22, 2018
Symtuza	TAF/emtricitabine/darunavir (cobicistat)	July 17, 2018
Delstrigo	TDF/lamivudine/doravirine	August 30, 2018
Dovato	Dolutegravir/lamivudine	April 8, 2019

[a]https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/21/58/fda-approved-hiv-medicines. Accessed 26 June 2020
[b]*TAF* tenofovir alafenamide, *TDF* tenofovir disoproxil fumarate
[c]Considered as a maintenance HIV treatment for adults, to be used only with those patients who have been undetectable (viral load in blood less than 50 copies/mL) for at least 6 months

Its use was recommended for adults with no antiretroviral treatment history and without known amino acid substitutions associated with resistance to either dolutegravir or lamivudine (reviewed in [19]). Since lamivudine is used as an antiviral drug against hepatitis B virus (HBV), patients who have both HIV and HBV must be closely monitored. Major threats to the current antiretroviral therapies are toxicity and drug resistance, and monitoring their evolution is important to consider if therapy switch is necessary.

2.3 Antiretroviral Drug Toxicity

The most common side effects of antiretroviral drugs are mild gastrointestinal events (e.g. nausea, diarrhoea and vomiting). However, abacavir (an NRTI analogue) can be responsible for life-threatening symptoms that may include very low blood pressure and even death, due to hypersensitivity reactions associated with a specific allele at the human leukocyte antigen (HLA) B locus, namely, HLA-B*5701 [20], whose prevalence varies depending on the race and origin of the human populations. Skin rash and other cutaneous adverse reactions are also observed with other drugs, most notably NNRTIs (e.g. etravirine and efavirenz) [21].

Drug toxicity is very important considering the lifelong duration of antiretroviral therapies. Although current regimens are generally safe, they are not benign. Increased risk of cardiovascular, kidney and bone disease has been associated with more commonly used drugs. In this context, the use of TAF instead of TDF ensures higher active metabolite concentrations in peripheral blood mononuclear cells and lower plasma tenofovir exposure. In consequence, the newer TAF-based formulations show reduced renal and bone toxicity [22]. Tenofovir-containing regimens comprise the preferred first-line antiretroviral therapy in many countries, and novel prodrugs (e.g. octadecyloxyethyl benzyl tenofovir) designed to facilitate a slow release of the active metabolite can be a significant advance towards safer therapies [23]. Continued exposure to efavirenz is associated with neuropsychiatric side effects, including anxiety, vivid dreams, psychosis and increased suicidality [24].

Recently developed integrase inhibitors may have long-term health consequences, including weight gain and obesity. This effect has been specifically demonstrated for dolutegravir. It has been shown in a clinical trial that triple-therapy combinations of emtricitabine and dolutegravir together with tenofovir prodrugs TDF or TAF showed similar efficacy than a standard-of-care

regimen (TDF/emtricitabine/efavirenz). However, patients receiving dolutegravir-containing regimens showed weight gains, especially when receiving TAF as part of the antiretroviral treatment [25]. Current evidence suggests that dolutegravir and bictegravir are associated with more weight gain than elvitegravir (boosted with cobicistat), although the physiological mechanisms involved are not known [26].

2.4 Acquired and Transmitted Drug Resistance

Highly effective antiretroviral drug combinations are now available in many parts of the world. However, the acquisition and transmission of HIV drug resistance still poses a major risk to the success of antiretroviral therapies. HIV has a high mutation rate (around 10^{-4} to 10^{-5} mutations per nucleotide and cycle of replication) and a high rate of recombination [27]. In vivo, the HIV replication cycle can be very short (1.2 days), and untreated patients can produce 1.03 x 10^{10} virions per day [28], revealing an enormous potential for the generation of drug-resistant variants. The selection of those viruses depends on several factors, most notably the genetic barrier to resistance and the viral fitness of the mutated strains emerging under drug pressure. The genetic barrier refers to the threshold above which clinically meaningful resistance develops [10].

Several RT inhibitors (e.g. lamivudine, emtricitabine, nevirapine and efavirenz) and integrase inhibitors such as raltegravir or elvitegravir have relatively low resistance barriers. One amino acid substitution is usually sufficient to confer high-level resistance to those drugs (Table 2.2). In contrast, two or more amino acid changes are usually required to confer high-level resistance to other drugs, such as zidovudine, abacavir and many protease inhibitors (e.g. darunavir, atazanavir, lopinavir). At present, emergence of resistance has been largely reduced in the clinical setting due to the increased potency of current regimens. However, there are still patients infected with drug-resistant strains that were selected after successive treatments with different antiretroviral drugs or individuals that were infected with drug-resistant HIV-1 (i.e. transmitted drug resistance). In addition, natural resistance to various antiretroviral drugs has been observed in several HIV-1 clades, as well as in HIV-2. Despite being considered less virulent than HIV-1, HIV-2 shows natural resistance to NNRTIs, the fusion inhibitor enfuvirtide and several protease inhibitors approved for the treatment of HIV-1 infection [31].

A remarkable example of multidrug resistance has been recently reported by Puertas et al. [32]. An individual diagnosed with HIV-1 on 1989 and starting antiretroviral treatment in 1995, 22 years later, was found to be infected with a pan-resistant HIV-1 group M/subtype B strain that showed broad genotypic and phenotypic cross-resistance to all approved antiretroviral drugs. Although the prevalence of pan-resistant HIV-1 strains is unknown and difficult to establish, this finding is a warning signal of alarming trends in emerging HIV drug resistance.

Prevention, monitoring and timely response to population levels of HIV drug resistance are very important to control and eventually eradicate the infection. Evidence collected from a large surveillance study carried out with patients recruited in the United States, Europe, Israel, Australia, South America, Mexico, Africa and Asia revealed that over 10.1% of HIV-infected patients had baseline drug resistance to at least one of the major families of antiretroviral drugs (i.e. NRTIs, NNRTIs and protease inhibitors) [33]. These numbers varied from country to country, with highest prevalences in Australia (17.5%), France (16.7%), the United States (12.6%) and Spain (12.6%). More recently, the World Health Organization (WHO) has noted that acquired and transmitted drug-resistant HIV-1 in ART-naïve individuals is increasing in resource-rich and resource-limited regions of the world, representing a major obstacle in the fight against the HIV-1 epidemic [34]. In the WHO report, prevalence of nevirapine and efavirenz resistance was found to be very high (>15%) in several countries of Central America and Southern Africa. Thus, in a survey carried out in Honduras

Table 2.2 Amino acid substitutions associated with HIV-1 resistance to currently prescribed antiretroviral drugs

Drugs	Amino acid substitutions associated with drug resistance
NRTIs	
Zidovudine (Retrovir)	M41L, **D67N**, **K70R**, V118I, L210W, **T215F/Y**, K219E/Q
Lamivudine (Epivir)	(E44D, V118I), (K65R, Q151M), **M184I/V** [a]
Abacavir (Ziagen)	K65R, L74V, Y115F, M184V, (M41L, D67N, K70R, L210W, T215F/Y, K219E/Q)
Emtricitabine (Emtriva)	(K65R, Q151M), **M184I/V** [a]
Tenofovir (Viread)	**K65R**, K70E
Combinations of mutations that confer resistance to various nucleoside analogues	
	(i) M41L, D67N, K70R, L210W, T215F/Y, K219E/Q; [b] (ii) A62V, V75I, F77L, F116Y, Q151M; (iii) insertions between codons 69 and 70 (i.e. T69SSS or T69SSG or T69SSA), M41L, A62V, K70R, L210W, T215F/Y
NNRTIs	
Nevirapine (Viramune)	L100I, K101P, K103N/S, V106A/M, V108I, **Y181C/I**, **Y188C/L/H**, **G190A**/C/E/Q/S/T
Efavirenz (Sustiva)	L100I, **K101P**, **K103H/N/S**, **V106M**, **V108I**, **Y188L**, **G190A/S**/T, **P225H**, M230L
Etravirine (Intelence)	V90I, A98G, L100I, K101E/H/P/Q, V106I, **E138A**/G/K/**Q/R**/S, **V179**D/**F/I/L**, Y181C/I/V, G190A/S, F227C, M230L, T386A, E399D
Rilpivirine (Edurant)	V90I, K101E/P, **E138A**/G/**K**/Q/**R**, V179F/**I**/L, Y181C/I/V, **M184I/V**, Y188L, V189I, H221Y, F227C, M230I/L
Doravirine (Pifeltro)	**V106A**/**I/M**/T, **Y188C/L**/H, G190E/S, F227C/L/R, M230L, L234I
Combinations of mutations that confer resistance to first-generation NNRTIs (nevirapine, delavirdine, efavirenz)	
	(i) K103N alone, (ii) V106M alone, (iii) Y188L alone and (iv) two or more amino acid changes of the group: L100I, V106A, Y181C/I, G190A/S, M230L and Y318F
Protease inhibitors	
Saquinavir (Invirase, Fortovase)	L10I/R/V, **G48V**, I54L/V, A71T/V, G73S, V77I, V82A, I84V, **L90M** and A431V [in p7(NC)/p1)] [c]
Ritonavir (Norvir)	L10I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54L/V, A71T/V, V77I, **V82A/F/S/T, I84V**, L90M and A431V [in p7(NC)/p1)]
Nelfinavir (Viracept)	L10F/I, **D30N**, M36I, M46I/L, A71T/V, V77I, V82A/F/S/T, I84V, N88D/S, **L90M** and Gag cleavage sites L449F and P453L [in p1/p6]
Amprenavir (Fosamprenavir, Lexiva)	L10F/I/R/V, V32I, M46I/L, I47V, **I50V**, I54V/M, **I84V**, L90M and Gag cleavage sites L449F and P453L [in p1/p6]
Lopinavir/r (Kaletra)	L10F/I/R/V, G16E, K20I/M/R, L24I, **V32I**, L33F, E34Q, K43T, M36I/L, **M46I/L**, **I47A/**V, G48M/V, I50V, **I54L/V**/A/M/S/T, Q58E, I62V, L63T, A71T, G73T, T74S, **L76V**, **V82A**/F/S/T, I84V, L89I/M, L90M and A431V [in p7(NC)/p1)]
Atazanavir (Reyataz)	L10F/I/V, K20I/M/R, L24I, L33F/I/V, M36I/L/V, M46I/L, G48V, **I50L**, I54L/V, L63P, A71I/T/V, G73A/C/S/T, V82A/F/S/T, **I84V, N88S**, L90M
Tipranavir (Aptivus)	L10I/S/V, I13V, K20M/R, **L33F/I/V**, E35G, M36I/L/V, K43T, M46L, I47V, I54A/M/V, Q58E, H69K, T74P, **V82L/T**, N83D, **I84V**, L89I/M/V, L90M
Darunavir (Prezista)	V11I, V32I, L33F, I47V, **I50V**, I54L/M, T74P, L76V, V82F, I84V, L89V and Gag cleavage sites A431V [in p7(NC)/p1)] and S451T and R452S [in p1/p6]
Combinations that confer resistance to multiple protease inhibitors	
	L10F/I/R/V, **M46I/L**, I54L/M/V, **V82A/F/T/S**, **I84V**, **L90M** [d]
Fusion inhibitors	
Enfuvirtide (Fuzeon)	G36D/E/S, I37T/N/V, V38A/E/M, Q40H, N42T, N43D/K/S (all in gp41)
Integrase inhibitors	
Raltegravir (Isentress)	G140S, **Y143C/R, Q148H/K/R, N155H**

(continued)

Table 2.2 (continued)

Drugs	Amino acid substitutions associated with drug resistance
Elvitegravir (component of Stribild and Genvoya)	T66A/I/K, L74M, E92Q/V, **Q148H/K/R**, V151L, **N155H**
Dolutegravir (Tivicay)	H51Y, E92Q, T97A, **G118R**, F121Y, E138A/K, G140A/C/S, Y143C, **Q148H/K/R**, N155H, S230R, **R263K**
Bictegravir (component of Biktarvy)	M50I, T97A, G118R, **Q148H**/R, **R263K**
CCR5 antagonists (entry inhibitors)	
Maraviroc (Selzentry, Celsentri)	Resistance usually develops through the selection of viruses that use the CXCR4 (X4) coreceptor. In addition, maraviroc resistance mutations have been selected in vitro in the sequences encoding the V2, V3 and V4 loops of gp120
Post-attachment inhibitors blocking CD4 receptors	
Ibalizumab-uiyk (Trogarzo)	Resistance acquired through the loss of glycosylation sites in the V5 region of gp120 (i.e. amino acid substitutions **N460Q** and **N464Q** alone or in combination). Potential glycosylation sites at position 386 and the side-chain length of residue 375 also correlated with resistance

For additional information, see ([29]; [30]) and the websites of the International Antiviral Society–USA (http://www.iasusa.org) and the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu). Major resistance mutations are shown in bold. Most protease inhibitors are usually prescribed in combination with a low dose of ritonavir that has a boosting effect on the protease inhibitor concentration in plasma

[a]Combinations between parentheses may decrease HIV drug susceptibility in the absence of M184I/V

[b]Three or more secondary resistance mutations of this group may confer resistance to all nucleoside analogues except lamivudine. In the case of AZT, resistance mediated by M41L, K70R, T215F/Y, etc., can be suppressed by antagonist mutations such as K65R, L74I/V, V75I, W88G, E89K, L92I, S117T, S156A, Q161L, M164I, Y181C or M184V. Suppression of AZT resistance by the mutation M184V depends on the sequence context (e.g. dual resistance to AZT and 3TC is observed in the presence of the substitution G333D/E and perhaps in the presence of T386I)

[c]In addition to amino acid substitutions in the viral protease, changes in the Gag polyprotein cleavage sites can be selected under drug pressure. HIV-1 protease-mediated processing of Gag occurs at cleavage sites indicated with asterisks: (MA * CA * p2 (SP1) * NC * p1 (SP2) * p6)

[d]Multiple protease inhibitor resistance can be achieved through the accumulation of 4 or 5 mutations, of those indicated in the list

in 2016–2017, researchers found that HIV drug resistance prevalence in pretreated individuals was 26.9% to any antiretroviral drug and 25.9% to NNRTIs [35]. In low-income countries, earlier combination antiretroviral therapies have been usually maintained and hardly implemented, and this has resulted in an increase in transmitted drug resistance, estimated in 12.3% in a span of 4 years [36].

Highest levels of transmitted drug resistance have been reported for antiretroviral drugs with a low genetic barrier to resistance that have been prescribed for many years. Examples are M184V/I associated with NRTI resistance and K103N/S, Y181C/I and G190A/S associated with NNRTI resistance (Table 2.2). The prevalence of those amino acid substitutions in naïve and treated individuals has been found to be high in many countries, including the United States [37], China [38] and Europe [39]. However, their prevalence appears to be decreasing where efavirenz is being substituted by novel NNRTIs (e.g. etravirine or rilpivirine) or integrase inhibitors, such as dolutegravir, characterized by their higher genetic barrier to resistance [37]. New drugs with different mechanisms of action will be needed to overcome challenges associated with the emergence of drug resistance and its prevalence in the infected population [40].

2.5 Novel Antiretroviral Drugs

Improved efficacy and simplification of drug regimens have guided the development of new anti-HIV-1 drugs. Progress has culminated with the recent approval of dolutegravir and bictegravir (integrase inhibitors), doravirine (NNRTI) or the monoclonal antibody ibalizumab-uiyk, directed towards the CD4 receptor of HIV-1. In addition, several molecules in the pipeline are being developed to expand the currently available classes of antiretroviral drugs, and compounds with new mechanisms of action (e.g. capsid assembly inhibitors, maturation inhibitors) have shown promised in preclinical and early clinical trials.

2.5.1 RT Inhibitors

Among NRTIs, islatravir (4′-ethynyl-2-fluoro-2′-deoxyadenosine, EFdA, or MK-8591) is an investigational drug (Fig. 2.6), currently in phase 1 clinical trials [41] that is being developed for extended administration in a subdermal drug-eluting implant [42]. The mechanism of action of islatravir is rather unique among NRTIs. After conversion into a triphosphorylated derivative, the drug acts as a translocation inhibitor due to its 4′-ethynyl group that, in combination with the 3′-hydroxyl group, results in chain termination [43, 44]. Islatravir-triphosphate has a long intracellular half-life in human and rhesus blood cells. In clinical trials of islatravir in combination with doravirine and lamivudine, daily oral administration resulted in high levels of virologic suppression in HIV-infected individuals [45]. The efficacy of islatravir in pre-exposure prophylaxis has been shown in the rhesus macaque simian/human immunodeficiency virus (SHIV) rectal challenge model and more recently in healthy HIV-1-uninfected humans that showed suppressive plasma levels of the drug for more than 1 year (reviewed in [46]).

Islatravir retains activity against a wide range of NRTI-resistant mutants; both clones generated by site-directed mutagenesis as well as clinical isolates, including the very highly resistant to AZT M41L/T69-insertion/T215Y mutants. However, M184V contributes to islatravir resistance particularly in combination with P119S and T165A [47, 48]. Although M184I or M184V might be involved in the loss of potency, other common drug resistance mutations in the RT-coding region (e.g. K65R, L74V and Q151M) confer hypersusceptibility to the drug. Despite being selected in treatments containing lamivudine or emtricitabine, M184I/V have a negative impact on the HIV-1 replication capacity. This observation and data from small clinical studies showing that selective withdrawal of lamivudine leads to modest increases in plasma viremia have been brought up to justify the inclusion of lamivudine or emtricitabine in second-line regimens even in the presence of M184V. However, in a recent study, Gregson et al. [49] showed that viral load at the time of virologic failure was in fact somewhat higher in the presence of M184I/V than in its absence. The authors also found that presence of high-level tenofovir resistance (conferred by K65R) and multiple non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations were more likely to occur when M184I/V were present. Interestingly, L74I was found to be a compensatory substitution that emerges in approximately 10% of viruses carrying M184I/V while restoring RT activity and HIV-1 replication capacity.

Rovafovir etalafenamide (known as GS-9131) is an oral prodrug of GS-9148 ([5-(6-amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl]phosphonic acid) that after phosphorylation acts as a chain terminator of DNA polymerization (Fig. 2.6). GS-9148 is a nucleoside phosphonate HIV-1 RT inhibitor with a unique resistance profile towards N(t)RTI resistance mutations. It shows efficacy against HIV-1 and HIV-2 strains and has low mitochondrial toxicity. GS-9131 has been shown to select for the very rare Q151L mutation in HIV-1 RT as a pathway to resistance, a characteristic that could limit its development into a novel antiretroviral drug [50]. A phase 2 study to evaluate the efficacy of GS-9131 in HIV 1-infected patients failing NRTI-containing therapies has been recently

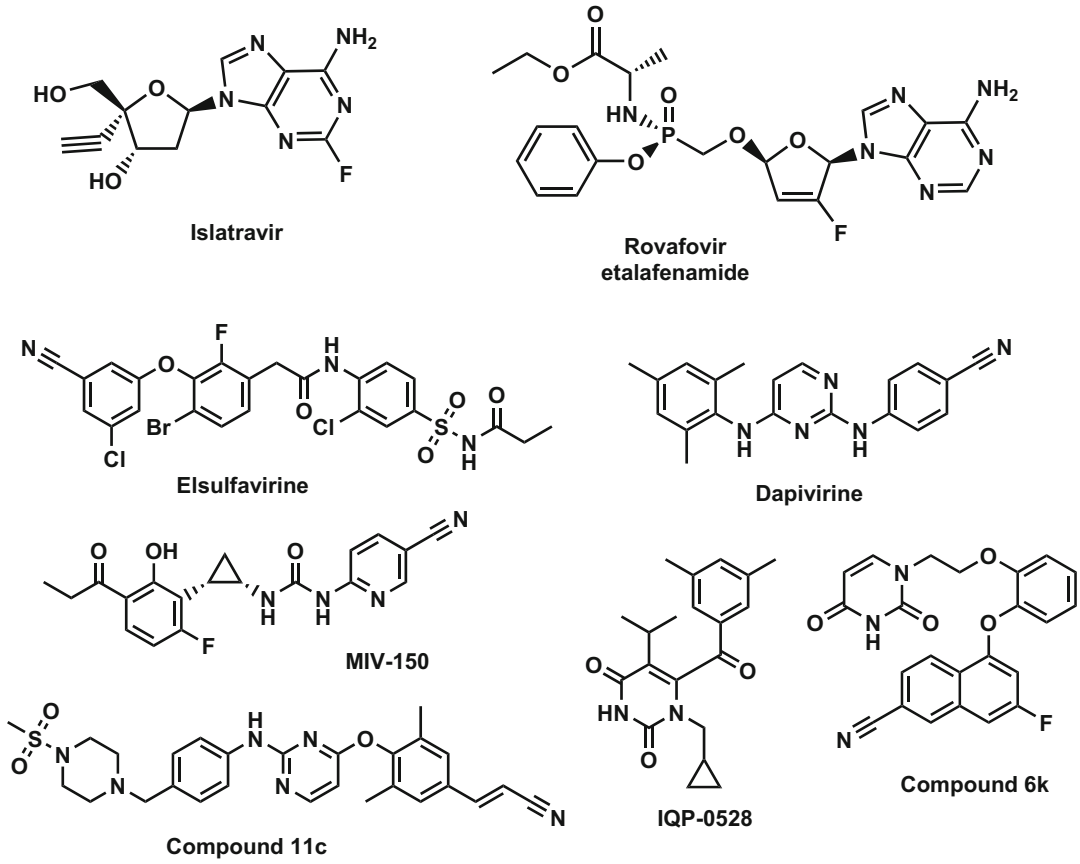

Fig. 2.6 Chemical structures of NRTIs and NNRTIs in preclinical development and clinical trials

terminated without meeting the targeted antiviral response (NCT03472326).

NNRTIs are essential components of HAART regimens. The latest NNRTIs approved for clinical use were two diarylpyrimidines (etravirine and rilpivirine) and doravirine. Doravirine was approved in 2018 and together with TDF and lamivudine is currently recommended as an initial regimen for treatment-naïve patients. In large phase 3 clinical trials, doravirine has demonstrated non-inferiority and improved tolerability when compared with darunavir/ritonavir and efavirenz-based regimens [51]. Unlike first-generation NNRTIs, doravirine binding to HIV-1 RT is less dependent on Lys103 and Tyr181, while hydrophobic interactions between the side chain of Val106 and the central phenyl ring of the drug are most relevant for RT–doravirine interaction. Doravirine resistance is usually associated with the combination V106A/G190A/F227L, and common resistance mutations such as K103N, Y181C or G190A were never detected in in vitro selection studies carried out with the drug (for a recent review, see [52]). The development of doravirine as a novel antiretroviral drug was based on its distinct resistance profile, but additional properties including excellent tolerability and reduced toxicity compared with other NNRTIs justified its approval as a new antiretroviral drug. Among its advantages, doravirine showed reduced central nervous system toxicity in comparison with efavirenz and less cardiotoxicity due to off-target effects on cardiac ion channels than rilpivirine. Doravirine also shows an optimal metabolic profile, tolerating food intake and minimizing the risk for drug−drug interactions.

Elsulfavirine (Fig. 2.6), the prodrug of VM-1500A, has been approved in Russia but not in Europe and the United States. It is currently under investigation in clinical trials to evaluate safety and pharmacokinetics (NCT03706898), and its efficiency as fixed-dose once-daily combination with TDF and emtricitabine has been determined in comparison with efavirenz-based therapies (NCT02489461 and NCT03706924 trials). Elsulfavirine has a long half-life, and preliminary data suggests fewer side effects than efavirenz [53].

Other investigational NNRTIs in the pipeline are IQP-0528 (studied as a drug preventing sexual transmission); MIV-150 that together with zinc acetate are co-formulated in a carrageenan gel called PC-1005 for prevention of sexually transmitted HIV infection; compound 11c, a diarylpyrimidine with strong activity against mutant E138K HIV-1 RT; and compounds 6k and 6l, naphthyl catechol phenyl ethers [54] with nanomolar activity in humanized mice models, showing inhibitory activity against NNRTI-resistant mutants Y181C and K103N/Y181C (Fig. 2.6) (reviewed in [55]).

2.5.2 Integrase Inhibitors

Second-generation integrase inhibitors such as dolutegravir and bictegravir offer important advantages in comparison with elvitegravir and raltegravir: good tolerability, once-daily dosing with no need for a pharmacological enhancer and relatively little cross-resistance with first-generation inhibitors. Thus, the most common resistance mutations in persons failing dolutegravir-containing therapies were G118R, Q148H/R, N155H and R263K, with G118R and R263K predominating in individuals naïve for integrase inhibitors. R263K reduced dolutegravir susceptibility by twofold, but phenotypic assays showed that the largest reductions in susceptibility occurred in viruses containing Q148H/K/R mutations in combination with G140S and/or E138K substitutions [56]. Bictegravir is even more effective than dolutegravir at inhibiting the known integrase inhibitor-resistant mutants. However, the triple mutant T97A/G140S/Q148H is known to confer a substantial reduction in bictegravir susceptibility [57, 58]. In addition, integrase amino acid substitutions R263K and M50I emerge successively, after passages of the virus in the presence of the inhibitor [58]. The higher barrier to resistance of second-generation integrase inhibitors has turned them into important players in antiretroviral therapy, both as part of successful first-line treatments and also as components of simplified therapeutic regimens and long-lasting antiretroviral drugs.

Cabotegravir is currently in advanced clinical trials. This integrase inhibitor is structurally related to dolutegravir and bictegravir (Fig. 2.7). Cabotegravir, dolutegravir and bictegravir showed similar efficacy against a panel of integrase inhibitor-resistant strains, isolated from patients exposed to raltegravir, elvitegravir and/or dolutegravir [59]. In this study, researchers showed that cabotegravir has reduced susceptibility against HIV variants containing amino acid substitutions Q148H/K/R plus one to two additional resistance-associated amino acid changes in the integrase. In a comprehensive study involving a large panel of mutant HIV strains, Smith et al. [60] showed that bictegravir was more efficient than cabotegravir and dolutegravir in inhibiting HIV replication of variants containing substitutions associated with resistance to integrase inhibitors. High-resolution cryo-electron microscopy studies have shown that second-generation integrase inhibitors cover the enzyme's active site more extensively than raltegravir or elvitegravir. The distal ring in the tricyclic moiety of dolutegravir and bictegravir makes multiple contacts with the β4-α2 connector, a structural element located at the edge of the integrase active site [61].

Cabotegravir has an exceptionally long biological half-life of 21–50 days after intramuscular injection and 40 hours after oral administration (about three times longer than dolutegravir) and can be formulated as an injectable nanosuspension for long-acting therapy [62]. A combination of long-acting cabotegravir and rilpivirine administered intramuscularly each month has been tested in clinical trials. Results

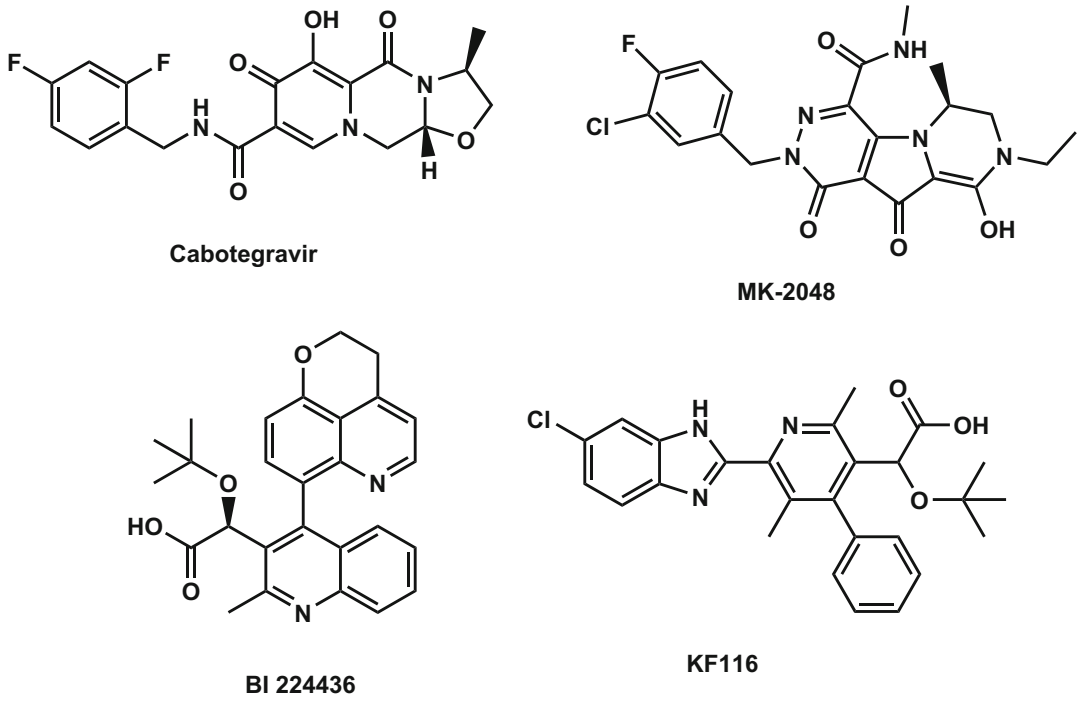

Fig. 2.7 Chemical structures of integrase inhibitors in preclinical development and clinical trials

of one of these trials showed that injection-related adverse events were common but unfrequently led to medication withdrawal [63]. Pharmacokinetics studies carried out in mice and rhesus macaques, with fatty acid ester nanocrystal prodrugs of cabotegravir, have recently shown that plasma drug levels can be maintained above the protein-adjusted 90% inhibitory concentration for up to a year [64]. The combination of cabotegravir and rilpivirine (marketed as Cabenuva™), developed by ViiV Healthcare and Janssen Pharmaceutica (Janssen) as a complete regimen for HIV infection, has been approved in Canada in March 2020, as replacement for current antiretroviral therapy in patients who are virologically stable and suppressed [65].

Among integrase inhibitors in preclinical development and clinical trials, MK-2048 (Fig. 2.7) is currently in early development for pre-exposure prophylaxis in combination with vicriviroc (a CCR5 coreceptor antagonist), and their safety in intravaginal rings has been demonstrated in clinical trials [66, 67]. Although G118R and E138K confer resistance to MK-2048, the compound retains significant activity against raltegravir- and elvitegravir-resistant HIV-1 strains (reviewed in [10]).

In addition to active-site targeted integrase strand transfer inhibitors like the ones described above, recent research has focused on allosteric integrase inhibitors (ALLINIs) that bind to a site located at the HIV-1 integrase dimer interface and recognized by a host cell transcription factor known as the lens epithelium-derived growth factor (LEDGF/p75). ALLINIs inhibit HIV-1 replication by inducing integrase hypermultimerization, which precludes integrase binding to genomic RNA and perturbs the morphogenesis of new viral particles (reviewed in [11]). Proof of this mechanism of action is also supported by experiments showing the selection of amino acid substitutions A128T, H171Q, K173Q and N254K in the HIV-1 integrase, after passage of the virus in the presence of ALLINIs. These amino acid changes decrease the multimerization potential of the integrase and facilitate propagation of ALLINI-resistant HIV-1 in cell culture [68]. Pyridine, thiophene, quinoline, isoquinoline and

thienopyridine are representative ALLINI chemotypes, with BI 224436 and KF116 among the best characterized compounds of this class (Fig. 2.7). BI 224436 was the first drug candidate of this class, but safety and pharmacokinetics studies were abandoned before entering clinical trials.

2.5.3 Protease Inhibitors

Despite achieving viral suppression, available protease inhibitors face major challenges derived from the long-term HIV therapies needed to combat the disease. Drug-related side effects and toxicity, together with the emergence of resistance and cross-resistance, are major limitations to the use of protease inhibitors in current therapies. Darunavir and atazanavir (both pharmacologically boosted with ritonavir) seem to be associated with increased cardiovascular risk. New classes of potent HIV-1 protease inhibitors with innovative ligands and functionalities have been developed. Major efforts have been focused on the design and synthesis of novel P2 ligands promoting enhanced backbone binding interactions to combat drug resistance. Further efforts have concentrated on developing nonpeptide protease inhibitors containing different structural scaffolds other than hydroxyethylsulfonamide isosteres, as found in darunavir (for a comprehensive review, see [69]).

Novel inhibitors (e.g. GRL-142, GRL-121, GRL-001 and GRL-003) have been designed to occupy a larger surface in the binding pockets of the HIV protease, making more extensive van der Waals contacts compared to darunavir [70, 71]. In addition, phosphate and amino acid prodrugs of the HIV-1 protease inhibitor atazanavir have been prepared [72], in an attempt to increase oral bioavailability and following the improved efficiencies shown by fosamprenavir versus amprenavir. Other advances in drug delivery led to nanoformulations based on ester prodrugs of darunavir. These long-acting drugs showed sustained antiretroviral response in human monocyte-derived macrophages [73].

2.5.4 Assembly and Maturation Inhibitors

The HIV-1 capsid protein (CA) forms the conical shell that protects the viral RNA. Despite its attractiveness, less than two dozen CA-targeting compounds have been described, including small molecules, peptides and a specific antibody [74]. Of these, PF-3450074 (PF74) has been extensively studied. PF74 is a peptidomimetic compound built around a phenylalanine core and capped with an aniline moiety at the carboxylate end and an indole-3-acetic acid at the amino end (Fig. 2.8). The compound affects the stability of the hexameric lattice in the mature HIV-1 capsid and binds to a pocket lined by helices H3 and H4 in the N-terminal domain of CA and helices H8 and H9 of the CA C-terminal domain in the adjacent monomer [75]. In addition, PF74 interferes with CA binding to host factors CPSF6 or NUP153 [76]. The compound demonstrated broad-spectrum inhibition of HIV isolates, with submicromolar potencies (EC_{50} values of 8–640 nM).

Recently reported HIV-1 CA inhibitors GS-CA1 and its analogue lenacapavir (formerly known as GS-6207 or GS-CA2) contain a PF74 scaffold (Fig. 2.8) and inhibit HIV-1 replication in T cells and peripheral blood mononuclear cells with picomolar activity. The location of resistance mutations and the results of structural studies further suggest that GS-CA compounds and PF74 share the same binding pocket, which is located between capsid monomers (reviewed in [77]). In vitro dose escalation studies identified CA variants with amino acid substitutions L56I, M66I, Q67H, K70N, N74D, N74S and T107N (alone and in different combinations), with reduced susceptibility to lenacapavir and reduced viral fitness [78]. The most frequent mutations in these assays were Q67H and N74D. In general, lenacapavir was found to be safe and well tolerated, after subcutaneous injection, and pharmacokinetics and phase 1 clinical studies support its development as a long-acting antiretroviral agent [78]. The drug is currently being tested in clinical trials to evaluate its safety and efficacy in

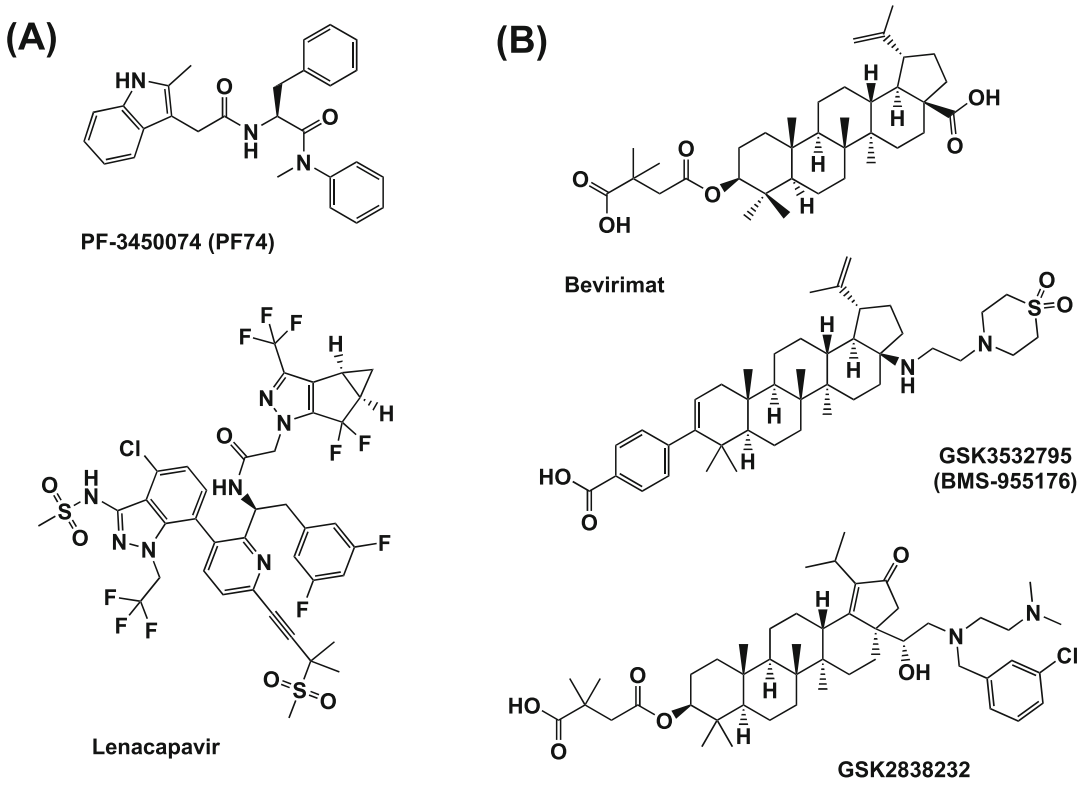

Fig. 2.8 Chemical structures of CA-binding inhibitors (**a**) and maturation inhibitors (**b**)

combination with an optimized background regimen in heavily treatment-experienced participants living with HIV-1 infection with multidrug resistance (NCT04150068).

As discussed above, the HIV protease is needed to convert immature capsids into the conical-shaped structures found in mature virions. This maturation process involves changes in the CA lattice that can be altered by impairing specific cleavages of the Gag polyproprotein precursor. Maturation inhibitors such as bevirimat (also known as PA-457) bind within six-helix bundles, formed by a segment that spans the junction between the CA and spacer peptide (SP1) within Gag [79]. Then, CA-SP1 is not properly processed, and an abnormal eccentric capsid is formed. This immature particle is not infectious. Bevirimat is a betulinic acid-like compound (Fig. 2.8), first isolated from *Syzygium claviflorum*, a Chinese herb. Although the drug entered clinical trials and showed efficacy in some patients, Gag polymorphisms and the emergence of drug resistance (mostly, amino acid substitutions around the CA-SP1 cleavage site) were determinant for interrupting further development of the drug [80].

Second-generation maturation inhibitors (Fig. 2.8) were initially represented by GSK3532795 (formerly known as BMS-955176). This compound was structurally related to bevirimat. Despite entering clinical trials, its development was also halted due to the rapid emergence of resistance in combination therapies with tenofovir and emtricitabine, in addition to gastrointestinal adverse effects [81]. Currently, the most advanced inhibitor in this class is GSK2838232. This compound has potent antiviral activity in vitro, with a mean 50% maximal inhibitory concentration (IC_{50}) of 1.6 nM, and retains activity across a broad spectrum of HIV isolates. GSK2838232 is currently in clinical trials, and available data indicate that

combined with cobicistat, the drug was well tolerated and exhibited efficacy as a short-term monotherapy in individuals infected with HIV-1, decreasing viral load by 1.7 logs depending on the administered drug dose [82].

2.5.5 Entry Inhibitors and Neutralizing Antibodies

Fostemsavir (BMS-663068, an oral prodrug of temsavir) is an attachment inhibitor approved by the FDA in July 2020. It prevents HIV entry into the CD4 T cell by binding to the viral envelope gp120 [83] (Fig. 2.9). The drug binds to gp120 at a location close to the CD4 binding site and is active against CCR5-tropic and CXCR4-tropic HIV. Previous studies have identified amino acid substitutions at four positions in gp120 (S375H/I/N/M/T, M426L/P, M434I/K and M475I) that affect the susceptibility of the virus to temsavir. Clinical trials have shown its efficacy in patients with multidrug-resistant HIV-1 infection and limited therapeutic options. Patients receiving fostemsavir experienced a significant reduction of the viral RNA levels during the first 8 days (compared to those receiving placebo), and the efficacy of the drug was maintained during 48 weeks [84].

The CD4-gp120 complex has been targeted by different compounds. Among them, NBD-556 and BMS-378806 are two representative small molecular chemical entities (Fig. 2.9). These compounds bind to the Phe43 cavity of gp120 (reviewed in [85]). BMS-378806 blocks CD4-induced conformational changes in Env, necessary for viral entry, and stabilizes the envelope glycoprotein in a pretriggered conformation (known as state 1). BMS-378806 stabilizes Env in this natural state for long periods of time, preventing shape changes occurring in the envelope HIV glycoprotein during viral entry [86]. Based on this evidence, BMS-378806 analogues are being developed as long-acting antiretroviral drugs that stabilize a state-1-like conformation of membrane Env for at least 21 days after a single application.

In addition to small pharmaceuticals binding to CD4, a humanized monoclonal antibody known as ibalizumab-uiyk (formerly TNX-355 and commercialized as Trogarzo) was approved in 2018 as a new antiretroviral agent effective against multidrug-resistant HIV-1. Ibalizumab binds to the N-terminal region of domain 2 in the CD4 receptor and has broad specificity neutralizing many HIV-1 strains [87]. However, resistance may appear due to the loss of the V5 glycan loop in the envelope glycoprotein.

Broadly neutralizing antibodies (bNAbs) have attracted a lot of interest as alternative therapies for preventing, treating and eradicating HIV infection. Most of the first-generation bNAbs were obtained by phage display and human hybridoma electrofusion (e.g. b12, 2F5, 4E10 and others). Although they were able to neutralize many HIV strains, their potency was not high, even when provided in combination. New technologies involving single B cell culture and sorting allowed the identification of more potent bNAbs useful for passive administration and effective in immunotherapy experiments carried out in macaques (reviewed in [88]). Several of these bNAbs have been evaluated in clinical trials. Examples include CD4 binding antibodies (3BNC117, VRC01, VRC01-LS and VRC07-523LS) and V3 glycan binders (10–1074 and PGT121) (Fig. 2.10). Clinical trials have shown the efficacy of some of those bNAbs in reducing viremia and maintaining viral suppression [93, 94]. However, the emergence of resistance and their suboptimal efficacy in preventing cell-to-cell viral transmission constitute important challenges to overcome in future developments. Infusion of VRC01 has been evaluated in patients chronically infected with HIV-1. Although patients showed different susceptibilities to the bNAb, there was no selection of VRC01 resistance after treatment interruption [95].

The combination of bNAbs (e.g. VRC01-PGT121/10E8v4 or VRC07-PG9–16) demonstrates increased antiviral activity, and clinical trials are in their initiation stages. Combinations of antibodies targeting nonoverlapping epitopes are expected to curb the development of HIV resistance in these therapies [96].

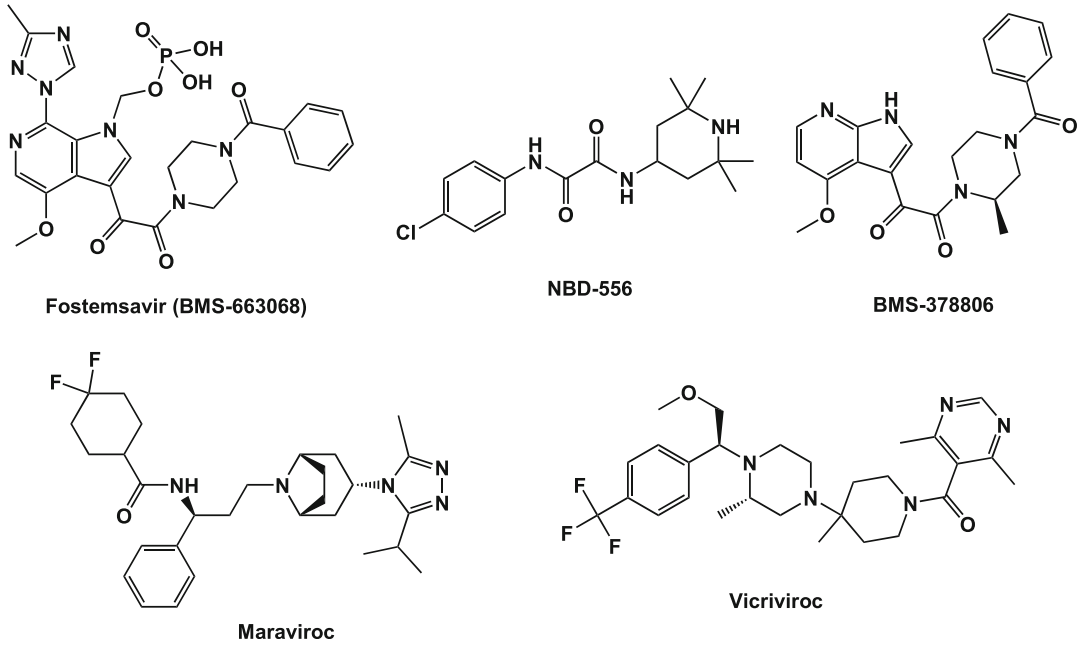

Fig. 2.9 Chemical structures of entry inhibitors

Leronlimab (formerly PRO 140) is a humanized IgG4 monoclonal antibody that prevents HIV-1 entry by blocking the viral coreceptor CCR5. The antibody has successfully completed nine phase 1/2/3 clinical trials in over 700 people, including a successful pivotal phase 3 trial in combination with standard antiretroviral therapies in HIV-infected treatment-experienced patients. In clinical trials exploring the use of leronlimab in monotherapy, researchers found

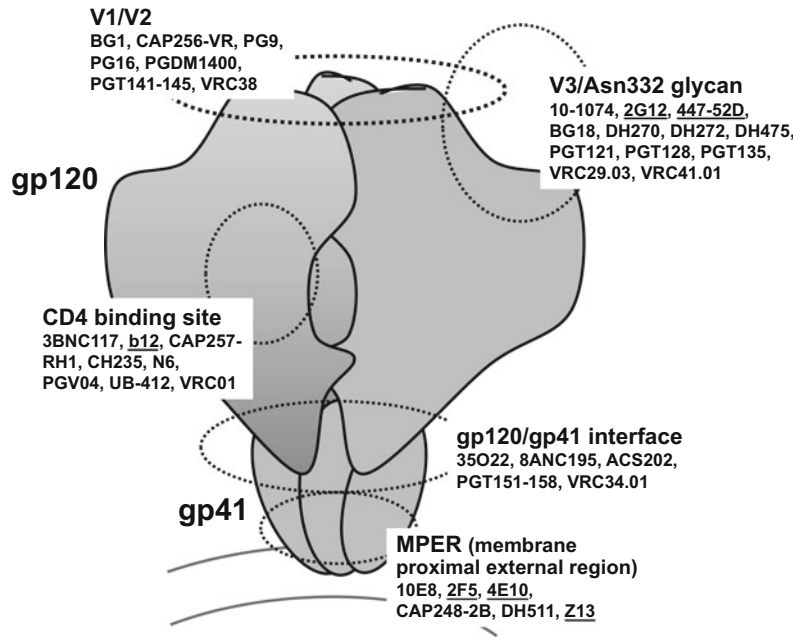

Fig. 2.10 Relevant bNAbs and location of their binding sites in HIV-1 Env [88–92]

that one fourth of the patients achieved viral suppression after approximately 1 year of monotherapy [97, 98]. Given the exceptional safety and efficacy profile, leronlimab appears to be a promising CCR5 antagonist and has the potential to become a best-in-class-treatment for HIV and perhaps other indications. So far, maraviroc is the only CCR5 antagonist approved for clinical use, although vicriviroc (MK-4176) is also in clinical trials (Fig. 2.9), co-formulated with an integrase inhibitor (MK-2048; see earlier discussion), for sustained delivery of antiretroviral medication in vaginal rings to prevent HIV infection in women [66, 67].

CXCR4 antagonists have been approved for hindering cancer progression, and specifically plerixafor has been approved by the US FDA to mobilize hematopoietic stem cells [99, 100]. Despite its efficiency in inhibiting HIV in cell culture, its use as an antiretroviral agent was not recommended after clinical trials [101]. Other small molecules, peptides and macromolecule inhibitors are now in preclinical development and attracting much attention as potential CXCR4-targeted anti-HIV-1 drug candidates (for a recent review, see [102]).

Finally, fusion inhibitors in development are represented by albuvirtide. This is a weekly injectable agent similar to enfuvirtide, with regulatory approvals in China, where it was originally developed [103]. It is a 3-maleimidopropionic acid-modified polypeptide of 32 amino acids, analogue of the fusion region of HIV gp-41. Clinical trials showed that in treatment-experienced patients, intravenous weekly administration of albuvirtide together with the protease inhibitors lopinavir/ritonavir was rather efficient in reducing viral load to less than 50 copies/mL [103]. These results have stimulated further studies to develop subcutaneous formulations of the drug allowing self-administration every 2–4 weeks.

2.5.6 Other Antiretroviral Targets

HIV replication and proliferation offer a number of additional potential targets still under investigation. Thus, the RNase H activity of HIV-1 RT can be inhibited by β-thujaplicinol and other inhibitors (for recent reviews, see [104, 105]). RNase H inhibitors are still far from entering clinical trials, but in some cases, they can be attractive molecules, as potential anti-HIV drugs with dual activity against RNase H and integrase [106], or RNase H and RNA-dependent DNA polymerase [107].

Another target of interest is the viral nucleocapsid protein (NC) that participates in multiple steps of the viral replication cycle. Although initial efforts to develop drugs targeting NC concentrated on small molecules destabilizing the zinc fingers of the protein, their development was halted due to their low specificity and potentially toxic mechanism of action. Nevertheless, some of them were further investigated as topical microbicides [108]. New compounds include more specific and less toxic noncovalent NC binders that inhibit the interaction between NC and nucleic acids and nucleic acid binders that compete with NC and block its functionality (for recent reviews, [109, 110]).

Interactions between host cell proteins and viral factors could also be targets of antiretroviral drugs. We discussed above on ALLINIs as compounds interfering in the interaction between HIV-1 integrase and LEDGF/p75. Other examples include inhibitors of the interaction between HIV-1 Vpu and host BST-2 that affect virus budding [111] or the HIV-1 Vif-APOBEC3 complex that could impact on the stability of the viral genome [112].

2.6 Implementation of Current Antiretroviral Therapies for Prevention

2.6.1 Pre-Exposure Prophylaxis

Individuals receiving antiretroviral therapy early during the course of infection are likely to maintain viral loads at undetectable levels. In this situation, the likelihood of HIV transmission between sexual partners was close to zero, as revealed in the PARTNER clinical trials

[113, 114]. These findings have been fundamental to support the 'U=U' (undetectable equals untransmittable) campaign and the benefits of early testing and treatment for HIV [115]. Therefore, present antiretroviral-based HIV prevention strategies concentrate on early treatment and the use of two-drug pre-exposure prophylaxis or three-drug postexposure prophylaxis of those uninfected individuals who are at risk for HIV infection (for a review, see [8]). In addition, the WHO has recommended the use of pre-exposure prophylaxis in pregnant and breastfeeding women to reduce HIV infection among women and vertical transmission [116, 117].

High adherence to a pre-exposure prophylaxis regimen of TDF and emtricitabine (one pill per day immediately before and following a sexual encounter) was found to be 99% effective in preventing HIV acquisition by sexual transmission and at least 74% through injection drug use. Currently, there are two FDA-approved medications for pre-exposure prophylaxis: (1) TDF and emtricitabine (sold under the brand name Truvada) and (2) TAF and emtricitabine (sold as Descovy). Despite these advances, the use of pre-exposure prophylaxis is still low among women, and recent efforts have concentrated on the development of strategies valid for both men and women.

In this context, HIV topical microbicides applied to the vagina or anus and rectum to prevent the sexual acquisition have been developed and tested in clinical trials. In 2016, a flexible silicone matrix vaginal ring containing dapivirine (an NNRTI), worn for a month at a time, was found to reduce HIV infection in women by ~30% compared to placebo, in two phase 3 placebo-controlled trials [118, 119]. The vaginal ring looks like a contraceptive ring but instead releases dapivirine slowly over the course of 1 month. After completing clinical trials, it is currently pending approval by health agencies, but pilot initiatives of extended use are currently in progress in several sub-Saharan African countries, whose selection was made attending to their public health needs.

Other antiretroviral drugs considered as potential microbicides include NRTIs (TDF), integrase inhibitors (cabotegravir) or entry inhibitors (maraviroc). The combination of maraviroc and dapivirine has been explored as pre-exposure prophylaxis against HIV, and recently its use in combination with emtricitabine has been proposed as an effective prophylactic option in both men and women [120].

2.6.2 Long-Acting Antiretroviral Drugs

Long-acting and extended-release formulations of antiretroviral drugs have the potential to revolutionize treatment and prevention. As discussed above, intravaginal rings facilitate the release of one or more anti-HIV drugs over time. Similar objectives can be achieved using implants. Still in early stages of clinical development, clinical trials have mainly tested the efficiency of islatravir, tenofovir prodrugs and dolutegravir [121]. However, there are other delivery options. For example, long-acting injectables containing cabotegravir have been tested for the prevention of HIV infection in young women (HPTN 084 trial currently in progress; https://www.hptn.org/research/studies/hptn084). Injection of 600 mg every 8 weeks was found to be well tolerated in both male and female study participants [122] and is being considered as a good pharmaceutical to be used in population groups with different levels of HIV risk. Recent studies have also shown that single subcutaneous doses of 450 mg of the capsid assembly inhibitor lenacapavir (GS-6207) produced a 2.2 mean-$\log_{10}$ transformed reduction of plasma viral load after 9 days while maintaining antiviral active concentrations of the drug for more than 6 months [78].

Finally, bNAbs are also considered to be promising strategies. Animal studies have shown that a single injection of a combination of two long-acting bNAbs (3BNC117-LS and 10–1074-LS) protected macaques against repeated mucosal challenges of SHIVAD8-EO for a median of 4–6 months [123]. The results of phase 1b clinical trials carried out with a

limited number of individuals have been reported [124]. In those individuals, HIV-1 viral load was 200-fold reduced, and this decrease was maintained during 3 months, after the first of three infusions. None of the studied patients developed resistance to any of both antibodies.

In addition to their relevant role in prevention, therapeutic strategies based on the use of long-acting antiretroviral agents are convenient and can improve adherence. Approved long-acting agents include the humanized IgG4 antibody ibalizumab and fusion inhibitor albuvirtide (although in this case, it has been approved only in China). Other long-acting antiretroviral drugs in preclinical or clinical development have been described in previous sections and include NRTIs (TAF, islatravir and rovafovir etalafenamide), NNRTIs (dapivirine, rilpivirine and elsulfavirine), integrase inhibitors (raltegravir and cabotegravir), protease inhibitors (darunavir boosted with cobicistat), assembly inhibitors (lenacapavir and GS-CA1) and entry inhibitors (maraviroc, vicriviroc and antibodies leronlimab, BMS-378806, UB-421, 3BNC117 and 10–1074) (reviewed in [125, 126]).

The combination of dolutegravir plus rilpivirine (first approved two-drug regimen) sustained virological suppression of HIV-1 and was associated with a low frequency of virological failure. In addition, it had a favourable safety profile (SWORD-1 and SWORD-2 trials at 48 weeks) versus standard three-drug or four-drug antiretroviral regimen for maintenance of virological suppression [127]. As mentioned earlier, the combination of cabotegravir and rilpivirine (Cabenuva™) has been approved in Canada, but at the end of 2020, US FDA approval was still pending. The latest results of the ATLAS and FLAIR clinical trials presented at the 2020 Conference on Retroviruses and Opportunistic Infections, in Boston, showed that once-monthly intramuscular injections of long-acting cabotegravir/rilpivirine were non-inferior to conventional oral antiretroviral therapy for maintenance of HIV-RNA suppression [128].

An important property shown by some long-acting antiretroviral agents is their presence in the blood after discontinuation. Clinical studies have shown that injectable cabotegravir might remain detectable after discontinuation for nearly 3 years in men and 4 years in women [129]. This is an important observation if the drug is considered for pre-exposure prophylaxis, since the presence of suboptimal concentrations of the drug poses a risk for selection of drug-resistant HIV after interrupting medication. Healthy individuals might be reluctant to use an antiretroviral drug that could persist for years after the final injection.

2.7 HIV Cure and Prospective Drugs

Despite the significant progress made in antiretroviral therapy, HIV infection remains as a lifelong chronic disease. So far, there are only two known instances of reported cure. The first is the case of the ‘Berlin patient’, an HIV-infected individual who suffered advanced leukaemia and needed a bone marrow transplant to survive. Physicians took advantage of the existence of a homozygous donor carrying a deletion in the CCR5 coreceptor that HIV uses to enter the cell. After destroying the immune- and blood-forming cells by radiation, drugs and antibodies, the bone marrow of the Berlin patient was repopulated with cells from a donor with the CCR5 mutation [130, 131]. Twelve years later, another patient in London went through a similar process. He had Hodgkin’s lymphoma and received a bone marrow transplant from a donor with the CCR5 mutation in May 2016. He also received immunosuppressive drugs, but the treatment was much less intense. In September 2017, he was able to stop taking antiretroviral drugs, with no sign of the virus returning 30 months later [132]. A possible third case was announced at the 2019 CROI Conference [133]. Treated in Düsseldorf, this patient also received a bone marrow transplant from a donor with the CCR5 mutation and showed no infectious HIV after 3 months off antiviral drugs. Currently, cord blood transplantation with CCR5Δ32 donor cells in HIV-1 infected subjects who require bone marrow transplantation is under clinical trial in the United States (NCT02140944).

Given the complexities and expenses associated with lifelong medication, developing an effective curative intervention is now a global priority. A cure for HIV would be the total eradication of the virus from the body, or a sustained virologic remission in the absence of antiretroviral therapy, defined as a functional cure. The existence of an HIV-1 reservoir is a major hurdle to a cure. There are five broadly defined approaches to achieving an eradicative cure or remission: early antiretroviral therapy, genetic modifications (including viral excision using clustered regularly interspaced short palindromic repeats (CRISPR) therapies for HIV-1 elimination), 'shock-and-kill', 'block-and-lock' and immunotherapy (e.g. bNAbs and cytotoxic T lymphocytes, including killer T cells engineered to have two surface receptors which recognize distinct parts of gp120) (reviewed in [134–137]).

From the point of view of medicinal chemistry, pharmacological approaches to HIV elimination concentrate on two major strategies: 'shock-and-kill' and 'block-and-lock'. In the 'shock-and-kill' therapy, latency-reversing agents (LRAs) are first used to reactivate latent HIV hiding in immune cells (the 'shock'), and the reactivated cells can then be targeted and killed by the body's immune system or antiretroviral drugs. Difficulties in this approach relate to the complex and highly heterogeneous nature of the proviral reservoirs that impedes pharmaceutical access to all hidden HIV (reviewed in [138]).

LRAs within the host cell can be divided into six primary categories: (1) histone post-translational modification modulators (e.g. inhibitors of histone methyltransferases and deacetylases [HDACs] inhibitors); (2) nonhistone chromatin modulators (e.g. BAF and BRD4 inhibitors); (3) NF-κB stimulators (e.g. protein kinase C agonists); (4) Toll-like receptor (TLR) agonists; (5) extracellular receptor binding compounds (e.g. maraviroc, interleukins 2 and 6 and tumour necrosis factor α); and (6) a miscellaneous group of molecules, including disulfiram, BMS-936559 and ixazomib, among others [138]. HDAC inhibitors (e.g. vorinostat, panobinostat or romidepsin) have been the most widely tested LRAs. Although clinical trials are still ongoing, progress in achieving HIV remission has been limited. LRAs seem to be unable to reduce the size of the latent reservoir, and studies show that only a minor fraction of the reservoir is reactivated upon treatment. In addition, many LRAs have undesired side effects and toxicity while affecting cellular homeostasis.

The limited success of eradication strategies has led scientists and clinicians to reevaluate the definition of HIV cure. A functional cure could be achieved by durably silencing the latent HIV provirus in infected cells and therefore preventing viral rebound [139]. The so-called 'block-and-lock' therapies would prevent HIV replication and propagation by targeting different factors of the HIV transcriptional machinery. Several research groups have described mechanisms acting on different factors of HIV transcription, potentially useful for designing effective 'block-and-lock' strategies (reviewed in [140]): (1) Tat inhibition by didehydro-cortistatin A; (2) LEDGINs; (3) curaxin CBL0100 inhibition of the FACT complex (FACT, stands for 'facilitates chromatin transcription complex'); (4) RNA-induced epigenetic silencing; (5) HSP90 Inhibitors; (6) *Jak*-STAT inhibitors; (7) BRD4 modulators; (8) mTOR inhibitors; (9) kinase inhibitors; and (10) triptolide, a diterpenoid epoxide obtained from a plant. Several of those compounds have been tested in clinical trials. Examples are trial NCT02219672 for triptolide and NCT01854034 and NCT01485536 for the HSP90 inhibitor AUY922. However, results are still not satisfactory, and efforts should be made to incorporate more of those treatments to in vivo models and clinical trials while monitoring their effect on the latent HIV reservoirs.

2.8 Conclusions and Future Perspectives

The extraordinary advances in the fight against HIV and AIDS include licensing of more than 30 antiretroviral agents and drug combinations demonstrating high potency and minimal toxicity. Nowadays, HIV infection can be considered a

treatable chronic disease although long-term toxicity, and acquired and transmitted drug resistance poses a risk to the success of lifelong therapy. Continued research efforts resulted in new drug formulations, most notably long-acting antiretroviral therapies that facilitate treatment compliance, and new pre- and postexposure prophylaxis that could play an important role in preventing the spread of the disease, particularly in populations more heavily hit by the pandemic. New antiretroviral agents are currently in development and in clinical trials. Some of them extend the existing families of antiretroviral drugs (e.g. new integrase inhibitors such as cabotegravir, or MK-2048, or NRTIs such as islatravir and rovafovir etalafenamide), while others interact with unexploited targets and processes (e.g. assembly inhibitors, such as the CA-binding agent lenacapavir). These new compounds are expected to help in simplifying dosage regimens while increasing the armamentarium against eventual multidrug-resistant HIV.

Current antiretroviral therapies can reduce viral load to undetectable levels, but, unfortunately, HIV cannot be eradicated, and significant efforts are now directed towards the development of potential cures. From a pharmacological point of view, 'shock-and-kill' and 'block-and-lock' strategies have gained a lot of attention. However, despite the identification of many compounds (including more than 160 LRAs) with potential efficacy to eliminate viral reservoirs, a deeper understanding of the regulatory processes involved in transcriptional activation of proviruses and a better knowledge of the fate, composition and location of latently HIV-infected cells will be needed to define successful curing strategies.

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Structural Insights to Human Immunodeficiency Virus (HIV-1) Targets and Their Inhibition

3

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Abstract

Human immunodeficiency virus (HIV) is a deadly virus that attacks the body's immune system, subsequently leading to AIDS (acquired immunodeficiency syndrome) and ultimately death. Currently, there is no vaccine or effective cure for this infection; however, antiretrovirals that act at various phases of the virus life cycle have been useful to control the viral load in patients. One of the major problems with antiretroviral therapies involves drug resistance. The three-dimensional structure from crystallography studies are instrumental in understanding the structural basis of drug binding to various targets. This chapter provides key insights into different targets and drugs used in the treatment from a structural perspective. Specifically, an insight into the binding characteristics of drugs at the active and allosteric sites of different targets and the importance of targeting allosteric sites for design of new-generation antiretrovirals to overcome complex and resistant forms of the virus has been reviewed.

Keywords

AIDS/HIV · Entry inhibitors · Nucleoside RT inhibitors · Non-nucleoside RT inhibitors · Integrase inhibitor · Protease inhibitors · Drug resistance · X-ray crystallography

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Abbreviations

3TC-TP	(−)-L-2′,3′-dideoxy-3′-thiacytidine triphosphate (or lamivudine triphosphate)
AIDS	Acquired immunodeficiency syndrome
ALLINIs	Allosteric integrase inhibitor
ART	Antiretroviral therapy
CA	Capsid protein
cART	Combined antiretroviral therapy
CCR5	CC chemokine receptor 5
CD4+	T helper cell
CXCR4	C-X-C chemokine receptor type 4

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DHBNH	Dihydroxy benzoyl naphthyl hydrazone
FTC-TP	Emtricitabine triphosphate
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IN	Integrase
INIs	Integrase inhibitors
InSTIs	Integrase strand transfer inhibitors
LEDGF/p75	Lens epithelium-derived growth factor
NC	Nucleocapsid protein
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
PDB ID	Protein data bank identifier
PFV	Prototype foamy virus
PLIF	Protein–ligand interaction fingerprint
RNase H	Ribonuclease H
RT	Reverse transcriptase
UNAIDS	Joint United Nations Programme on HIV/AIDS
USFDA/FDA	Food and Drug Administration

3.1 Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by a human retrovirus, human immunodeficiency virus (HIV), and is considered as a deadly disease since its discovery in the year 1983 [1]. To date, 76 million are infected by the virus globally with around 33 million deaths. According to the UNAIDS report in 2019, 37.9 million people were living with HIV, among them 36.2 million are adults and 1.8 million are children, indicating these infections as a major threat to humans all over the world [2]. Young adults in the age group of 15–24 and women are at a higher probability of risk. There are approximately 6200 new infections every week arising amid the young. The human immune system weakens after 10–15 years of infection due to the entry of HIV into the host cell followed by replication with the help of host cell mechanism and ultimately killing the CD4 T-cells, giving way to opportunistic infections such as tuberculosis, pneumonia, herpes simplex, Kaposi's sarcoma, and coccidioidomycosis. The devastated CD4+ T-cells are the characteristic feature of AIDS that leads to the death of a patient infected with HIV [3].

Although there have been intense efforts toward the development of drugs used effectively for the treatment of HIV infections, however, several drugs fail to function due to the acquired mutation in the virus and the development of resistant strains. To overcome the viral mutation and drug resistance, combination treatment regimens called combination antiretroviral therapy (cART) are being explored. For effective treatment of resistant strains, several promising clinical trials are currently in progress. One of the rational approaches to effectively combat resistant strains of HIV is by using the structural information of the target protein. Through the knowledge of the binding pocket and the important amino acid residues at the HIV target site, drugs could be designed effectively to treat mutant strains, resistant to the routine drug therapy.

HIV-1 is a *Lentivirus* of the family *Retroviridae* and contains a genomic RNA of approximately 9800 base pairs. The development of mature viral and multiplication are carried out in various stages in the replication cycle of HIV. The key enzymes/receptors/proteins involved in the replication are glycoproteins, gp120 and transmembrane gp41, C-C chemokine receptor type 5 (CCR5 or R5) or CXCR4 co-receptor, reverse transcriptase (RT), integrase (IN), and protease (PR) [4]. Blocking of targets located on the human cell surface or viral envelope prevents entry of the viral DNA into the human cells. The surface proteins of HIV, gp120 and gp41, which can bind to the human cell surface receptor(s) are targeted by the so-called entry/fusion inhibitors that prevent the entry of viral genetic material into the human cells. The fusion inhibitors are also used to target receptors on the human cell surface, including CD4 (a protein receptor on helper T-cells), CCR5, and CXCR4. The entry of viral nucleocapsid into the host cell liberates three key enzymes including RT, IN, and

PR which play a vital role in viral replication; hence, these individual enzymes are essentially targeted in HIV therapy as reverse transcriptase inhibitors (RTIs), nucleoside RTIs (NRTIs), non-nucleoside RTIs (NNRTIs), integrase strand transfer inhibitors (INSTIs), and protease inhibitors, respectively. Budding inhibitors stop new HIV from leaving of the CD4 cell, while maturation inhibitors block the final proteolytic processing process.

3.2 Structural Information for Rational Drug Design

The process of drug discovery was initiated from hit identification, hit-to-lead generation, lead optimization, and clinical trials. The drug discovery process involves several challenges in addition to the improvement of potency, for instance, optimization of physicochemical properties, pharmacological profile, dosage regimen, and reduction of the overall toxicity of the drug candidate. Recent advances in high-throughput and fragment-based screening techniques are applied in searching a hit molecule that can be further optimized to target a protein or receptor. This is well supported by the knowledge of protein structure obtained through novel techniques and advancement in X-ray crystallography, intense synchrotron radiation, and phase determination. X-ray crystallography has a central role in the structure elucidation of the protein and in particular the complexes with drug-like ligands by providing a deeper understanding of molecular recognition. Additionally, mutagenesis studies allow confirmatory evidence into the importance of these amino acids in the binding pocket of the target protein.

Given the three-dimensional (3D) structure of the target protein, the structure-based drug design paves the way for great improvement and speeds up the drug discovery process. This provides an opportunity to computationally screen a large library of molecules, identify novel scaffold, and predict ligand interaction with the amino acid residues in the binding pocket and binding affinity and specifically for the viral targets, which are the major source of information to design molecules against the mechanism of drug resistance [5]. As a part of the lead optimization process, the 3D structure of the protein provides a guide for a rational modification of molecules by applying medicinal chemistry approaches such as molecular hybridization, scaffold hopping, or bioisosteric replacement, to establish high-quality interaction with residues within the binding pocket of the protein or receptor. Thus, understanding the binding mode of ligands with the 3D structure of the target protein supports the design of efficient drug candidates [6–8].

3.2.1 Entry and Fusion Inhibitors

Currently, targeting viral entry is one of the interesting avenues in the area of antiretroviral drug discovery. The gp120 surface and gp41 transmembrane subunits on the trimeric envelope glycoprotein allow entry of HIV into the host cells. The attachment toward host cells is through non-specific electrostatic interactions found between the positive charge of the CD4 molecule and the negative charge of the gp120 pocket. The subsequent exposure of CCR5 or CXCR4 on the cell surface causes further modification in the conformation of gp120. The N-terminal hydrophobic fusion peptide of gp41 is detached from the hold of gp120 and inserts into the host cell membrane. Consequently, trimeric N-terminal repeats are folded by three C-terminal heptad repeats in an antiparallel orientation ensuing in the construction of a hairpin-like six-helix bundle (6-HB) that drags the viral and cellular membranes concurrently and permits for the fusion reaction. Three distinct crucial steps involved in the entry process allow for entry inhibition: cell surface attachment, co-receptor binding, and fusion [9–13].

Enfuvirtide is a linear 36 L-amino acid synthetic peptide fusion inhibitor that was approved by the FDA in the year 2003 used in combination therapy for the treatment of HIV-1/AIDS. Due to its sequence similarity to the helical heptad repeat (HR2) at the C-terminal fragment of gp41, enfuvirtide binds as such to helical heptad repeat (HR1) from N-terminal and prevents HR2

binding by blocking the formation of a six-helix bundle structure, which is a vital process for the viral and host–cell membrane fusion reactions. Continuous therapy with enfuvirtide leads to the development of drug resistance by the selection of changes in the 10-amino-acid (residues, 36–45) segment within the HR1 region of gp41 [14, 15].

Maraviroc was approved as an HIV entry inhibitor in the year 2007. The drug binds to the cell surface of the human chemokine receptor. It changes the CCR5 molecular shape and avoids interaction in the viral attachment site. Before starting therapy using maraviroc, the existence of R5 viruses has to be tested in individual patients, because maraviroc is only effective against R5 viruses that use CCR5 receptors for their entry into their target, i.e., the immune system cells [16].

In 2018, FDA approved ibalizumab as the first humanized IgG4 monoclonal antibody for the treatment of multidrug-resistant HIV-1 infection, in combination with other forms of antiretroviral therapy for adults who have failed treatment with the conventional antiretroviral regimen. It binds with the CD4 extracellular domain 2 residues L96, P121, P122, and Q163 along with E77 and S79 on domain 1 which prevents conformational modification in the complex of CD4–HIV envelope glycoprotein (gp120), a crucial step for the viral access [17–19].

Fostemsavir is a phosphonooxymethyl prodrug which is hydrolyzed to the active metabolite temsavir by alkaline phosphatase that received FDA approval, granted in July 2020 [20]. The temsavir binding to gp120 prevents the gp120 conformational change required for attachment of HIV-1 to the host CD4 cell surface receptor on the host immune system and prevents its attachment. Hence, HIV virus does not enter the host cell while in the deficiency of efficient attachment of HIV gp120 with the host CD4 receptor [21]. It is the first attachment inhibitor used in combination with other classes of antiretroviral drugs in vastly treatment-experienced patients with multidrug-resistant HIV-1 infection who are failing their current therapy [22]. However, fostemsavir activity gets reduced against HIV AE subtypes by the occurrence of prevalence gp120 resistance-associated polymorphisms at key sites L116Q, S375H/M/T, M426L, M434I, M426R, and M475I [23]. At present, numerous compounds such as PRO-140, cenicriviroc, monomeric DAPTA, and albuvirtide, belonging to the group of entry and fusion inhibitors that target the HIV gp120 either at the CD4 T-cell receptor surface or at the CCR5/CXCR4 co-receptors, are under clinical trials [24].

CCR5/CXCR4 binds to one of the co-receptors CD4–gp120 with the support of variable loop region V3 from gp120 (Fig. 3.1), and the other two variable loop regions V1/V2 and CD4 are involved in electrostatic, van der Waals, and hydrogen bond interactions that assist in the formation of CD4–gp120 complex. These two co-receptors are the entry point for HIV-1 into human CD4+ cells, thereby demonstrating as an important target for possible interference to stop the infection [26–28].

3.2.2 Crystal Structures and Ligand-Binding Site of CCR5

In 2013, the first X-ray crystal structure of the CCR5 chemokine receptor in complex with HIV entry inhibitor maraviroc (PDB ID: 4MBS, Fig. 3.2) was resolved [29]. The structure reveals allosteric inhibition mechanism of chemokine signaling and viral entry. Further in 2018, Peng et al. reported two crystal structures (at 2.8 Å resolution) of CCR5 in complex with propane diamine derivatives (PDB ID: 6AKX and 6AKY) that showed a similar binding mode and interaction to the protein as noted for maraviroc [30].

Maraviroc binds to the largest hydrophobic cavity on the membrane of CD4 cells (T-cells) between the extracellular loop and upper transmembrane domain and prevents the interaction of HIV-1 gp120 and CCR5, which is required for CCR5-tropic HIV-1 to enter the host cells. The drug was approved for clinical use as an HIV-1 entry inhibitor and showed additional efficiency

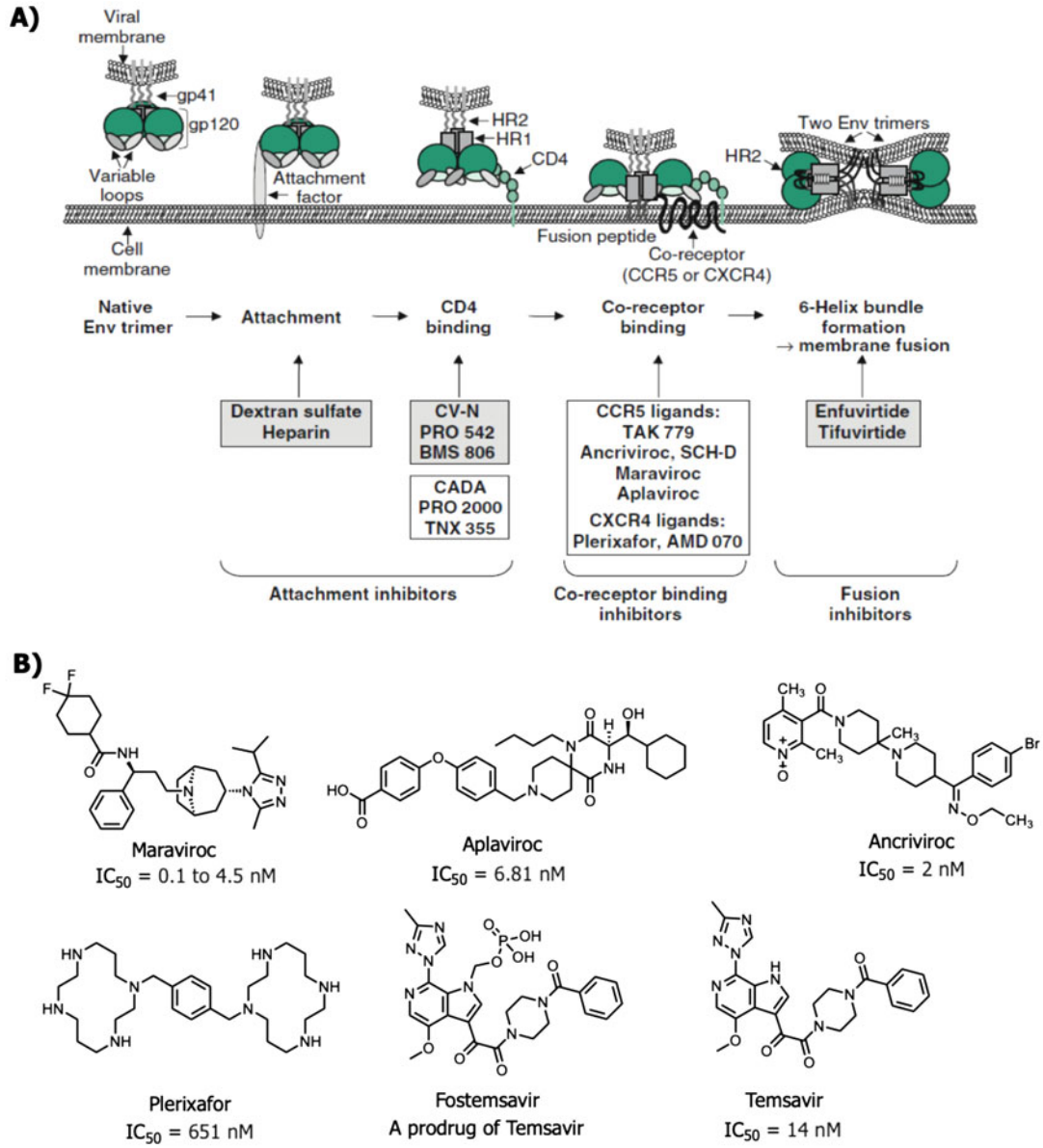

Fig. 3.1 Potential inhibitors involved in the entry mechanism of HIV-1 inhibition and their biological activities. (**a**) Different steps involved in the viral entry process, and inhibitors targeting the different steps are represented (Reprinted by permission from Springer publishing company [25]. (**b**) Chemical structures of various entry inhibitors along with their IC_{50} values

in antiretroviral-pretreated patients [29]. Maraviroc interacts with CCR5 via hydrophobic and polar pocket containing residues Y37, Y89, W85, Y108, F109, F112, K191, F182, Q194, T196, I198, W248, Y251, L255, T259, M279, E283, T284, and M287. Specifically, hydrogen bond interactions occur with Y37, E283, and Y251 (Fig. 3.3) which is important for blocking the gp120–CCR5 interaction after binding to the co-receptor [29, 31–33]. Binding of this drug also

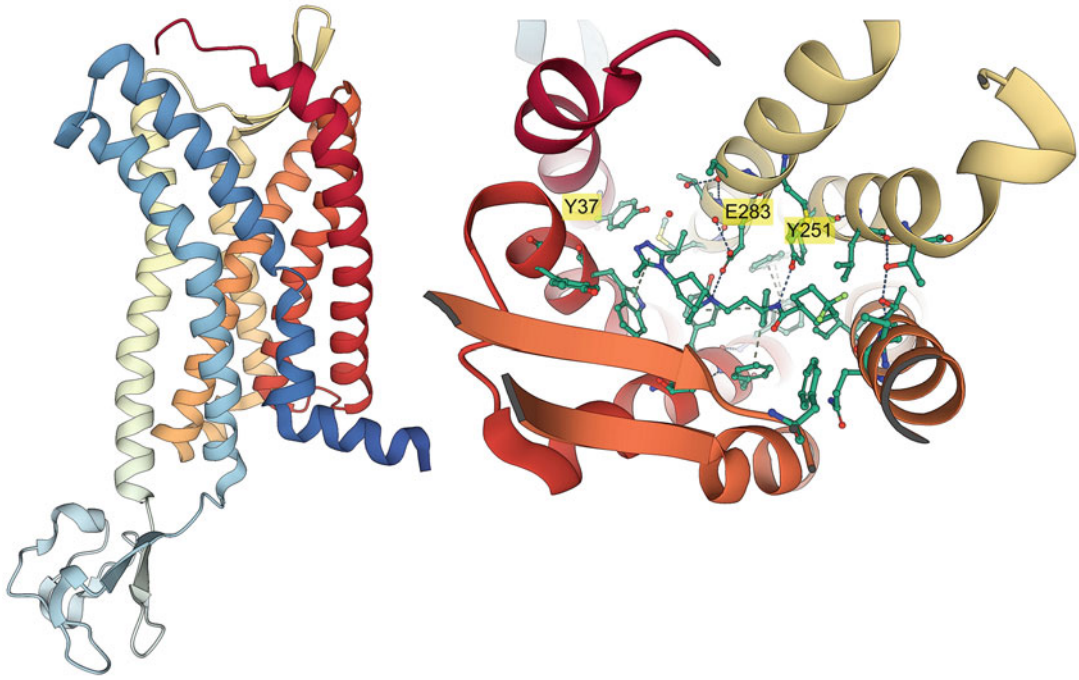

Fig. 3.2 Structure of the CCR5–maraviroc complex (PDB ID: 4MBS). The crystal structure of CCR5 shown in cartoon representation and the ligand-binding pocket of CCR5 with maraviroc including the important amino acid residues (green) interacting with the inhibitor. The key interacting amino acid residues are highlighted in yellow

leads to the altered conformation of the second extracellular loop of the receptor, which prevents the interaction with the V3 stem-loop of HIV's gp120 envelope glycoprotein.

3.2.3 Resistance to Entry and Fusion Inhibitors

It has been shown that every entry inhibitor is susceptible to drug resistance, which may be unique to individual inhibitor subclass. For instance, several point mutations are reported at the V3 loop region of P120 glycoprotein, and different inhibitors (aplaviroc, maraviroc, vicriviroc, TAK-779, SCH-C, and a benzyloxy-carbonyl-aminopiperidin-1-yl-butane derivative) bind to the CCR5 target such as E283A, W86A, Y37A, Y108A, A316T, K305R, R315Q, K319T, I323V, and I198M. Some minor mutations, A29G and Y251F, at the binding pocket of CCR5 may also lead to drug resistance. Further, amino acid mutations may take place either inside or outside of the V3 loop; therefore, synergistic interactions between substitutions in the V3 loop and other parts of envelope (Env) changes may also contribute to the resistance [34–36].

3.2.4 Reverse Transcriptase Inhibitors

Reverse transcriptase (RT) is the central enzyme that reverse transcribes the viral single-stranded RNA genome to linear double-stranded proviral DNA, needed for insertion into the genome of the host (Fig. 3.3a). Three distinct functions are involved in the RT enzyme, including RNA-dependent DNA synthesis, RNase H activity, and DNA-dependent DNA synthesis. The catalytic performance of these enzymes originally involved in the synthesis of the (−) DNA strand that depends on RNA genome by way of hydrolytic cleavage of the template RNA and

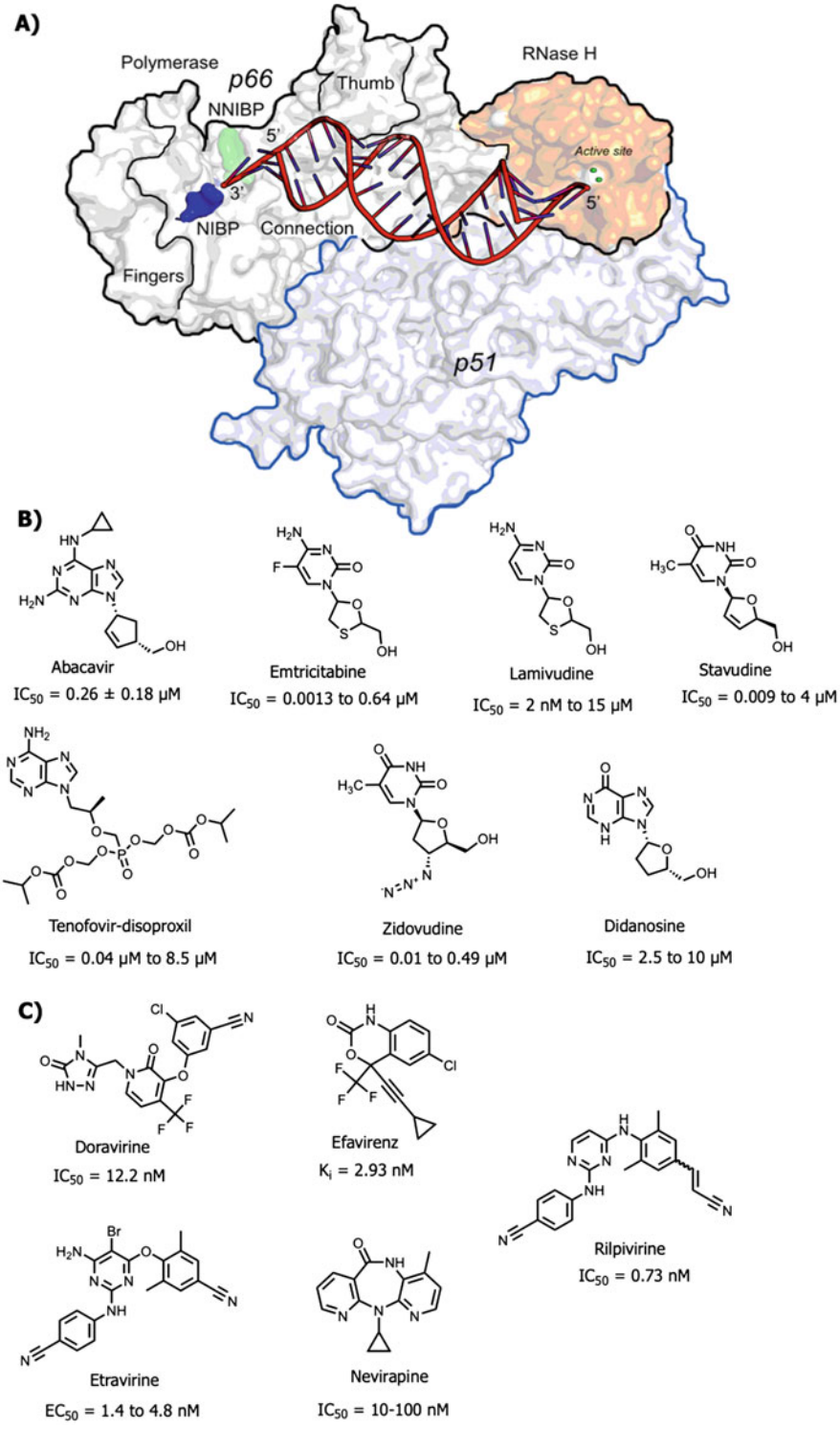

Fig. 3.3 HIV-1 reverse transcriptase enzyme and its approved NRTI and NNRTIs. (**a**) HIV-1 RT template–primer complex showing the fingers, thumb, palm, and connection subdomains of the DNA polymerase domain and the RNase H domain located at the carboxy-terminal region of the enzyme. (**b**) Chemical structures of various NRTIs commercially available and their biological activities. (**c**) Chemical structures of various NNRTIs commercially available and their biological activities

subsequent synthesis of (+) DNA that depends on the DNA fragments [37]. The asymmetric heterodimer of HIV-1 RT enzyme contains two subunits, namely, p66 subunit (560 amino acids) and p51 subunit (440 amino acids). These subunits are obtained by cleavage of the Gag–Pol polyprotein, precursor by the viral protease. Both subunits are encoded by sharing the first 440 identical sequences in the viral genome. The RNase H domain includes residues between 441 and 560 of p66 subunit and consists of eight connecting loops that link the major five-stranded mixed β-sheet surrounded by four α-helices. The three-dimensional crystal structures of HIV-1 RT have shown that both subunits are composed of four common polymerase subdomains represented as "fingers" (residues 1–85 and 118–155), "palm" (residues 86–117 and 156–236), "thumb" (237–318), and "connection" (319–426). The DNA polymerase catalytic residues such as D110, D185, and D186 are located within the p66 subunit. For catalysis, two divalent cations (Mg^{2+}) are necessary. The fingers, palm, and thumb subdomains of p66 and the thumb subdomain of p51 form the nucleic acid binding cleft, which together with the connection subdomains of both subunits contribute to the "floor" of the cleft. The RNase H domain responsible for nonspecifically hydrolyzing the RNA strand of the RNA/DNA hybrid results in the formation of integration-competent double-stranded proviral DNA from the RNA genome of the infecting particle and also facilitates the formation of the RNA primers called as polypurine tracts (PPTs), key elements essential for initiation of DNA synthesis. The catalytic activities of HIV-1 RNase H domains depend on the occurrence of prerequisite cofactor Mn^{2+} metal ions. HIV-RT is a principal targeting enzyme for antiretroviral agents due to its pivotal role in the life cycle of HIV-1 infectious disease. So far, all the clinically approved (e.g., US FDA) RT inhibitors/drugs inhibit only the polymerase activity of RT enzyme, not the RNase H domain. RT inhibitors are classified into three types: nucleoside RT inhibitors (NRTIs), nucleotide RT inhibitors (NtRTIs, Fig. 3.3b), and non-nucleoside RT inhibitors (NNRTIs, Fig. 3.3c) [38].

All FDA-approved NRTIs act as chain terminators that are converted into active metabolites of triphosphates catalyzed by cell-derived kinases. Further, it competes with natural dinucleotide triphosphates (dNTPs) for binding at the RT polymerase active site and gets incorporated into viral DNA elongating primer strand leading to inhibition of further DNA synthesis [39]. The blockade of NRTIs reverse transcription takes place by the lack of a hydroxyl group at the 3′-position of the ribose ring in NRTIs. As of now, seven NRTIs have been approved by the FDA, which include zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, and emtricitabine for the treatment of AIDS.

The mode of action of NtRTIs is very similar to the NRTIs that requires two phosphorylation steps for converting into the active metabolite by cellular kinases and to terminate the DNA polymerization. In contrast, NNRTIs act by noncompetitively binding to a hydrophobic allosteric pocket which is 10 Å away from the polymerase active site (Fig. 3.4). NNRTIs block the chemical step involved in the polymerization reaction of DNA synthesis [40].

Many HIV-1 RNase H inhibitors have been reported as active site-directed inhibitors and allosteric inhibitors that bind to the RNase H catalytic center and a region outside the RNase H domain, respectively [41]. Several classes of compounds including N-hydroxyimide, diketo acid, α-hydroxy tropolone, hydroxy pyrimidine, naphthyridinone, nitrofuran-2-carboxylic acid, carbamoyl methyl ester, thiocarbamate, and triazole derivative have been reported as active site-directed inhibitors and bind to the catalytic site residues through metal ions. On the other hand, derivatives such as hydrazones, anthraquinones, naphthalene sulfonic, vinylogous ureas, thieno pyrimidinone. and benzo

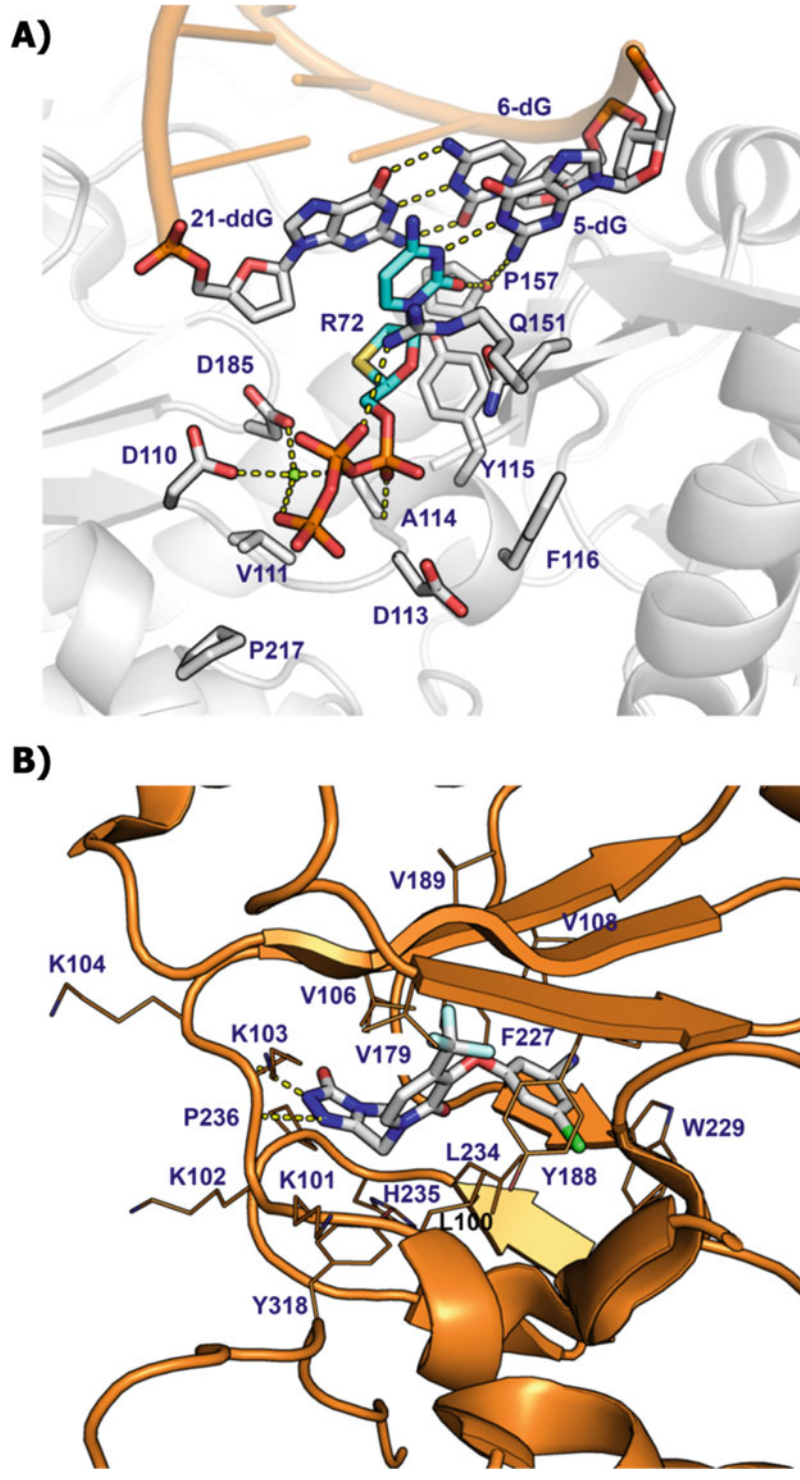

Fig. 3.4 Crystal structure of HIV-1 RT complex with lamivudine triphosphate and doravirine. (**a**) The binding pockets of RT in complex with dsDNA primer/template and lamivudine triphosphate (PDB ID: 6OUN), a NtRTI, and (**b**) doravirine (DOR), an NNRTI, (PDB ID: 4NCG) with key amino acids in the binding pocket

pyrimidinone are allosteric RNase H inhibitors binding to the site which is distant to the active site [42].

3.2.5 Crystal Structures and Ligand-Binding Site of RT and RNase H

In 1992, Kohlstaedt et al. reported the first crystal structure of HIV-1 RT in complex with nevirapine (PDB ID: 2HVT, 3.50 Å) [43]. Subsequently, Smerdon et al. refined the available crystal structure to a better resolution (PDB ID: 3HVT, 2.90 Å) and defined the polymerase domains of HIV-1 RT which is the structural basis of asymmetry in the HIV-1RT heterodimer [44].

As of now (July 2020), >260 X-ray crystal structures of HIV-RT are available in the Protein Data Bank that could be used for understanding detailed molecular biology of HIV-RT and binding mechanisms of drugs from NRTI and NNRTI classes. In addition, these structures also provide a basis for understanding drug resistance and to facilitate the design of new inhibitors for this enzyme. To date, widespread X-ray crystallographic studies were reported to explain the mechanisms of RT initiation and binding/incorporation/scission of nucleoside analog RT inhibitors (NRTIs) related to drug susceptibility/resistance. Notably, Bertoletti et al. elucidated the first crystal structures of reverse transcriptase inbound with a dsDNA primer template and the active triphosphate forms of lamivudine [(−)3TC-TP] and emtricitabine [(−)FTC-TP] to understand the molecular basis of recognition for NRTIs by RT (PDB ID: 6OUN, 6OR7, and 6UJY). The insight of the binding modes of (−)3TC-TP, (+)FTC-TP, and (−)FTC-TP showed a Watson–Crick base pair constructed between cytosine base moieties of the compounds and guanine base (5-dG) of the template DNA oligo through a π–π stacking with the guanine base 21-ddG of the prime and triple H-bonding interactions to the 5-dG (Fig. 3.4a). The structures also showed the three phosphate groups, namely, alpha, beta, and gamma, buried in the active site residues of R72, D110, D113, A114, D185, and K220 and formed coordination with Mg^{2+} ion forming several H bonds [45].

The binding mode of DOR complex with RT shows that there are two hydrogen bonding interactions between methyl-triazolone ring and backbone of K103 (Fig. 3.4b). The side chain of Y181 twists about 90° and doesn't interact with the Y188 residue, long distance in the interaction of the cyanochlorophenol moiety of DOR with Y181. Similarly, K103 and Y181 residues are least involved in the binding of DOR with RT. This makes DOR an important profile against common resistance mutations (K103N and Y181C) at these positions. Y188 residue forms π–π stacking interactions with the cyanochlorophenol moiety of DOR and is pivotal for DOR binding with RT. There are van der Waals interactions between the central ring of DOR and the isopropyl group of V106 within a distance of 3.5 Å distance, at the NNRTI binding pocket. The virus becomes resistant to DOR by tenfold caused by the mutation of V106A, thereby losing the hydrophobic interactions and weakening the binding of DOR with RT [46, 47].

A large number of HIV-RT crystal complexes with clinically approved drugs and diverse chemical compounds which are currently under investigation have been resolved with the wild-type (e.g., PDB ID: 6DTX, 3T19, 2WON) [48–50] and mutant forms of RT including single and multiple mutations (e.g., PDB ID: 6DUG, 6DUF, 6CGF, 6C0J, 6C0R, 6C0O). Wild-type and mutant HIV-1 RT structures have been explained with both apo and ligand-bound conformational states toward the RNase H primer grip site, polymerase catalytic site/dNTP binding site, *gap* and bulge RNA/DNA, knuckles site, and NNRTI site, some including bound drugs or varied chemical scaffold of small-molecule and inbound aptamers [51]. Few of the X-ray crystal studies reveal that the dislodgment of the β12–β13–β14 sheet at the polymerase primer grip occurs, while binding of an NNRTI resulted into inappropriate positioning of the nucleic acid that influences the conformation of the polymerase catalytic site. The structurally dissimilar NNRTI shows common pharmacophoric features capable of forming hydrophobic interaction and a few

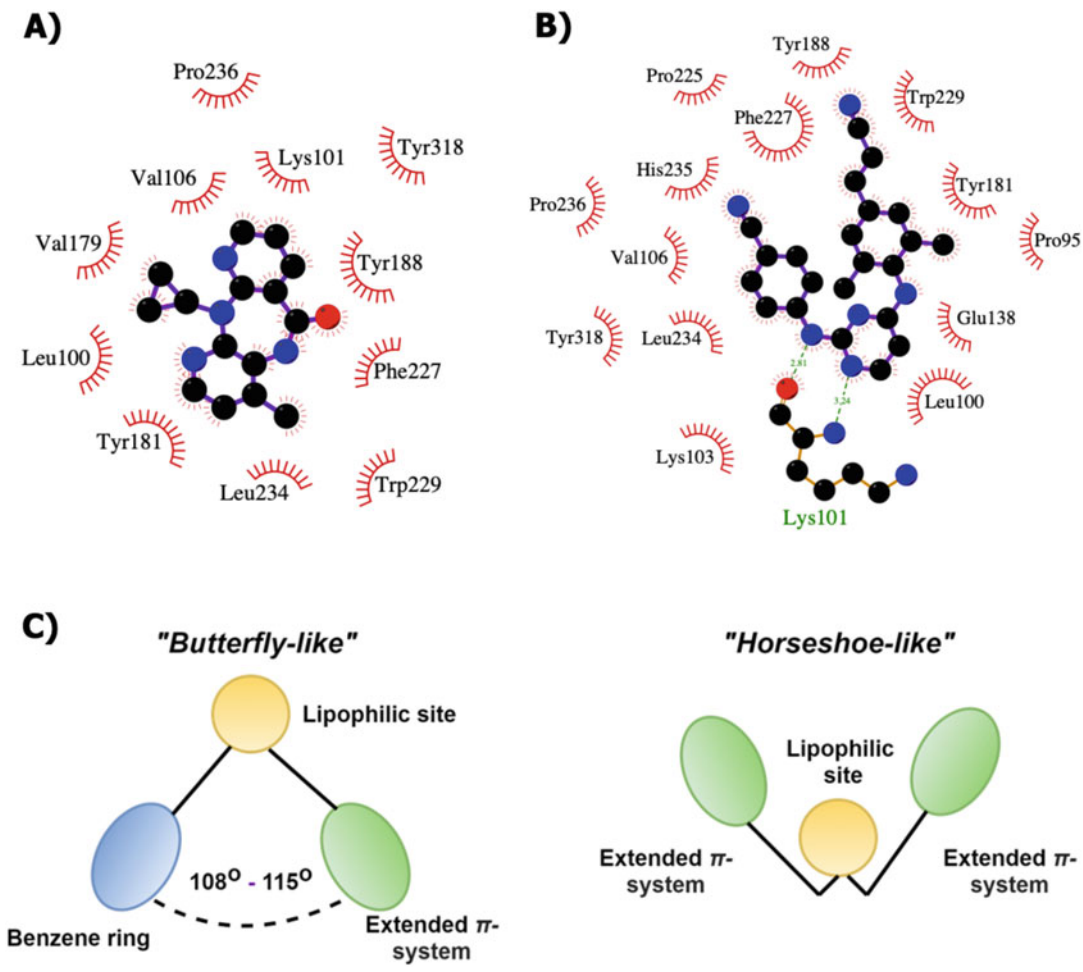

Fig. 3.5 Interaction diagram and schematic representation of NNRTI drugs. (**a**) The 2D interaction diagram of nevirapine (4PUO), (**b**) rilpivirine (4G1Q) positioned in the NNRTI binding pocket, and (**c**) illustration of "butterfly-like"- or "horseshoe"-shaped conformation of NNRTIs

H-bonding interactions at the binding pocket of RT (Fig. 3.5a, b) [52]. The NNRTIs adopts a "butterfly-like"- or "horseshoe"-shaped conformation (Fig. 3.5c) based on the arrangement of the chemical linkers and aromatic rings. The NNRTI binding pocket largely constitutes the p66 subunit of RT. The hydrophobic pocket of HIV-1 RT encloses several aromatic and lipophilic amino acid residues Y181, Y188, F227, W229, Y318 P95, L100, V106, V108, V179, L234, and P236 from the p66 subunit which stabilizes hydrophobic interactions including van der Waals (L100, V106, V179, Y181, G190, W229, L234, and Y318 residues), electrostatic forces (K101, K103, and E138 residues), and π–π interactions (Y181, Y188, W229, and Y318 residues) with NNRTI. In addition, K101 is also shown to form a hydrogen bond with several NNRTIs. Further, E138 of the p51 subunit contributes toward NNRTI binding [53–55].

Crystallographic studies showed that a novel site of RNase H is located ≥50 Å away, between the NNRTI-binding pocket and the polymerase active site of RT (Fig. 3.3a) [56, 57]. It contains Mg^{2+} ions that form a coordinated complex with the four conserved carboxylates, comprising of

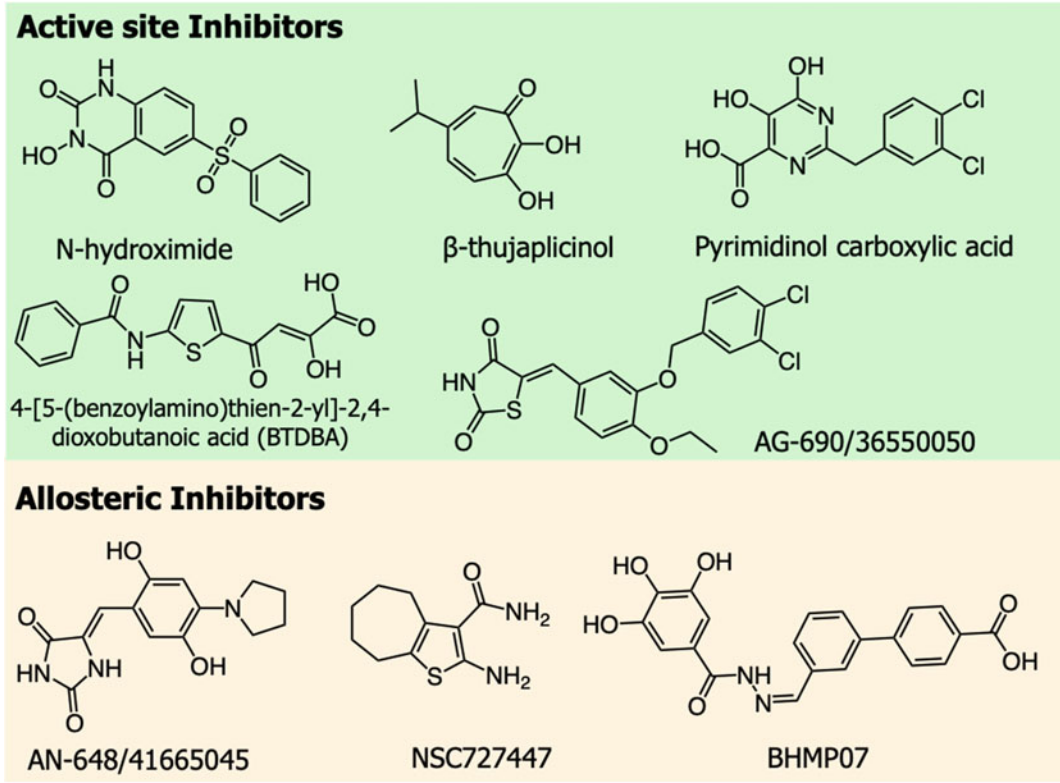

Fig. 3.6 Chemical structures of active site and allosteric RNase H inhibitors

negatively charged amino acids D443, E478, D498, and D549 (DEDD motif). In addition, the existence of histidine residue also contributes to the RNase H catalytic efficiency. If the divalent metal ion doesn't bind favorably to the active site of the RNase H domain, then there will be reduced or no catalytic activity [58]. Numerous X-ray crystal structures of RNase H inhibitor complexes have been deposited in the PDB. The reported structures are either RNase H inhibitors in complex with full-length RT or the isolated HIV-1 RNase H domain, including one allosteric inhibitor and several RNase H active site inhibitors. For instance, N-hydroxy quinazolinediones, hydroxylated tropolones (-β-thujaplicinol), manicol, dihydroxycoumarins, N-hydroxy thienopyrimidine-2,4-diones, and galloyl forms coordinate bonding with the metal ions at RNase H active site and also consequently contributes to hydrogen bonding/ionic and edge-to-face π–π stacking interactions with H539 residue [59–63]. Recently, Kirby et al. identified 2-hydroxyisoquinoline-1,3-dione which blocks polymerase activity as both noncompetitive inhibitor ($IC_{50} = 2.6$ μM) and competitive inhibitor of RNase H functions ($IC_{50} = 0.65$ μM) of RT through binding at the RNase H active site in multiple conformations (PDB ID: 5UV5). This data was confirmed by X-ray crystallography, nuclear magnetic resonance (NMR) analysis, and molecular modeling approaches. In both RNase H-active sites, 2-hydroxyisoquinoline-1,3-dione is involved in chelation of the active site metals and also directly engaged in conserved residue H539 by forming an H-bond [64]. One of the most potent classes N-acyl hydrazone (NAH) analog dihydroxy benzoyl naphthyl hydrazone (DHBNH) as HIV-1 RT RNase H inhibitor effectively inhibits the HIV-1 RNase H activity with an IC_{50} of 0.5 μM. The co-crystal structure of HIV-1 RT with the (NAH) analog was resolved by Himmel et al. at 3.15 Å resolution. DHBNH

binds ≥50 Å far from the RNase H active sites, oriented with its benzoyl ring partially toward non-nucleoside RT inhibitor (NNRTI) binding pocket residues of W229 and Y188, and the naphthyl ring system appears to be located near the polymerase active site and the polymerase primer grip (PDB ID: 2I5J) [65].

Structure-guided screening coupled with similarity search using Specs database was used to identify new scaffolds as HIV-1 RNase H inhibitors. Interestingly, the molecules identified form multiple interactions with RNase H domain through Mg-dependent/Mg-independent mode of actions. Among the identified molecules, thiazolidinediones showed potent inhibition with an IC_{50} of 5.1 μM (AG-690/36550050, Fig. 3.6) and exhibited an allosteric inhibition. However, another structurally similar compound AN-648/41665045 (Fig. 3.6) with an IC_{50} of 9.35 μM slightly showed increased absorbance in the UV–Vis spectrum from Mg^{2+} titration experiment. In addition, other compounds showed IC_{50} values <10 μM against HIV-1 RNase H enzyme. Structural studies reveal that the most active compound forms polar interactions with R557, highly conserved RNase H primer grip residue of N474, and the wide-ranging hydrophobic interactions at the site A502, K503, and W (406, 426, and 535) residues [63].

Over the last decade, many structurally diverse scaffolds of HIV-1 RNase H inhibitors have been developed, but none of the compounds entered clinical trials as agents specifically inhibiting the ribonuclease H (RNase H) function of RT (Fig. 3.6). The clinical drawback of the active site-directed inhibitors leads to nonspecific metal chelation, which exerts higher cellular toxicity and limited cellular permeability. So far, all the clinically USFDA-approved RT inhibitors/drugs target only against the polymerase activity of reverse transcriptase enzyme, not on the RNase H domain.

3.2.6 Resistance to RT Inhibitors

The early successes of these HIV-1 RT classes of inhibitors have gradually been susceptible to the variants leading to drug resistance. The mutants K65R, L74V, Q151M, or M184V at several positions have led to a reduction in affinity specific NRTIs toward RT, with little or no change in the affinity toward corresponding dNTP substrate. Mutations enhancing primer unblocking activity including those selected by zidovudine (ZDV) and stavudine (d4T) are known as thymidine analog mutations (TAMs): M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E [66–68].

Frequently, mutations are observed in the NNRTI binding pocket (NNIBP) of RT, including L100I, K103N, V106A, Y181C, Y188L, and G190A, during the treatment of first-generation NNRTIs against the viral strains. The most widespread NNRTI-resistant mutations are K103N, Y181C, and Y188L, which initiate steric hindrances, electrostatic dissimilarity, and elimination of significant π–π stacking interactions between NNRTIs and the pocket residues [69–71]. Steric conflict of the methyl side chain and a bound NNRTI develops G190A mutation. The K103N mutation appears to affect the kinetics of the inhibitor-binding process by stabilizing the unbound state of RT. In the second-generation NNRTIs, etravirine (ETR, also known as TMC125) and rilpivirine (RPV, also known as TMC278) are designed with conformational flexibility and positional adaptability to overcome the drug-resistant RT mutants. Both the second-generation drugs exhibit potent antiviral inhibition against wild-type (WT) HIV-1 as well as HIV-1 variants, displaying significant resistance to first-generation NNRTIs. However, the following mutations, namely, V90I, L100I, K101E/P/T, V106A/I, V108I, E138A/G/K/Q/R, V179F/I/L, Y181C/I/V, Y188I, G190E, H221Y, F227C/L, and M230I/L, decrease the effectiveness of ETR and RPV against these mutant viral strains [72, 73]. Doravirine exhibited potent antiviral inhibition against the K103N, Y181C, and K103N/Y181C mutants than those with RPV and EFV against other prevalent NNRTI-associated mutants, with the exception of Y188L and V106A (Fig. 3.7) [74].

3.2.7 Integrase Inhibitors

Integrase strand transfer inhibitors (INSTIs) act by blocking the integration of viral DNA into the

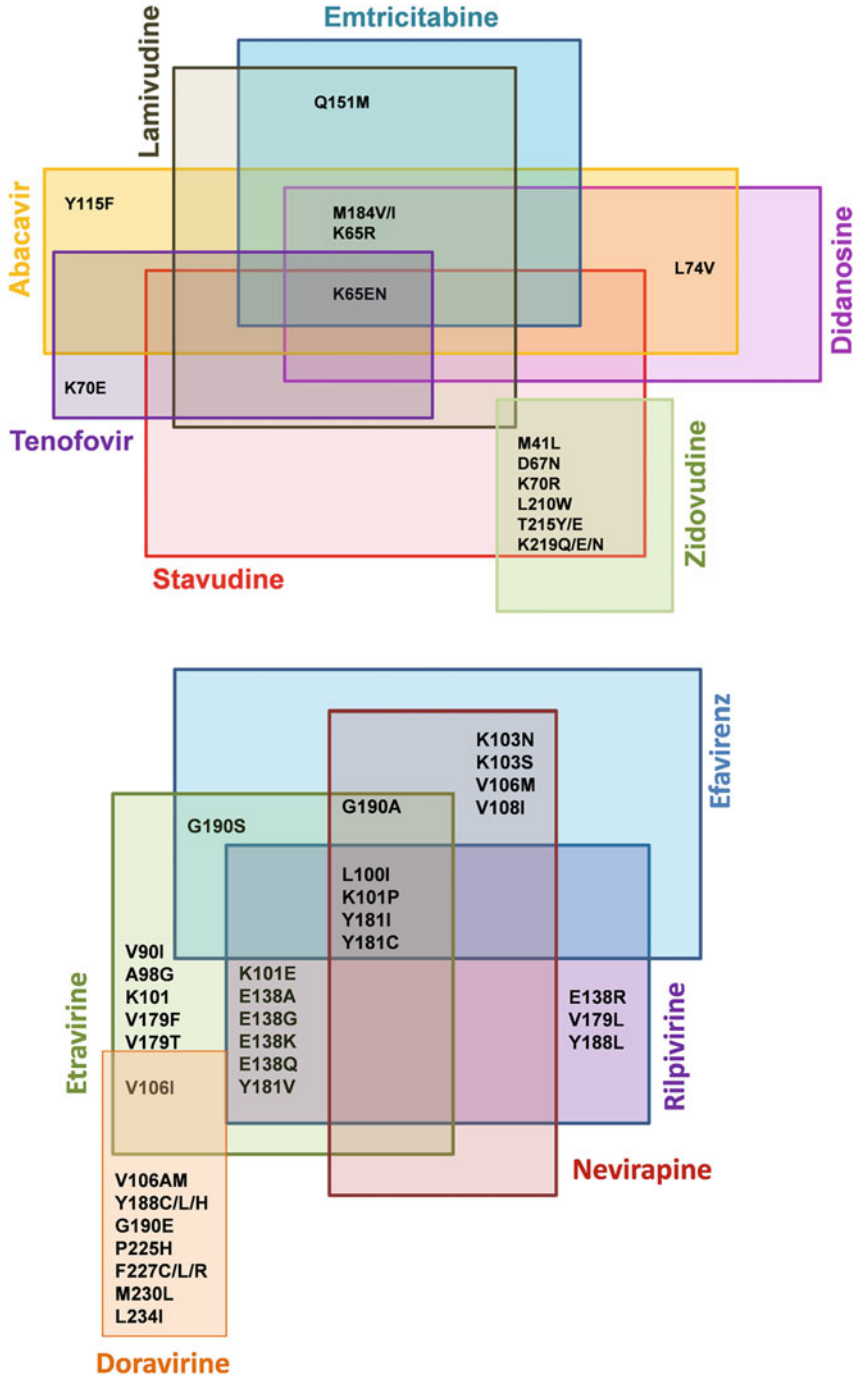

Fig. 3.7 Venn diagram showing the most common clinically significant resistance mutations for NtRTI- and NNRTI-approved drugs

host genome, thereby preventing the strand transfer reaction in the target cell [75]. The retroviral integration is accomplished by a two-step process: the first step is the removal of a dinucleotide from each of the 3′-end of the long terminal repeat (LTR) of the viral DNA which occurs by binding of integrase first to a short sequence located at either end of LTR. Further, the second step,

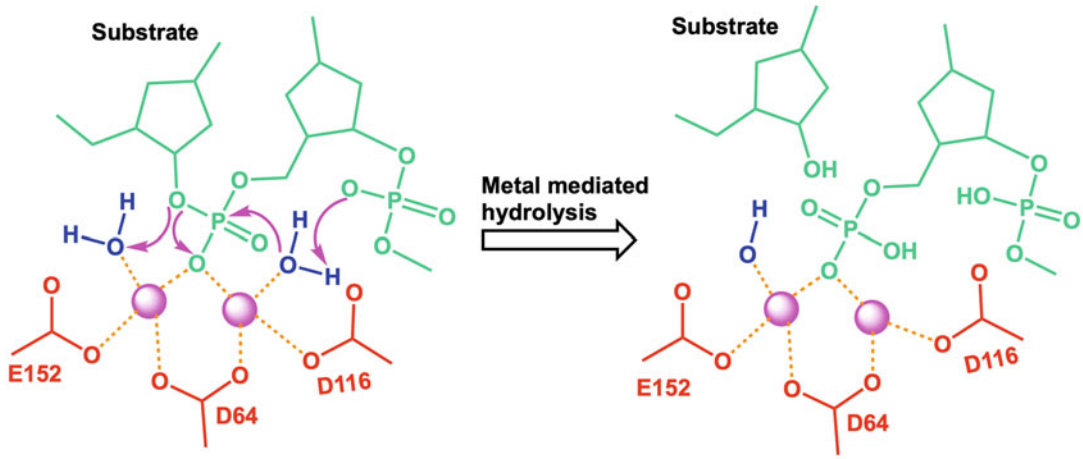

Fig. 3.8 Schematic representation of metal-mediated hydrolysis mechanism of substrate by integrase enzyme with the key amino acids involved in the hydrolysis process

transfer of DNA strand, occurs by a pair of phosphodiester bonds in the target DNA, attacked with the hydroxyl groups at the 3′-end of the processed viral DNA. Further, intermediate integration undergoes elimination at 5′-end of the viral DNA resulting in the exclusion of unpaired nucleotides, followed by DNA polymerase and ligation, filling the single strand gaps between viral DNA and host which are carried out by cellular enzymes [76, 77]. The hydrolytic catalytic activity of the IN enzyme is carried out by conserved DDE carboxylate residues D64, D116, and E152 that coordinates with the Mg^{2+} metal ions and water molecules (Fig. 3.8).

The integrase enzyme has become an attractive target in drug development and is extensively used in clinical practice for the treatment of HIV infections. Raltegravir (RAL, licensed 2007) was the first approved integrase inhibitor for the treatment of HIV infection by the US Food and Drug Administration (FDA) [78]. In 2012, Elvitegravir (EVG) was approved for clinical usage in the AIDS treatment [79]. The first-generation INSTIs, RAL and EVG, are clinically less useful as these drugs have a relatively low barrier for resistance development. In addition, RAL has problems with dosing, requiring a higher dose of 400 mg twice daily (BID), while EVG needs the use of a pharmacokinetic (PK) boosting agent such as ritonavir or cobicistat to inhibit CYP3A4, which is the primary cytochrome responsible for the metabolism of EVG. However, it is susceptible to widespread cross-resistance with RAL. To combat the rapidly emerging resistance to the first-generation INSTIs, newer second-generation compounds were developed and approved as bictegravir (BIC), dolutegravir (DTG), and cabotegravir (CAB) in the year 2013, 2018, and 2020, respectively. Second-generation INSTIs have high potencies, good tolerability, a high genetic barrier to resistance, once-daily dosing without the need of a pharmacological enhancer, and comparatively low cross-resistance with RAL. The structural modification of bicyclic to tricyclic ring on the central pharmacophore scaffold leads to further improvement of the metal chelation for a higher genetic barrier to resistance strains than EVG and RAL. However, second-generation INSTIs (DTG and BIC) are ineffective against the prevalent double-mutant G140S/Q148H as noted in more complex VRV strains [80–82]. Most of the USFDA-approved INSTIs target exclusively the substrate-binding region of the IN active site. Because all drugs share the same mechanism of action, INSTIs experience resistance and cross-resistance issues. Recently, numerous compound developments focused on targeting novel allosteric binding site to overcome resistance and cross-resistance problems,

which has led to the development of allosteric INIs (ALLINIs). Many compounds belonging to diverse classes, such as benzoic acid, 2-(quinolin-3-yl) acetic acid, 3-hydroxypicolinamide, quinoline, N-aryl-naphthylamines, 3-quinolineacetic acid, 8-hydroxyquinolines, flavonoid, etc., were reported as allosteric IN inhibitors [83]. ALLINIs reduce the protein–protein interactions of HIV IN and its essential cellular cofactor lens epithelium-derived growth factor (LEDGF/p75) by binding to an allosteric site topologically separate from the catalytic site. In this way, ALLINIs make the IN turn out to be catalytically inactive that is termed as abnormal IN multimerization [84–87].

3.2.8 Crystal Structure and Ligand-Binding Site of Integrase

The HIV-1 integrase enzyme is 32 kDa proteins that are encoded by *pol* gene C-terminal part of the HIV-1 genome. Until 2008, only partial retroviral integrase structures (25) were available in the Protein Data Bank (PDB). In 2009, the first full-length integrase from the prototype foamy virus (PFV) in complex with its cognate DNA was deposited in the PDB by academic researchers (PDB ID: 3L2Q) after the FDA approval of raltegravir in 2007 [88]. The 3D crystal structure shows the association and locality of the canonical integrase domains (N-terminal, core catalytic, and C-terminal) and its involvement in the extensive protein–DNA and protein–protein interactions for insertion of viral DNA into a host cell chromosome [89–91]. As of July 2020, more than 46 wild-type and mutant full-length PFV crystal structures were deposited with drugs and other chemicals (PDB ID: 3L2V, 3L2W, 3S3O, 5NO1, 4ZTF, 4BE2, 3OYJ, etc.) for modeling of the HIV-1 integrase and the development of next-generation strand transfer inhibitors [92–96].

HIV-1 IN enzyme is a 288-amino-acid protein with a size of 32 kDa and consists of three structurally independent functional domains as defined by NMR and X-ray crystallography methods. It comprises the N-terminal domain that includes 1–49 amino acids and contains two histidine (H12 and H16) and cysteine residues (C40 and C43), all of which are conserved and form an HHCC zinc-finger motif involved in the stable folding and proper multimerization of the integrase subunits. The catalytic core domain is the central active site of IN, which contains 50–212 amino acids. There are three negatively charged conserved amino acids; D64, D116, and E152 present at the catalytic core domain. The divalent metal ion (Mg^{2+}) forms a coordinate bonding with these amino acids forming a motif that is required for the catalysis of integration. In catalytic core domain, flexible loops are formed by varied five β-sheets and six α-helices that allow conformational changes for 3′-processing of the viral DNA and strand transfer in the integration reaction. The C-terminal domain has 212–288 amino acids, involved in binding with viral and cellular DNA, nonspecifically required for IN 3′-processing and strand transfer activities during interaction with other domains of N-terminal and core catalytic domains. The active sites of the integrase enzyme defined in the 3’--processing reaction involving residues are D64, C65, T66, H67, E92, N120, F121, and D116 and strand transfer cavity committing residues of Q62, I141, P142, Y143, Q148, I151, E152, N155, K156, and K159.

INSTIs compete with host DNA binding into the catalytic core domain of integrase. INSTIs have two common groups, namely, halogenated benzyl and three coplanar oxygen atoms. The crystal structures of PFV IN complex with the RAL and EVG at the active site showed that the β-hydroxy ketone moiety forms chelation with divalent Mn^{2+} metal ion that is coordinated by the DDE motif of the IN active site, thereby impeding their involvement in the DNA strand transfer reaction. The halogenated benzyl moiety blocks the catalytic DDE triad activity through interactions with the penultimate viral DNA G·C base pair and a 310 helix in IN (P145–Q146 in HIV-1 IN), ejecting the viral 3′-dA (with its associated 3′-hydroxyl nucleophile) from the active site [97, 98]. RAL has additional contacts with N117, Y143, N144, and P145 residues, but the EVG makes only one additional contact with

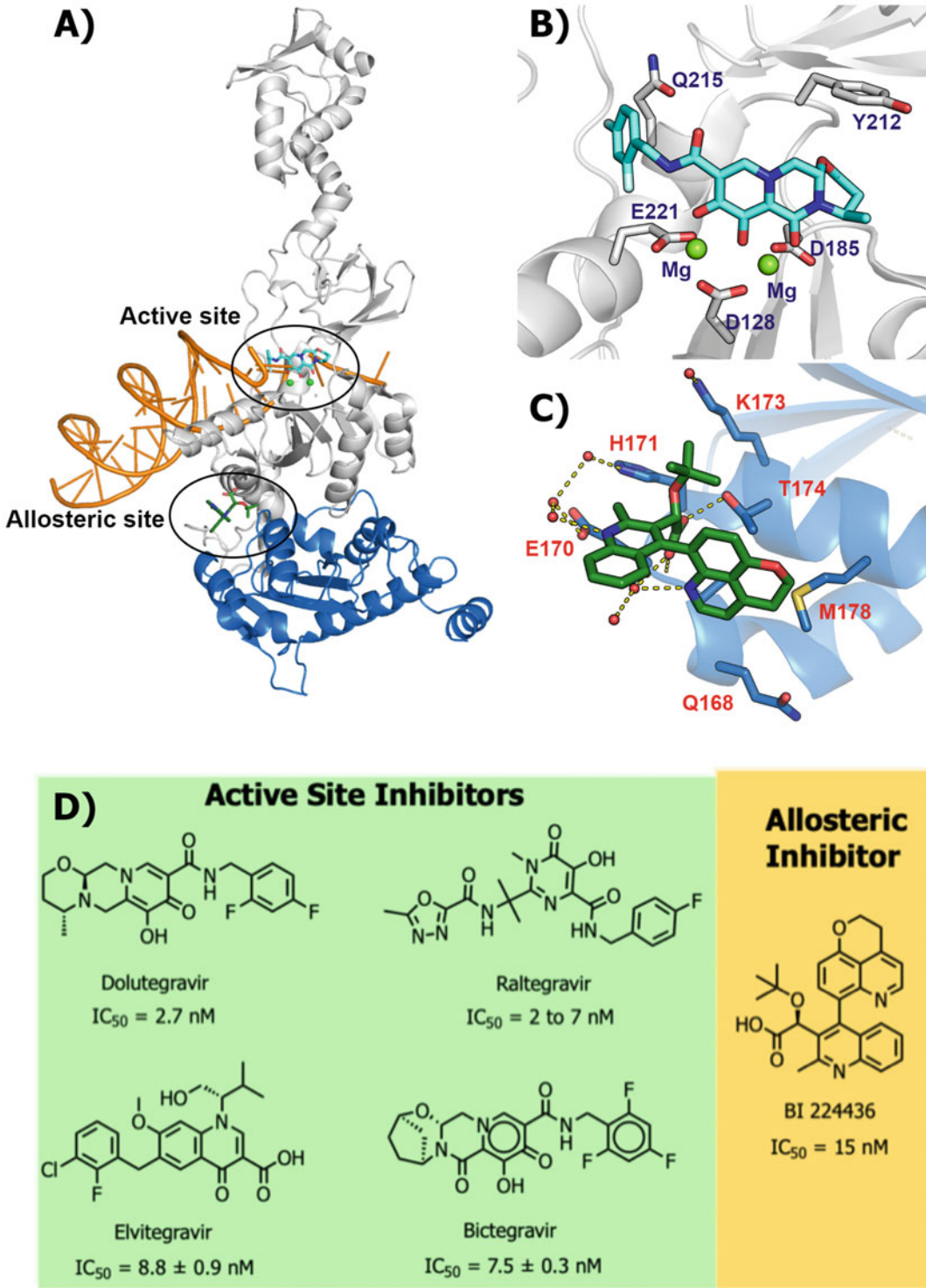

Fig. 3.9 Integrase structure and inhibitors approved and in clinical development. (**a**) Crystal structure of the retroviral prototype foamy virus intasome depicting the IN domains in complex with raltegravir (PDB ID: 3OYA) at the active site and the inhibitor, BI 224436, at the allosteric binding site. (**b**) Crystal structure of raltegravir bound to the active site of HIV-1 IN. (**c**) Interaction of BI 224436 with the amino acid residues at the allosteric binding site of HIV-1 IN (PDB ID: 6NUJ). (**d**) 2D representation of IN inhibitors (active and allosteric binding site) and their biological activities

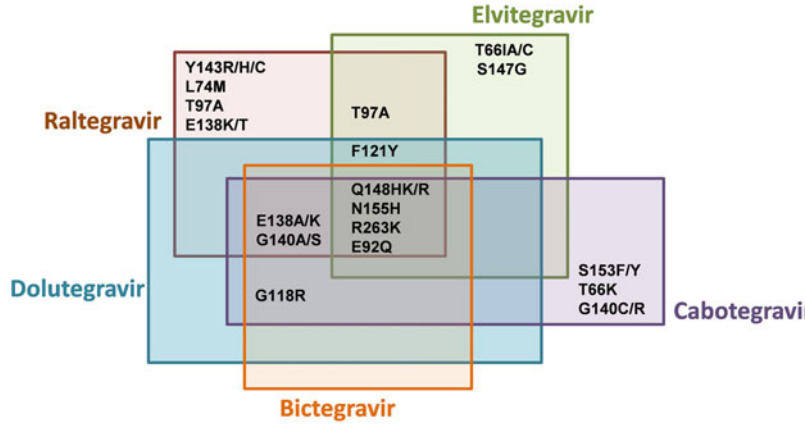

Fig. 3.10 Venn diagram showing the most clinically significant resistance mutations for IN inhibitors as approved drugs

C65. Contacts in residues Q146 and R231 are commonly shared with both drugs. X-ray crystallographic structures of DTG in complex with wild-type and mutant IN have shown that DTG adjusts its conformation and position in the form of well-built bound mode inside the active site, whenever certain resistance mutations appear in the substrate-binding pocket. The DTG makes additional optimal coordination toward the metal cations by the existence of oxygen-derived lone pairs on the rigid coplanar aromatic fraction of the tricyclic ring system. This rigid chelation characteristic in DTG differs from the other drugs of RAL, CAB, and BIC which have elastic linkers, amide oxygen atom that contributes to the chelation of the metal atoms. This allocates the DTG to assemble into the hydrophobic pocket of the IN active site, leading to the stronger interaction between the halogenated benzyl ring and protein interactions [80]. The two-metal chelating scaffold linked with the hydrophobic component of these compounds is essential for inhibition and enhancing the overall affinity along with the specificity of the inhibitor in the integrase DNA complex, respectively. A promising class of new compounds is "allosteric integrase inhibitors" that target a part of the integrase enzyme. The new inhibitors that are currently under investigation prevent the formation of infectious viruses by building integrase protein bunch jointly.

Among several classes of compounds, BI 224436 is one of the first LEDGINs belonging to 3-quinolineacetic acid derivative, which proceeded to the phase IA clinical trials due to its excellent bioavailability, tolerability, plasma levels, pharmacokinetics, and profile. BI 224436 displays an IC_{50} = 15 nM in an LTR-cleavage assay measuring the 3′-processing hydrolysis reaction of a dinucleotide from the DNA 3′-end of each viral long terminal repeat. Moreover, BI 224436 exhibits excellent antiviral potency (EC_{50} = 11–27 nM) in a panel of wild-type and recombinant viruses with different aa124/aa125 variants of IN. Crystallography studies revealed that BI 224436 bind to a noncatalytic site and disrupt chromatin and IN from interacting with LEDGF/p75. It binds to the highly conserved allosteric pocket of the core catalytic domain of IN, where LEDGF binds by modulating IN multimerization and interfering with IN-lens epithelium-derived growth factor (LEDGF)/p75 binding [99, 100]. Koneru et al. solved the X-ray crystal structure of BI224436 bound to IN core catalytic domain (PDB ID: 6NUJ) and illustrated that the carboxylic acid moiety of BI224436 interacts with the backbone amides of E170 and H171 through hydrogen bonding interactions. The ether oxygen of the *tert*-butoxy interacts with the conserved residue side chain of T174. At the core catalytic domain dimer, the hydrophobic regions occupied fully with tricyclic (dihydropyranoquinoline) moiety of BI224436 and also expand toward the caps of the pocket residue of W132. In addition, the V-shaped pocket region has been enclosed and interacts with quinoline moiety of the BI224436 (Fig. 3.9) [101].

Aryl diketo acid (DKA), pyrrolyl DKA, and quinolonyl DKA derivatives were investigated as dual inhibitors against the targets, RT (RNase H domain) and IN (substrate active site). Three main pharmacophore domains are critical for their binding with both the targets, viz., chelator, hydrophobic, and aromatic domains. The following catalytic site residues D64, D116, and E152 for the IN and the residues D443, E478, D498, and D549 for RNase H along with two Mg^{2+} as cofactors coordinated the catalysis of the RNase H and IN enzymes. This bifunctional mode of multi-target inhibition avoids drug–drug interactions, dose complication, and adverse drug events [102].

3.2.9 Resistance to Integrase Inhibitors

In the HIV IN, the most common mutation leading to resistance occurs at the residue position of Y143, Q148, and N155 (Y143H/R/C, Q148H/R/K, or N155H). Further, the combination of secondary mutation at position such as G140 (G140S) along with the primary mutation Q148K/R/H drastically increases drug resistance [103–105]. Several primary and secondary mutations arise during raltegravir- and elvitegravir-based therapies. The primary raltegravir-associated mutations are Y143R, Q148H/K/R, and N155H, and secondary mutations are L74M, E92Q/A, T97A, E138K/A, G140S/A, Y143C/H, V151I, K156N, E157Q, G163K/R, T206S, and D232N. The primary elvitegravir-associated mutations are T66I, E92Q, S147G, Q148H/K/R, N155H, H51Y, T66K/A, L68I/V, S119R/G, E138K, G140S/C, E157Q, K160N, R166S, E170A, S230R, and D232N. The residues mutated in the conserved area of protein stability, multimerization, DNA binding, and catalytic activity make the inhibitors less active [36, 106]. In phenotypic assays, DTG retains full or partial inhibitory activity against RAL- and EVG-resistant HIV variants including T97A + Y143C/R, N155H, G140S, and Q148H and G140S + Q148H, T92A + G140S + Q148H, and G140S + Q148H + G163R (combinations of mutations) [107]. The mutations G140S + G147S + Q148K were highly resistant to CAB, EVG, and RAL but vulnerable to DTG and BIC (Fig. 3.10). To all five INSTIs, L74M + G140S + S147G + Q148K combination mutation was associated with high resistance [108].

3.2.10 Protease Inhibitors

Therapeutic inhibition of HIV-1 protease is an attractive target enzyme in HIV therapy and plays a critical role in catalytic hydrolysis of the *gag* and *gag-pol* gene into essential viral proteins required for the formation of new virulent virions [109]. In 1995, saquinavir was the first protease inhibitor to be approved by the USFDA. Since then, nine protease inhibitors were approved by the FDA and are available in the market for HIV treatment [110]. The first-generation protease inhibitors include saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir. All five of the first-generation HIV protease inhibitors were based on a non-cleavable peptidomimetic scaffold containing hydroxyethylamine, hydroxyethylene, and dihydroxyethylene cores presented to the catalytic active site. The limited use of first-generation PIs in the clinic was due to its peptidic nature resulting in high metabolic inactivation, low half-life with bioavailability, high pill burdens, GIT adverse reactions, and the emergence of drug-resistant strains of HIV [111]. Various research efforts developed to address these issues led to the development of second-generation PIs, namely, lopinavir, atazanavir, fosamprenavir, tipranavir, and darunavir. There are currently ten FDA-approved protease inhibitors on the market for HIV treatment (Fig. 3.11). Lopinavir is available as a combination pill with ritonavir to boost the pharmacokinetics [112]. After that, atazanavir became the first protease inhibitor to be an effective once-daily dosed regimen, approved in the year 2003 [113]. Tipranavir was approved in late 2005 used in salvage therapy and extension to pediatric use [114]. Darunavir is one of the most recently FDA-approved drugs (2006) that exhibits potency against multidrug-resistant strains of

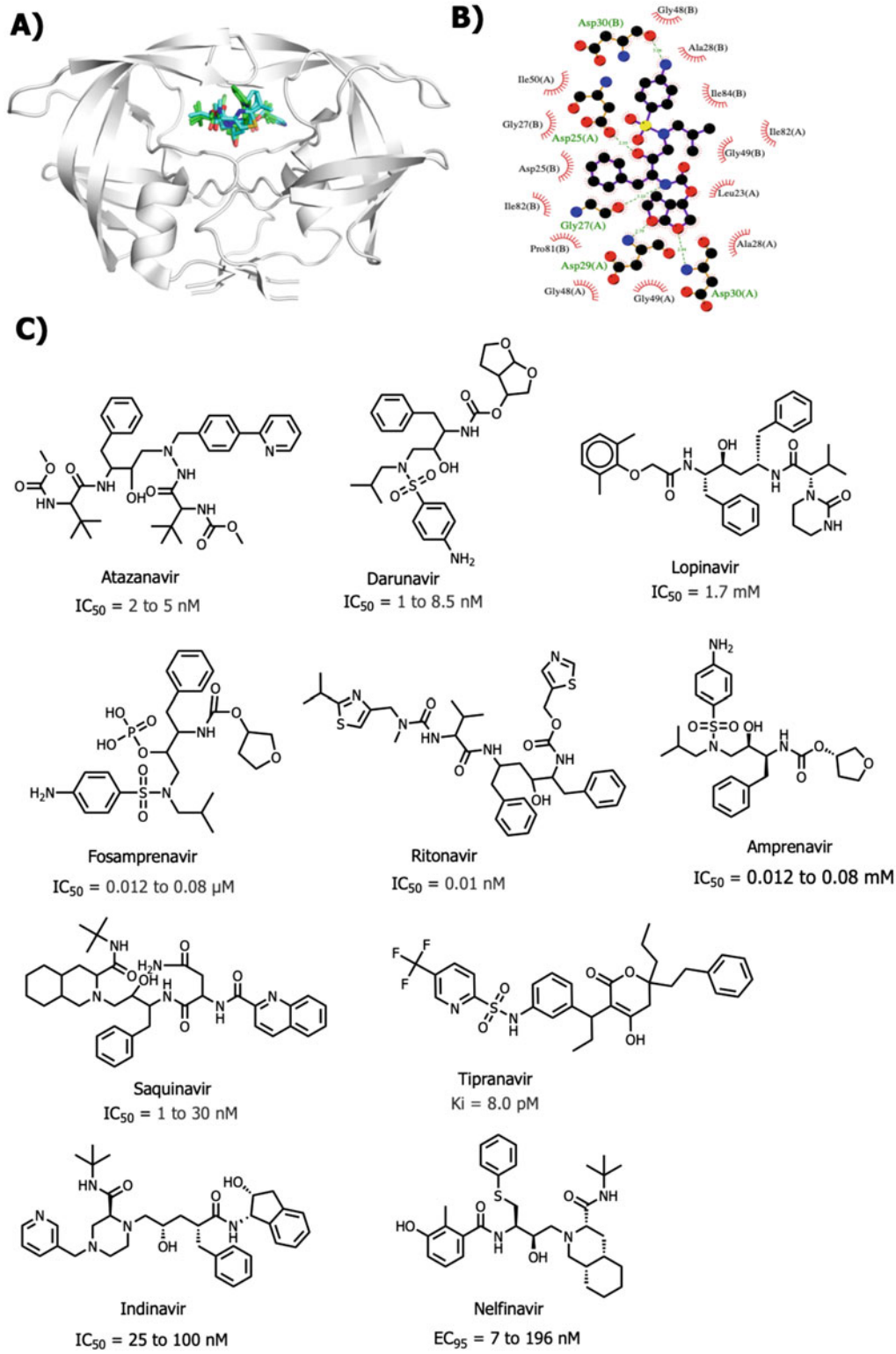

Fig. 3.11 The structure of HIV-1 protease enzyme and their inhibitors with biological activity. (**a**) The crystal structure of the HIV-1 protease mutant V82I with different subunits in complex with protease inhibitors in the binding pocket. (**b**) Protein–ligand interaction for darunavir with the important amino acid residues in the binding pocket (PDB ID: 6DH3). (**c**) 2D structures of protease inhibitors of HIV-1 along with their biological activity

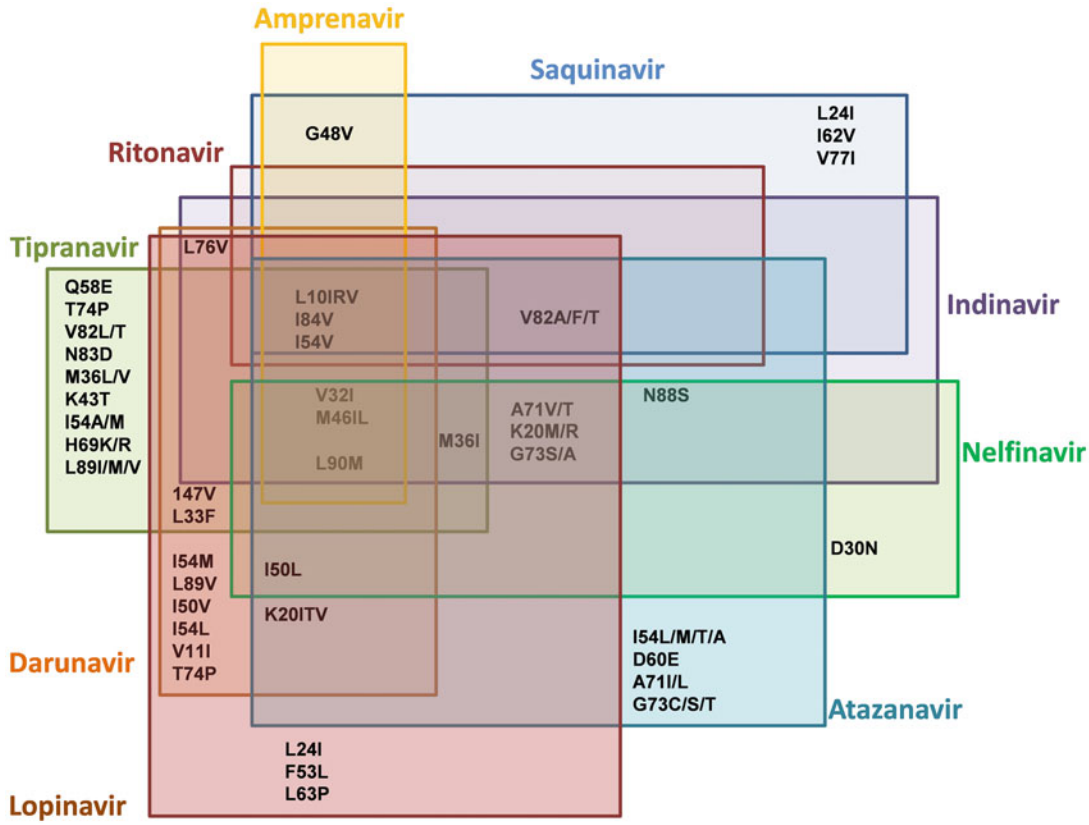

Fig. 3.12 Venn diagram showing the most common clinically significant resistance mutations for protease inhibitors as approved drugs

HIV with a high genetic barrier for the development of resistance in preclinical studies, and also it is used for the treatment of pediatric and young patients [115].

3.2.11 Crystal Structure and Ligand-Binding Site of Proteases

The first PDB structure of synthetic HIV-1 protease enzyme was determined in 1989 at 2.8 Å resolution and was based on the Rous sarcoma virus protease (PDB ID: 3HVP, 4HVP, and 7HVP) [109, 116, 117]. After 4 years (1993), the crystallographic structure of the protease from HIV-2 was reported (PDB ID: 1IVQ, 1IVP) with two synthetic peptidic transition state analog inhibitors [118]. To date (1993–2020), more than 700 HIV-1 proteases were deposited in the PDB. All the deposited crystal structures of HIV-1 protease complexes were primarily made with substrate-based peptidomimetic inhibitors/drugs and few small organic molecule inhibitors (PDB ID: 2NNP, 3CYW, 3EL9, 3EL0, 1G2K, 1XL5, 1XL2, 2HS2, etc.) targeting against wild-type and 22 different multidrug-resistant mutants (L24I, I50V, V82A, A71V, V82T, I84V, V32I, and G73S) in HIV-1 protease enzymes.

Protease is composed of two 99-amino-acid subunits. Two common catalytic sequences Asp-Thr-Gly (D25, T26, and G27), one from each subunit, meet together to form a long tunnel

catalytic active site of the enzyme. On top of the active site, the flap region is formed by two flexible glycine-dense β-sheets. Two flexible protein "flaps" experience conformational shift to close over the active site up to 7 Å when the enzyme is bound to a substrate. The native substrate containing a minimum of seven amino acid residues binds to the enzyme in the form of extended conformation denoted by standard nomenclature subsite of P4 to P1 and P1′ to P4′. The hydrolysis of the amide bond at P1 and P1′ residues by the protease is highly specific. Each subsite has a preference for specific types of side chains that can be accommodated. The S1, S1′, S3, and S3′ subsites prefer only hydrophobic residues, while the S2 and S2′ subsites can accommodate both polar and hydrophobic side chains [119, 120]. Direct inhibitor reveals stronger hydrogen bonds (average length of 3.11 Å) with the flap regions (residues 48−50), over the other rigid active site consisting of residues 25−29 [121]. PR inhibitor competes with the substrate and binds to the active site and causes inappropriate cleavage of protein polypeptides which results in an inviable and ineffective HIV virion formation. The crystal structure of saquinavir bound in an extended conformation against the HIV protease binding pocket through the transition state hydroxyl group is positioned between the catalytic aspartic acid residues D25 and D25′. The structural fragments of saquinavir comprise of decahydroisoquinoline, *tert*-butylamide, P1 phenyl, and quinadyl moieties that occupy S1′ (flap region contact), S2′ (polar contacts), and S1 (hydrophobic contacts) pockets in addition to the S3 subsite. The P2 carboxamide forms H-bonding interactions with the backbone amide of D29 and D30 [122].

The X-ray co-crystal structure of HIV protease–nelfinavir illustrates the phenylthio group partially buried in the S1 binding site, which further extends toward the S3 site. The hydrophobic S1′ pocket is occupied with lipophilic dodecahydroisoquinoline ring moiety, and the catalytic aspartates of the enzyme interact with the central hydroxyl group. The 3-hydroxy-2-methylbenzamide group adopts a non-planar conformation allowing the phenolic hydroxyl and NH of benzamide group to form a hydrogen bond interaction with the D30 and G27 residues in the S2 pocket. The carboxamide -NH of the *tert*-butyl group interacts with the NH of D29′ via a bridged water molecule at the S2′ subsite [123].

The structurally different amprenavir binding to the enzyme reveals that the main interaction with the flap water preserves by aminobenzene-sulfonamide attaining a precise conformation of the SO_2–N bond. The isobutyl chain and the arene ring system are engaged in the S1′ and S2′ hydrophobic binding regions, respectively. The tetrahydrofuranyl oxygen atom interacts with hydrogen bond donors of the main chain D29NH and D30NH with distances of 3.50 Å at the nonprime side of the binding site of the enzyme [124]. Vincent Stoll et al. described the crystal structure of ABT-378 (lopinavir), bound to the active site of HIV-1 protease. The cyclic urea carbonyl forms two bidentate hydrogen bonds from the backbone and side chain of the D29 residue of the protease. The isostere amidic core NH interacts with the carbonyl oxygen of G27 and G27′ residues. The two phenyl groups at P1 and P1′ position into the S1 and S1′ hydrophobic subsites. In the flap region, bridging interactions occur between two carbonyl amide fragments and the NH bonds of I50 and I50′ residues [125].

The crystal structure atazanavir-bound protease revealed lipophilic phenyl at P1 and pyridylbenzyl at P1′ residue in the S1 and S1′–S3′ hydrophobic subsites, respectively. An intense hydrogen bonding network exhibits between the *tert*-Leu N-carbamate moieties with protease backbone skeleton. Similarly, the terminal urethanes (carbonyl and NH component) form several hydrogen bonding interactions with the amide NH of the D29 and D29′ residues and also in the carbonyl oxygens of G48 and G48′ residues in the protease flap region. In the S2 and S2′ subsites, they are completely integrated with two *tert*-butyl bulky groups of the *tert*-Leu moieties. The carbonyl piece in two *tert*-Leu is gathering hydrogen bonds in association with a water molecule that arbitrate binding toward the Ile50 and Ile50′ residues of the flap region [126]. Analysis of the X-ray crystal structure of

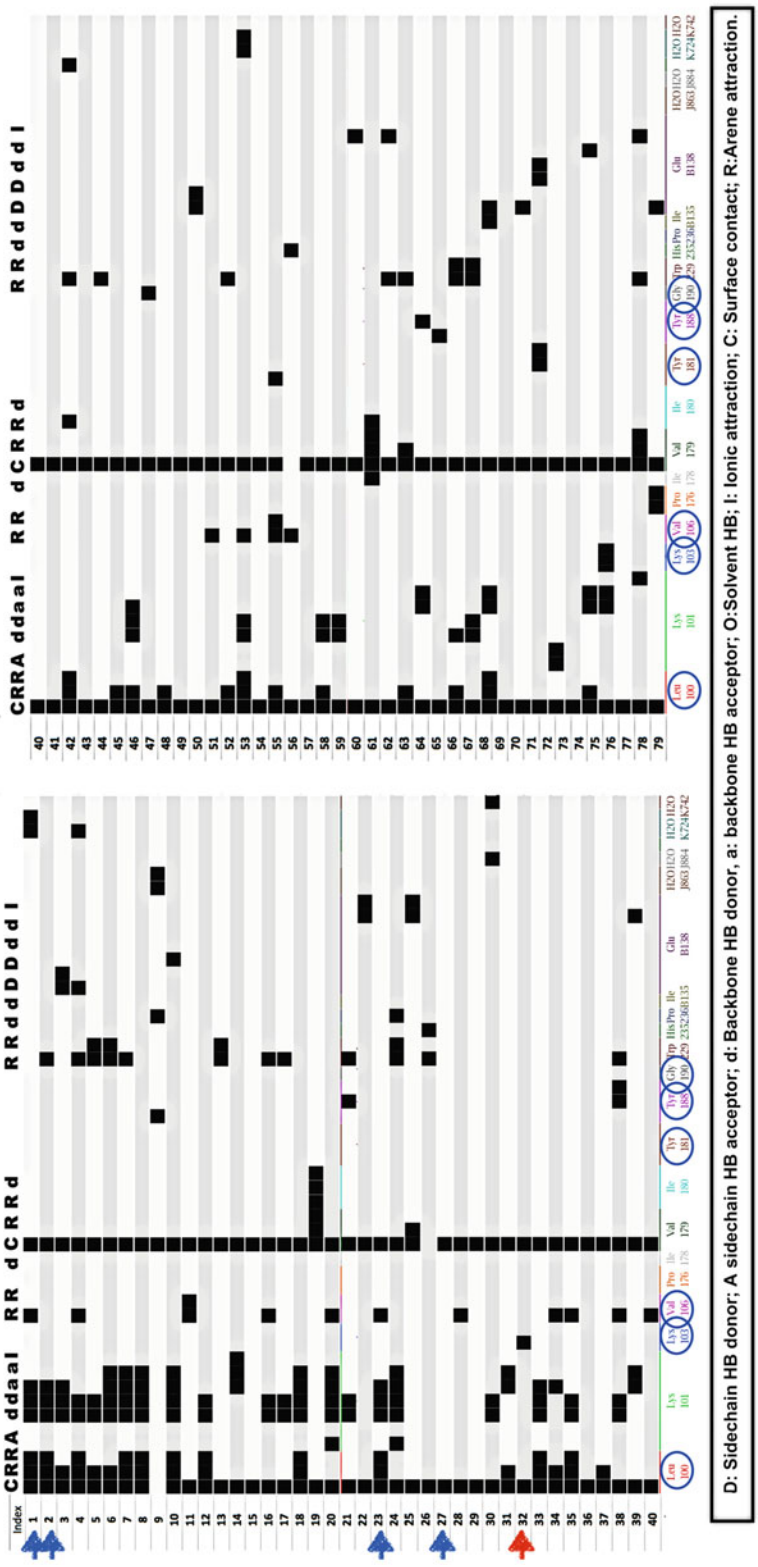

Fig. 3.13 The protein–ligand interaction patterns of 65 series of NNRTIs in the binding pocket of HIV-1 RT enzymes. The important amino acid residues in the binding pocket and the different types of interaction formed between the NNRTIs and the amino acid residues are shown

the non-peptidic tipranavir-bound protease complex revealed that several hydrophobic moieties such as phenethyl and propyl groups occupy within the S1′ and S2′ subsites, ethyl and aromatic groups at C3a lay in the S1 and S2 subsites, and arylsulfonamide fragment occupies region traversing from S2 to the S3 subsite of the protease. The 4-hydroxy group and carbonyl group at pyrone ring interact with two catalytic residues of D25 and D25′ and flap region backbone NH amides of I50 and I50′ utilizing four strong hydrogen bonding contacts, respectively. Two key hydrogen bonds involved between sulfone oxygen atoms with the Asp30 NH backbone amide and Gly48 amide NH are mediated by a water molecule. The trifluoromethyl-substituted pyridine nitrogen also accepts a hydrogen bond from the D29 amide. The outstanding antiviral activity and resistance profile of tipranavir are due to the several H-bonding network and numerous hydrophobic interactions which occurs within the subsites of protease enzyme [127].

Yunfeng et al. reported the X-ray crystal structure darunavir complexed with HIV-1 protease at high resolution (1.3 Å), providing detailed insight into darunavir binding [128]. The free OH group of the isostere forms two hydrogen bonding contacts with the D25/D250 catalytic dyad. Three close-up hydrogen bonds take place between the P2 bis-THF ligand's ring oxygens and the backbone NHs of D29 and D30 in the S2 subsite. In the S2′ subsite, hydrogen bonds are observed between the NH_2 group of the P'2 sulfonamide and backbone residues D30′s NH and with carboxylate side chain D30′. The urethane NH of darunavir forms hydrogen bonds with the carbonyl of G27 in the S2 subsite. Additionally, there is a tetracoordinate water-mediated interaction formed by the backbone NHs of I50/I50′ residues which donate hydrogen bonds to DRV urethane carbonyl, while one of the sulfonamide oxygen accepts the hydrogen bonds from the water molecule. These interactions are preserved in a majority of other HIV-1 protease–inhibitor complexes. The rationalization of DRVs with excellent resistance profile is because of the highly conserved nature of the hydrogen bonding with the backbone atoms appearing in the protease active site. Subsequent analyses of ultra-high resolution (0.84 Å) X-ray crystal structure DRV-bound mutant HIV proteases have recently uncovered the structures of TMC114 bound at two distinct sites, one on the surface of the flexible flaps in the PR dimer and the another one in the active site cavity [129, 130]. All the HIV protease inhibitors except tipranavir contain a central transition state isostere consisting of a hydroxy ethylene scaffold, which mimics the transition state of the enzyme's catalytic cleavage. In the binding site, the hydroxyl group on the core motif forms a hydrogen bond with the carboxylic acid on the D25 and D25´ residues in the binding site (Fig. 3.11).

3.2.12 Resistance to Protease Inhibitors

There are two types of HIV-1 protease mutations (primary and secondary mutations) that generally reduce the binding affinity of PIs. Primary mutations take place at the substrate-binding site of the enzyme, specifically at regions 25−32, 47−53, and 80−84, whereas secondary mutations are located away from the active site and are usually compensatory mutations to downgrade the detrimental effects of primary mutations on binding to the protease's natural substrate [131–133]. Primary mutations directly alter the binding pockets by influencing the favorable binding of PI; contrastingly, substrate-binding remains unaffected, thereby retaining the natural proteolytic activity. The effects of the active site double mutation V82F/I84V reduce the Van der Waals contacts of PIs resulting in modification of the site of the binding pocket to an unfavorable conformation for PIs [134]. A total of 45 mutations were observed in the monomer of HIV-1 protease which is 99 residues long. Among these, only 11 mutations have been considered as primary mutations, and the remaining mutations come under secondary mutations (Fig. 3.12). These secondary mutations are situated at the dimerization interface and in the flap region that changes the shape of flaps (open or closed) to maintain access to the binding

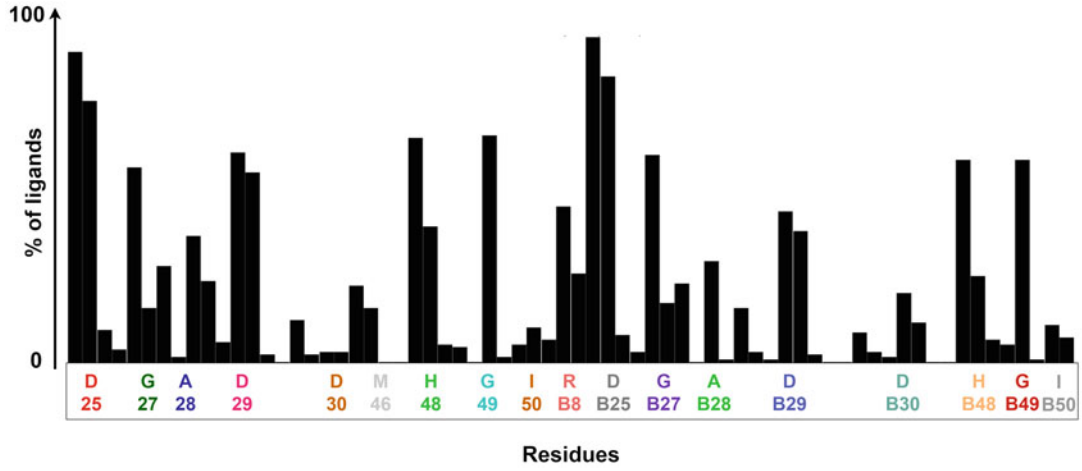

Fig. 3.14 PLIF analysis of protease–ligand complexes. The percentage of ligands from the total of 156 unique inhibitors selected for the analysis interacting with the amino acid residues in the binding pocket of protease are shown

pocket, therefore reducing the capability of the PIs to favorably bind to the active site. For instance, the G48T and L89M mutations alter the binding pattern of saquinavir due to the flap being in a more open conformation. Another common mutation I84V influences the binding of several PIs, including fosamprenavir, indinavir, atazanavir, tipranavir, and darunavir. The substitution of the bulkier amino acid side chain to a relatively smaller size reduces more optimum van der Waals contacts between the PI and the protease, thereby reducing the affinity [135–137].

3.2.13 Budding and Maturation Inhibitors

HIV-1 capsid protein (CA) is an attractive target as it plays a vital function in the replication of virus. CA is expressed in *gag* and *gag-pol* polyproteins and imparts the interactions between the proteins required for virion assembly. Once in the virion, CA is released by the precursor cleavage mediated by HIV-1 protease and self-assembles as a conical capsid comprising of about 250 CA hexamers and 12 pentamers. The capsid formation and its integrity are crucial for further infection of virus. Once a new cell is infected, the interactions with the host cell regulate the intracellular transport and disassembly of the capsid of virus supporting the reverse transcription and proviral DNA integration [138].

HIV-1 CA is made by 1000−1500 copies of the CA monomeric protein assembling into an asymmetric fullerene-shaped cone within the virion which consists of approximately 250 hexamers and 12 pentamers. The CA protomer has two domains, N-terminal domain (residues 1–145) and a C-terminal domain (residues 150−231), both interlinked by a flexible linker. The CA is essential for viral infection process as it is important in viral replication pathway.

CA networks with few host cell factors include CypA (cyclophilin A), NUP153 (nucleoporin 153 kDa), CPSF6 (cleavage and polyadenylation specific factor 6), TNPO3 (transportin-3), and RanBP2 (also known as NUP358), which are critical for infection by viral preintegration complex entry into the nucleus and avoiding the innate immune surveillance. Mutational analysis has shown that abnormal morphologies or altered stability of the HIV-1 CA is detrimental for viral infectivity [139].

3.3 Protein–Ligand Interaction Fingerprint Analysis

The interaction patterns of the ligand in the binding site of protease can be better understood by analyzing the residues in the protein involved in the binding of the protease inhibitors. Protein–ligand interaction fingerprint (PLIF) is one of the valuable tools which analyzes residue interactions with ligands in a high-throughput screening mode. It summarizes the different non-covalent interactions including hydrogen bonds (acceptor and donor), ionic interactions, and surface contacts of ligand–protein complexes and is useful to analyze the binding mode of ligands and important amino acid residues in the binding pocket contributing for the ligand binding. In this chapter, the particular section focuses on PLIF analysis for NNRTI and protease inhibitors, since these two targets are well characterized with a high number of crystal structures and diverse chemical class of compounds, in comparison to other targets including CCR5, RT-nucleoside/nucleotide-binding pockets, and IN active or allosteric binding site.

3.3.1 PLIF Analysis of NNRTIs

PLIF analysis was performed for 65 series of NNRTIs using the PLIF module implemented in Molecular Operating Environment (MOE) [52]. Among them, one highly active compound from each series was energy minimized and docked using the Glide module implemented in Schrödinger, and the first ranked pose was selected for the PLIF calculation. There were a total of 50 interactions between the RT and the selected compounds from the 65 series of NNRTIs which were investigated in this study. The important interactions were surface contact (C), π–π interaction (R) backbone donor and/or acceptor (d/a), ionic interactions (I), and solvent donor and/or acceptor (O) interactions (Fig. 3.13).

The study reveals that the approved drugs like rilpivirine, etravirine, efavirenz, and dapivirine have a similar binding mode which includes π–π interaction between phenyl groups with Y181 and backbone H-bond interaction with K101 (as shown in "butterfly-like"), and the X-ray structures show that the ligand surfaces are exposed to nonpolar residues such as W229, F227, Y188, L234, V318, L100, and V179. Majority of the compounds considered for the calculations showed similar interactions to that of the approved drugs, with the residues including L100, V106, and G190, and restricted interaction with K103, Y181, and Y188 which are common mutants in RT. Lersivirine (ViiV Healthcare (UK), a second-generation NNRTI, showed better mutant resistance property, though the phase II study was withdrawn in 2013 because of pharmacokinetic issues. Loviride (Janssen, now part of Hanssen-Cilag) entered phase III trials and was withdrawn due to poor potency due to incompetence against K103N mutation.

Binding mode analysis suggests that most of the compounds in the class possess a common structural feature. The "body" or the parent nucleus is surrounded by two hydrophobic aryl moieties which are so-called "butterfly-like" conformations. The protein NNIBP contains nonpolar residues such as Y188, W229, Y181, and I180 on one side and charged residues such as K101, K103, L100, E138, and H235 on the other side. Most of the derivatives show no interactions with the mutation prone residues and possess strong interactions with K102, W229, and I135 providing hydrophobic environment. Thus, these derivatives could be potential NNRTI series in the future.

3.3.2 PLIF Analysis of Protease Inhibitors

The abundant source of structural information of proteases in complexes with different ligands including small molecules, peptide, and peptidomimetic inhibitors with more than 700 crystal structures provides a great source of information to understand the binding site and their amino acid residue interaction. However, the protein–ligand complexes ($n = 302$) from

the PDB-bind database were collected, and interaction fingerprint analysis was performed for the co-crystallized ligands using the PLIF module implemented in MOE 2019.01. From the protein–ligand complexes, only one conformation of the ligand was considered, and this resulted in 156 unique inhibitors for the analysis. The result shows the ligand interaction with 18 amino acid residues in the binding pocket of protease (Fig. 3.14). The important amino acid from the binding pocket D25 from chains A and B forms interaction with 134 and 128 inhibitors, respectively, and other contributions are from D29, H28, and G49 with approximately 80–100 protease inhibitors. The major interactions are side chain hydrogen bond donor (D), backbone hydrogen bond donor surface contact (d), and ionic interaction (I) with the ligand selected for the study.

3.4 Conclusion

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV), is considered as a deadly disease. Since the first infection reported in 1959, there have been >76 million infections with around 33 million deaths. According to the UNAIDS report in 2019, 37.9 million people were living with HIV globally, which is one of the major threats to humans. An HIV infection leads to a compromised immune system that weakens over 10–15 years from its first entry. The replication of the virus occurs by using the host cell mechanism that ultimately leads to the death of CD4 T-cells, giving way to opportunistic infections such as tuberculosis pneumonia, herpes simplex, Kaposi's sarcoma, and coccidioidomycosis. The key inhibitors of HIV are entry inhibitors/fusion inhibitors (blocks envelope protein, gp41 or gp120, and CD4 receptor or co-receptor CCR5), NRTIs/NtRTIs/NNRTIs (reverse transcriptase enzyme inhibitors), integrase (strand transfer) inhibitors (viral DNA), protease inhibitors, budding inhibitors, and maturation inhibitors. The incredible suppression of HIV viral load followed by a reduction of AIDS-related mortality has been achieved through cART. The knowledge of protein structure is obtained through novel techniques and advancement in crystallography, intense synchrotron radiation, and phase determination. X-ray crystallography has a central role to reveal the structure of the protein and in particular the complexes with ligands and provides a clear understanding of molecular recognition. The available crystal structure for the key targets of HIV-1 in both wild type and mutants facilitates to understand the significant failure of the drugs and their interaction with the residues in the binding site and in particular progressing in clinical trials and development. Additionally, mutagenesis studies allow for visualizing the importance of these amino acids in the binding pocket of the target protein. Structural information of the target protein regarding interactions with the ligands, mutation, and drug resistance is very important and gives a clear picture to the medicinal chemist for effective drug designing.

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4 LEDGINs, Inhibitors of the Interaction Between HIV-1 Integrase and LEDGF/p75, Are Potent Antivirals with a Potential to Cure HIV Infection

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Abstract

A permanent cure remains the greatest challenge in the field of HIV research. In order to reach this goal, a profound understanding of the molecular mechanisms controlling HIV integration and transcription is needed. Here we provide an overview of recent advances in the field. Lens epithelium-derived growth factor p75 (LEDGF/p75), a transcriptional coactivator, tethers and targets the HIV integrase into transcriptionally active regions of the chromatin through an interaction with the epigenetic mark H3K36me2/3. This finding prompted us to propose a "block-and-lock" strategy to retarget HIV integration into deep latency. A decade ago, we pioneered protein–protein interaction inhibitors for HIV and discovered LEDGINs. LEDGINs are small molecule inhibitors of the interaction between the integrase binding domain (IBD) of LEDGF/p75 and HIV integrase. They modify integration site selection and therefore might be molecules with a "block-and-lock" mechanism of action. Here we will describe how LEDGINs may become part in the future functional cure strategies.

Keywords

LEDGF/p75 · HIV · Integrase · Protein–protein interactions · LEDGINs · Latency · Integration site

Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALLINI	Allosteric integrase inhibitor
ATH	AT-hook-like motifs
bDNA	Branched DNA imaging
B-HIVE	Barcoded HIV Ensembles
cART	Combination antiretroviral therapy
CC_{50}	Concentration that is toxic for 50% of cells
CCD	Catalytic core domain
CTD	C-terminal domain
CRISPR	Clustered regularly interspaced short palindromic repeats
EC_{50}	Concentration that inhibits for 50%
EC_{95}	Concentration that inhibits for 95%
HDAC	Histone deacetylase
HDGF	Hepatoma-derived growth factor
Gps	Global positioning system
HATH	Homologous to the N-terminus of HDGF
HIV	Human immunodeficiency virus
HRP	HDGF related
IBD	Integrase binding domain
IBM	IBD-binding motif

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IN	Integrase
LEDGIN	Inhibitor of the interaction between LEDGF/p75 and integrase
LEDGF/p75	Lens epithelium-derived growth factor of 75 kDa
LRA	Latency reversing agent
LTR	Long terminal repeat
MACCS	Molecular ACCess System
MNase	Mung bean nuclease
MLL	Mixed lineage leukemia
NCINI	Non-catalytic site integrase inhibitor
NLS	Nuclear localization signal
NTD	N-terminal domain
PIC	Pre-integration complex
PSIP1	PC4- and SFRS-interacting protein 1
qVOA	Quantitative viral outgrowth assay
SIV	Simian immunodeficiency virus
SRD	Supercoil recognition domain
Tat	Transactivator of transcription
Tscm	T central memory stem cells
Tfh	Follicular helper cells
WHO	World Health Organization

4.1 Introduction

The HIV/AIDS pandemic imposes an important threat to public health despite important improvements in antiviral therapy. The World Health Organization (WHO) states that worldwide 38 million people are infected and 1.7 million new infections occurred in 2019 [1]. In 2019, 25.4 million people worldwide received antiretroviral therapy. Broadened access to cART (combination antiretroviral therapy) in particular in resource-limited regions has reduced the overall incidence of HIV-associated deaths to about 690,000 in 2019 in comparison to 1.9 million in 2004 [1]. However, the 90–90–90 HIV treatment goals of UNAIDS have not been achieved as in 2019 only 81% of people living with HIV (PLHIV) knew their HIV status; among those, only 82% received therapy, and among the patients on therapy, 88% were virally suppressed [1]. The most vulnerable populations, in particular, such as young females in sub-Saharan Africa, have no access to antiviral therapy. Moreover, cART fails to eradicate HIV since proviral DNA remains present in long-living cellular reservoirs, resulting in viral rebound upon interruption of cART. As a result, cART has to be given lifelong, and strict adherence is required to prevent antiviral resistance that emerges under suboptimal therapy. In addition, increased access to therapy in developing countries poses significant financial, infrastructural, and logistic challenges. Hence, the effort to rise our understanding the molecular virology of HIV has to be maintained. More in particular the mechanisms of HIV persistence in infected cells ought to be better understood to validate novel therapeutic targets and strategies to cure HIV infection [2].

4.2 Obstacles on the Road Toward an HIV-1 Cure

The existence of a persistent, replication-competent HIV proviral pool in patients, even when on optimally controlled cART, was already shown in 1997 [3, 4]. Yet, the study of HIV persistence has proven to be quite complicated. First, integrated HIV DNA is present in many anatomical sites including the lymphoid system, blood circulating cells, the brain, the gut-associated lymphoid tissues, and other tissues [5, 6]. Next, HIV can infect both lymphoid and myeloid cells. Yet, the relative contribution of various cell types in the HIV reservoir remains debated (for a review, see [6]). Additionally, the persistent viremia in patients under cART can be explained in two different ways: ongoing low-grade viral replication due to suboptimal tissue penetration of drugs in the so-called sanctuary sites [7–10] and reactivation of HIV expression from pools of latently infected cells [11–13]. Both latently infected cells and sanctuary sites are called reservoirs in the literature, since either contain replication-competent virus that contributes to viral persistence and viral rebound after treatment interruption. Latently infected cells contain intact, replication-competent HIV DNA but do not produce new virions before reactivation. Importantly, many integrated proviruses are deficient and do not reactivate even under suboptimal

treatment. Pre-integration latency relates to non-integrated HIV DNA leading to limited expression of viral proteins or integration after stimulation but is unlikely to contribute to long-term persistence in treated patients [14, 15]. Here, we will discuss post-integration latency of cells that contain an integrated provirus producing little to no viral mRNA, proteins, and/or viral progeny. However, upon reactivation of the provirus, transcription is restored and active viral replication resumes [14]. We thus refer to the reservoir as the pool of latently infected cells, since the focus of this chapter is on post-integration latency.

Today, memory CD4+ T cells [16] including long-lived CD4+ T central memory stem cells (Tscm) [17] as well as T follicular helper cells (Tfh) [18] are believed to be the main cell types composing the latent reservoirs. Apart from their long half-life, memory CD4+ T cells are maintained through homeostatic proliferation [16]. Depending on their HIV-1 integration locus, they can also undergo clonal proliferation [19–21]. As a consequence of low or absent expression of viral proteins, the immune system does not clear these cells, and they persist lifelong. Hence, memory CD4+ T cells constitute a permanent source for virus reactivation, and they probably cause the rapid rebound of plasma viral load observed after treatment interruption. In conclusion, the presence of latent reservoirs carrying replication-competent provirus is the major impediment toward eradication of HIV in infected individuals [2]. It follows that a more profound insight in viral reservoir formation and maintenance is necessary to design novel approaches targeting these latent reservoirs. Diverse strategies are being investigated [2, 22]. To date, most efforts have been dedicated to the "shock-and-kill" approach, for which latency reversing agents (LRAs), such as histone deacetylase (HDAC) inhibitors, are given to reactivate the transcription of the latent provirus [23, 24] (Fig. 4.1a). The strategy is based on the concept that reactivated cells will express viral proteins, triggering an immune response and cell killing. Although the strategy resulted in exploratory clinical trials, the first results show limited efficacy [25–30]. Moreover, the treatment is not very specific and therefore results in an overall activation and clear toxicity. Gene editing has been pursued as an alternative approach. Autologous T or stem cells are modified to provide an HIV-1-resistant pool, e.g., by deleting part of the CCR5 gene with CRISPR/Cas or zinc-finger technology [31]. Furthermore, researchers try to eradicate HIV-1 provirus from latently infected cells using gene editing [32, 33]. Yet, this approach is limited to cell culture settings so far. Delivery of gene editing tools to all cells of the reservoir in the patient remains an exceptional bottleneck. Moreover, gene editing comes with unknown risks due to possible off-target effects reducing the likelihood to achieve an HIV cure. A last strategy is the creation of a cellular reservoir resistant to reactivation, therewith preventing viral rebound [22], known as "block-and-lock" strategy, which will be explained in detail in this chapter (Fig. 4.1b). The distinct block-and-lock strategies were recently reviewed by Vansant [34]. A typical example is the Tat inhibitor didehydro-cortistatin A preventing HIV-1 reactivation in cell culture and mouse models [35].

4.3 Molecular Drivers of HIV Persistence

Several molecular determinants of HIV latency have been described though a deeper understanding on the underlying mechanisms controlling viral gene expression in resting CD4+ T cells is warranted. While latent HIV-1 proviruses integrate preferably in actively transcribed genes [36, 37], numerous HIV-1 proviruses are transcriptionally repressed at the level of initiation of transcription and/or elongation, resulting in post-integration latency. Latency is associated with a transcriptional block at the main HIV-1 promoter, the 5′ LTR (long terminal repeat). While the HIV-1 transactivator of transcription (Tat) activates viral transcription and poor Tat transactivation is associated with latency, additive mechanisms such as epigenetic silencing and the absence of transcription factors mediate the suppression of viral transcription (for a detailed

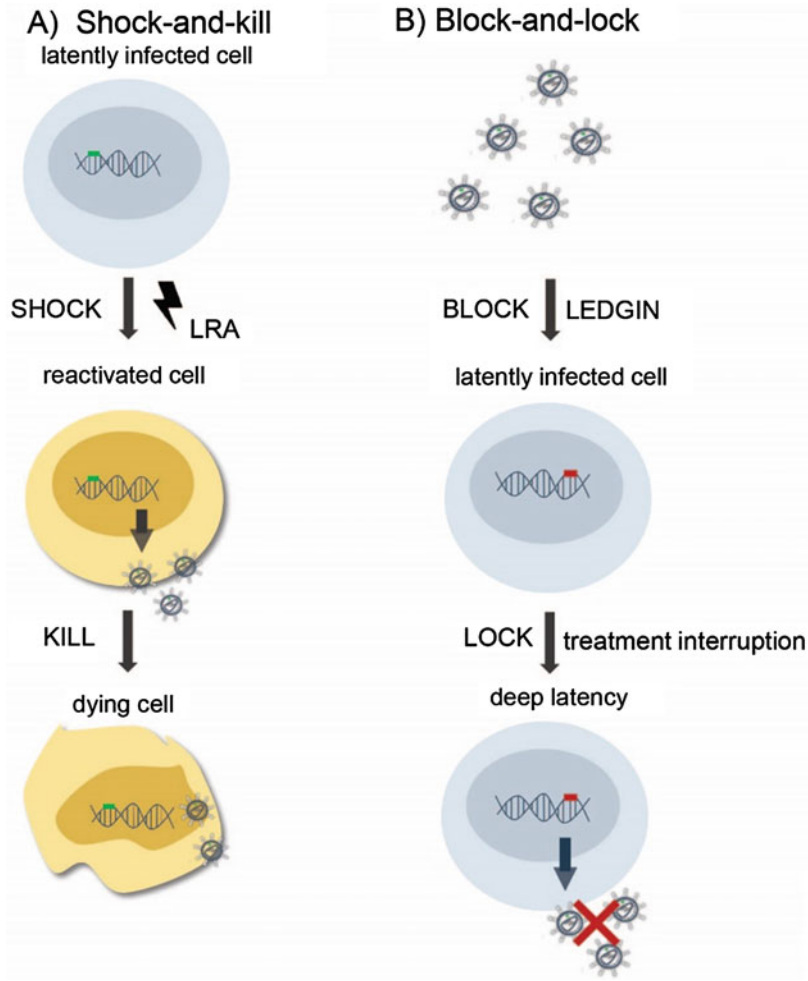

Fig. 4.1 Prototype HIV cure strategies. (**a**) In the ''shock-and-kill" approach, reactivation of the latent provirus is followed by the killing of the activated cells by viral cytopathic effects or the host immune system. (**b**) In the ''block-and-lock" functional cure strategy, the latent reservoir is permanently silenced, for example, by LEDGIN-mediated retargeting of integration to sites that are less susceptible to reactivation after interruption of cART

review [13, 24, 38]). Furthermore, not only the silencing of HIV transcription has to be overpowered to efficiently reverse latent infection. The interplay between the viral Rev protein, involved in HIV mRNA splicing and export, and latency is not yet decoded to detail. Also, the role of other cellular processes such as translation, viral antigen expression, and/or processing in HIV latency, in particular in resting $CD4^+$ T cells, is not well understood. Moreover, the possible role of exosomes in HIV-1 latency requires investigation since they mediate HIV infection and modulate immune responses by presenting viral proteins [39, 40]. Recent evidence even indicates that exosomes from (un)infected cells reactivate latent HIV [41–43]. Finally, a better insight in host factors that enable or restrict latency is required as well. We will first explain HIV-1 integration highlighting its relation with transcription and latency.

4.4 LEDGF/p75, the "Global Positioning System (GPS)" of HIV, Mediates HIV-1 Integration

While the enzymology of integration reaction has been well studied, the link between integration site selection and transcription remains poorly understood. In recent years, evidence has accumulated that the transcriptional state of the

provirus depends on the site of integration. In fact, HIV integration does not happen random but is directed toward the periphery of the nucleus and active transcription units in gene dense regions [37, 44–49]. Of note, all primate lentiviruses (HIV-1 and HIV-2, as well as simian immunodeficiency virus (SIV)) share this pattern [50–52]. The variations between these viruses are modest and relate to the preferred orientation of integration (sense or antisense) and the identity of specific integration hot spots. Lentiviruses employ cellular cofactors to achieve these specific integration patterns. More in particular, LEDGF/p75 is a major determinant of the selection of HIV integration sites (reviewed in [53]). In 2003, LEDGF/p75 was identified as a cellular cofactor of HIV integrase [54]. Further research demonstrated that interaction with LEDGF/p75 is a conserved feature among all lentiviral integrases such as feline immunodeficiency virus, equine infectious anemia virus, SIV, and HIV-2, pointing to its importance during evolution. This conserved feature explains the similarity between lentiviral integration patterns [55].

The ubiquitously expressed LEDGF/p75 has been implicated in lens epithelial cell gene regulation, stress responses [56], neuroepithelial stem cell differentiation and neurogenesis [57], and the regulation of embryogenic gene expression [58]. Next to its role in cell biology, LEDGF/p75 has been associated with various disease states such as prostate cancer [59], autoimmune disease [60], HIV infection [54], and mixed lineage leukemia (MLL) [61]. LEDGF/p75 acts as a molecular tether both in health and in disease that couples proteins and protein complexes with chromatin. Nucleosomes associated with actively transcribed genes are recognized by the chromatin reader function of LEDGF/p75 [62, 63]. As such it provides HIV with a molecular "global positioning system (GPS)."

4.4.1 The Domain Structure of LEDGF/p75

LEDGF/p75 is encoded by the PC4- and SFRS-interacting protein 1 (PSIP1) gene on chromosome 9 [64]. As a transcriptional coactivator, it recognizes di- and trimethylated lysines on histone 3 (H3K36) [58, 62, 63, 65, 66]. LEDGF/p75 is a member of the hepatoma-derived growth factor-related (HDGF) family [67]. Next to LEDGF/p75 and HDGF, four additional members belong to this class of proteins (HDGF-related proteins 1-4 (HRP1-4)). A conserved N-terminal HATH (homologous to the N-terminus of HDGF) domain with a typical structural domain, PWWP (Pro-Trp-Trp-Pro) [67], is shared by the HDGF family of proteins. The PWWP domain interacts with methylation marks on histones, DNA, and negatively charged molecules in general such as heparin. Although conserved in function, the primary sequence of PWWP comes with some variability, namely, the P type (PHWP) and the A type (AHWP). Next to the HATH domain, HDGF proteins contain a nuclear localization signal (NLS) enabling nuclear import of PWWP proteins and access to the chromatin [68].

The PSIP1 gene encodes LEDGF/p52 and p75, products from alternative splicing [56, 64]. Both splice variants share their N-terminus (aa 1-325) that carries the PWWP domain, the nuclear localization signal (NLS), two AT-hook-like motifs (ATH), and the supercoil recognition domain (SRD) [68–71] (Fig. 4.2). LEDGF/p52 contains a short C-terminal extension of only eight amino acids, whereas LEDGF/p75 carries a largely unstructured sequence of an additional 205 aa. Within this region, solely the integrase binding domain (IBD) adapts a discrete structure. The domain is named after its interaction with HIV integrase (IN) [69], although later work has shown that this domain mediates interaction of several cellular proteins with LEDGF/p75 [72, 73].

4.4.2 The Interaction Between the IBD of LEDGF/p75 and HIV Integrase

The IBD domain of LEDGF/p75 interacts with integrase via its catalytic core domain (CCD) and the N-terminal domain (NTD) [69, 74, 75], thus

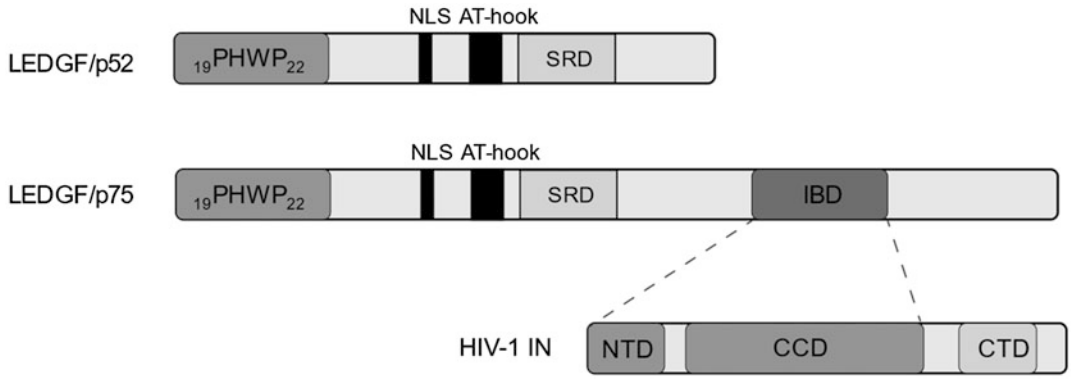

Fig. 4.2 Schematic representation of the LEDGF isoforms and HIV integrase. Both LEDGF/p52 and LEDGF/p75 contain a PHWP sequence (residues 19–22) in the PWWP domain (residues 1–93), a nuclear localization signal or NLS (residues 148–156), two AT-hook motifs (residues 178–198), and the supercoil recognition motif or SRD (residues 200–274). LEDGF/p75 harbors an integrase binding domain or IBD (residues 346–426) that modulates binding to HIV integrase (IN). HIV IN carries an N-terminal domain (NTD), a catalytic core domain (CCD), and a C-terminal domain (CTD)

tethering the integration complex chromatin of the host cell [76]. As a consequence, it facilitates the integration into actively transcribed genes and promotes viral replication [77–80] (Fig. 4.2). In addition to the tethering function, LEDGF/p75 also promotes the catalytic activity of IN and protects it from degradation in proteasomes [54, 81, 82]. In human cells depleted of LEDGF/p75 by RNAi, in embryonic knockout mice-derived fibroblasts, and in stable human LEDGF/p75 knockdown and knockout cell lines, the absence of LEDGF/p75 significantly impairs HIV replication [77–80, 83]. HRP-2, a homologue of LEDGF/p75, can substitute for LEDGF/p75 function in its absence [78, 83]. Ectopic expression of the IBD in cell culture potently competes with endogenous LEDGF/p75 and inhibits HIV replication [77, 84]. This provided proof of principle that the LEDGF/p75–integrase interaction is a genuine target for antiviral treatment.

4.4.3 Interaction of LEDGF/p75 with Chromatin

The PWWP domain belongs to the Tudor domain "Royal Family." Despite being conserved, minor sequence variations are possible. For instance, LEDGF/p75 harbors a PWHP sequence [85]. PWWP interacts with methylated lysine 36 in histone H3 (H3K36me2 and H3K36me3), epigenetic markers associated with actively transcribed chromatin [62, 63, 65, 66]. Lysine 36 is located at the site where the tail of H3 protrudes the nucleosome [86]. The PWWP structure is defined by a five-strand antiparallel β-barrel, a 3_{10} helix between β_4 and β_5, and a C-terminal region of two α-helices [62]. The size and shape of the hydrophobic cavity of the PWWP domain enable cation–π interactions with methylated lysine side chains from histones. Negatively charged DNA binds to a highly basic protein surface, located N-terminally of the hydrophobic cavity. Of note, the PWWP domain interacts both with methylated histone 3 lysines (H3K36) and nucleosomal DNA with low affinity (Fig. 4.3). The combination of both is the driver of specific binding of LEDGF/p75 to methylated H3K36 nucleosomes. In conclusion, the hydrophobic cavity and the basic surface act synergistically to ensure high-affinity binding of the PWWP domain of LEDGF/p75 to mononucleosomes [62, 63].

While PWWP domains are present in 23 other human proteins, besides LEDGF only HRP-2

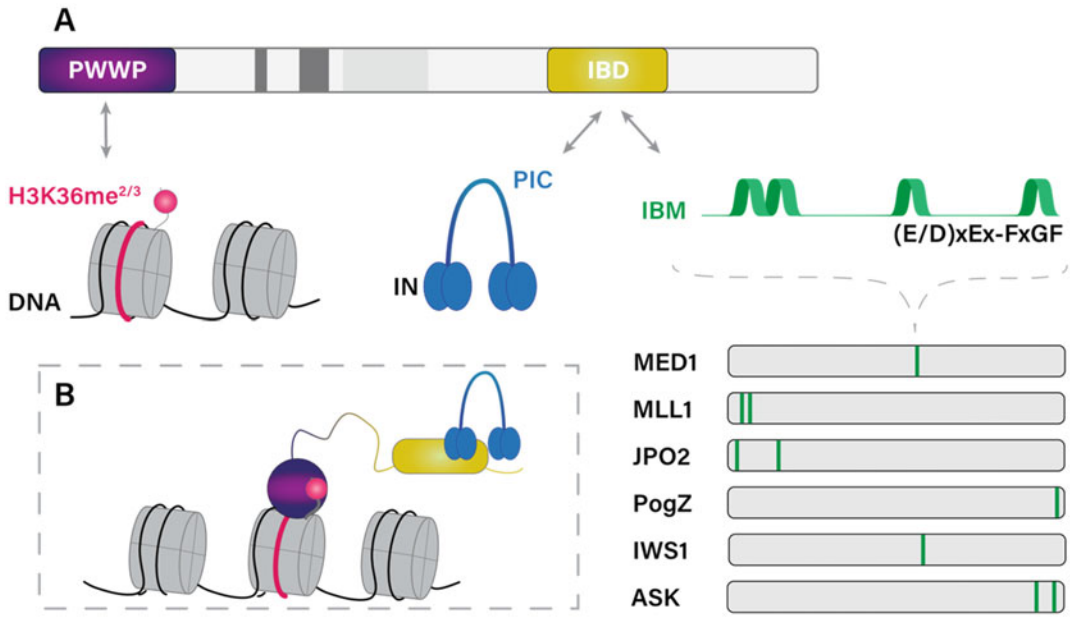

Fig. 4.3 Representation of LEDGF/p75 PWWP and IBD interactions. (**a**) Via the PWWP domain (purple), LEDGF/p75 interacts with methylated lysine 36 on histone 3 (pink ball, H3K36me$^{2/3}$) and nucleosomal DNA (pink). Via the integrase binding domain (IBD, yellow), LEDGF/p75 interacts with HIV integrase (IN, blue) or cellular binding partners harboring an IBD-binding motif (IBM, green). Several known IBD-interaction partners are listed, and IBM motifs are indicated by green bands. PIC; pre-integration complex. (**b**) Schematic representation of LEDGF/p75 that is bound to DNA and the methylation mark via the PWWP domain while tethering the PIC to the DNA via its IBD domain

(also called HDGF2) contains both a PWWP and an IBD [69]. The combination of chromatin tethering with the anchoring function of IBD is thus rather unique and indicates certain redundancy in the cellular roles of both proteins. Indeed, overexpression of HRP-2 can rescue HIV replication in cells depleted of LEDGF/p75 [78].

4.4.4 LEDGF/p75 Represents Two Distinct Drug Targets

Interference with the regulation of transcription through pharmacological inhibition of chromatin readers, writers, and erasers presents an emerging strategy for future therapies. Precedence was provided with the discovery of bromodomain inhibitors (JQ1 and I-BET762). Bromodomains are epigenetic readers of histone acetylation, implicated in a diverse set of diseases including cardiovascular diseases, inflammation, and cancer [87]. Since LEDGF/p75 plays an essential role in HIV-1 integration and MLL-rearranged leukemia, inhibitors of the PWWP–chromatin interaction could potentially inhibit HIV infection and MLL-rearranged leukemia. To date, knowledge on small molecule inhibitors of trimethyl-lysine binders is scarce [88–90]. One may target the hydrophobic pocket of the PWWP domain of LEDGF/p75 by mimicking the trimethyl-lysine side chain of histone 3. Targeting the positively charged patch adjacent to the hydrophobic pocket represents a second option. Targeting the PWWP–chromatin interaction displaces LEDGF/p75-protein complexes from the chromatin and as a consequence can downregulate co-transcriptional activity. But also other domains of the chromatin reader LEDGF/p75 could provide druggable targets. Targeting the IBD aims at inhibiting the interaction between LEDGF/p75

and its cellular interaction partners or HIV integrase, aborting protein complex formation. Either strategy may be successful in discovery of anticancer agents or anti-HIV drugs. The major challenge in inhibiting these interactions is to modify the disease phenotype while minimizing interference with their role in cell biology. In fact, the discovery and further development of LEDGF/p75–HIV IN inhibitors [91] (referred to as LEDGINs), discussed in more detail below, provide a proof of concept for the development of highly selective inhibitors targeting the protein–protein interactions of LEDGF/p75. In respect to PWWP, the recent discovery of specific inhibitors of the NSD3 PWWP domain supports such strategy [92].

4.5 LEDGINs Are Antivirals Blocking the Interaction Between LEDGF/p75 and HIV-1 Integrase That Display a Multimodal Mechanism of Action

In 2010, structure-based drug design targeting the interface between HIV IN and LEDGF/p75 resulted in the discovery of 2-(quinolin-3-yl) acetic acid derivatives that inhibit HIV-1 replication [91]. A diverse, commercial library (200,000 compounds) was the starting point. This library was filtered with a series of 2D descriptors defining the small molecule PPI inhibitor chemical space. A consensus pharmacophore was constructed after analysis of available (co-)crystal structures of HIV-1 IN. This pharmacophore described the molecular recognition patterns in the LEDGF/p75 binding site of HIV-1 IN [91]. Next, molecules identified through the pharmacophore query were docked into the binding pocket and scored via a consensus scoring algorithm. The best scoring molecules were ranked by visual inspection for agreement with the pharmacophore hypothesis. Twenty-five commercially available compounds were selected, and their inhibition of the interaction was determined in an in vitro bead-based IN-LEDGF/p75 protein–protein interaction assay (AlphaScreen, PerkinElmer). One compound (LEDGIN 1, **1** Fig. 4.4) modestly reduced the AlphaScreen signal by 36% at 100 μM. Commercial analogs were sought using MACCS (Molecular ACCess System) structural fingerprints, which resulted in the discovery of LEDGIN 2 (**2**) that displayed an IC_{50} of 27 μM in the AlphaScreen assay. Medicinal chemists replaced the tetrazole by a carboxylic acid and removed the unstable secondary ketamine, which resulted in 2-(quinolin-3-yl) acetic acid LEDGIN 3 (**3**). LEDGIN 3 inhibited the in vitro interaction with an IC_{50} of 12.2 μM and viral replication with an EC_{50} of 41.9 μM. No apparent cellular toxicity was observed (CC_{50} > 150 μM). The compound was successfully soaked into the LEDGF/p75 binding pocket in crystals of the HIV-1 IN catalytic core dimer (CCD). Comparison to the structure of the IBD–CDD complex revealed that LEDGF/p75 residues I365, D366, and L368 were mimicked by the phenyl, acid, and chlorine functions of the compound, respectively, proving the validity of the pharmacophore model. Supported by insight in the structure, hydrophobic bulk was introduced on the acetic acid 2-position to occupy a hydrophobic region of the binding site [91, 93]. Indeed, the potency of compounds was increased into the low micromolar range (CX05168, **4**; IC_{50} = 1.4 μM, EC_{90} = 5.4 μM, and CC_{50} = 60 μM). Interestingly, together with increased potency of the compound inhibiting the LEDGF/p75–IN interaction, allosteric inhibition of IN catalytic activity in the absence of LEDGF/p75 appeared as CX05168 showed an IC_{50} = 55 μM against strand transfer activity [91, 93]. The quinoline benzene was replaced by thiophene, various substitutions were made on the 4-phenyl group, and the hydrophobic bulk in the acetic acid 2-position was optimized [93]. This yielded CX05045 (**5**; IC50 = 0.58 μM, EC_{90} = 1.86 μM, and CC_{50} = 72 μM) and CX14442 (**6;** IC_{50} = 0.046 μM, EC_{90} = 0.114 μM, and CC_{50} = 96 μM). Interestingly, CX14442 inhibited the IN catalytic activities (ST and 3P) with IC_{50} values of 146 nM and 727 nM, respectively, when the compound was preincubated with HIV-1 IN before addition of LTR or transfer DNA substrate [93]. The

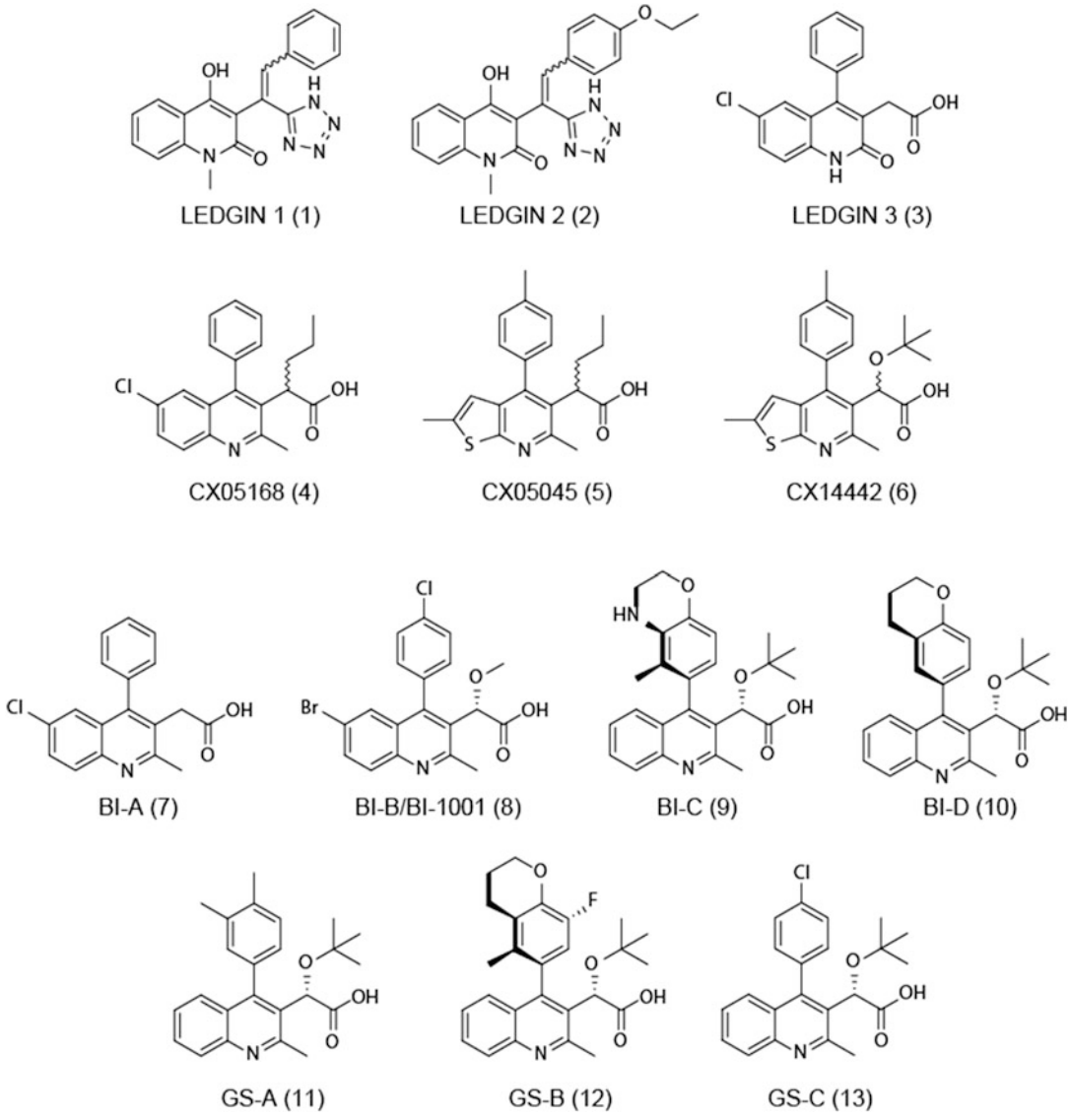

Fig. 4.4 Chemical structures of LEDGINs. The compounds are discussed in the text

LEDGINs were licensed to Pfizer Inc./ViiV Healthcare for preclinical development.

Independently, a high-throughput IN 3′ processing screen was performed by investigators at Boehringer Ingelheim GmbH aiming at the discovery of novel classes of IN inhibitors. They identified the 2-(quinolin-3-yl)acetic BI-A (**7**) as a hit ($IC_{50} = 9$ μM and $EC_{50} > 40$ μM) [94]. Structure-guided medicinal chemistry resulted in the development of BI-B (**8**; IC_{50} in 3′ processing = 28 nM, IC_{50} for IN-LEDGF/p75 interaction = 1.5 μM, $EC_{50} = 0.45$ μM, and $CC_{50} > 50$ μM) and BI-C (**9**; IC_{50} in 3′ processing = 3 nM, $EC_{95} = 10$ nM, and $CC_{50} > 80$ μM). Of note, differences in the potency of all LEDGINs with respect to the IN–LEDGF/p75 interaction or IN catalytic activities are likely attributable to differences in order-of-addition, preincubation, or general assay format [95]. BI-C showed excellent ADME (absorption, distribution, metabolism, and excretion) characteristics: favorable metabolic stability, low cytochrome P450

inhibition, high permeability, and excellent physicochemical properties [94]. One compound with excellent PK in preclinical animal species advanced into phase I clinical trials [94]. BI 224436 (**14,** $EC_{95} = 22$ nM and $CC_{50} > 90$ μM) was given to 48 healthy subjects in escalating doses and displayed good tolerability and no serious adverse events [94]. However, clinical studies on BI 224436 were discontinued. Gilead Sciences took a license on the program for further development [96]. In the following, Gilead has published three more compounds from this series (GS-A to C, **11**–**13**) [97]. Not only Gilead and Pfizer have continued development of these inhibitors; patent literature reveals that almost all major pharmaceutical companies active in the treatment of HIV/AIDS maintain a significant interest in this class (an extensive overview is given in [98]).

Since the initial discovery, other compounds have been found that bind to the same LEDGF/p75 binding pocket of HIV-1 IN [99–105]. In reference to the common mechanism of action, this class of antivirals is collectively referred to as LEDGINs [98]. The term LEDGINs is preferred over other terms used in the later literature, to avoid confusion. The term ALLINIs (allosteric integrase inhibitors), for example, refers to a more general mechanism of action since not all allosteric integrase inhibitors bind to the LEDGF/p75 binding pocket [98]. LEDGINs interfere with the binding of LEDGF/p75 to HIV IN by binding to the IN dimer interface and allosterically inhibit the catalytic activity of IN (the so-called early effect) [93, 95]. Later it was found that LEDGINs also inhibit viral maturation (the so-called late effect) [93, 97, 106–108]. Virions generated in the presence of LEDGINs show morphological defects due to LEDGIN-induced multimerization of integrase, resulting in aberrant maturation. As a result, the majority of particles present with a delocalized ribonucleoprotein outside the capsid core or even fail to establish a core. These crippled viruses are impaired for the subsequent round of infection at the level of reverse transcription, nuclear import, and integration.

4.5.1 A Block-and-Lock Strategy for a Functional Cure of HIV Infection

LEDGF/p75 is the main determinant of HIV integration site selection, and LEDGINs provide an elegant research tool to interfere with LEDGF/p75-mediated choice of integration sites. The hypothesis was raised that LEDGIN therapy may retarget residual provirus away from the integration sites of choice. As such, LEDGIN retargeted integration could impact proviral gene expression and latency and provide the rationale for a "block-and-lock" functional cure strategy (Fig. 4.1b). In 2016, Vranckx et al. reported on such a strategy [109]. LEDGIN treatment of infected cells clearly shifted residual HIV integration out of active genes in a dose-dependent manner [109]. In addition, the integrated provirus relocalized away from the nuclear periphery to the inner nucleus. A virus carrying two distinct reporters was designed by the Verdin lab [110] and used to demonstrate that LEDGIN-mediated retargeting increased the proportion of latent provirus. Moreover, this residual reservoir was less susceptible to HIV reactivation with TNFα. Of note, Chen et al. reported at the same time on a barcoded HIV vector—Barcoded HIV Ensembles (B-HIVE). They experimentally provide that provirus transcription and reactivation are affected by the proximity of the integration locus to enhancers [111, 112]. These data corroborate older findings whereby distinct HIV-infected clones express varying HIV RNA levels [47]. Taken together, both approaches independently demonstrate that modifying the integration site pattern can influence the transcriptional activity of the HIV provirus and thus HIV latency.

LEDGINs thus provide a research tool to study the underlying mechanisms of latency. As told, LEDGINs lead to IN multimerization in the late stages of replication. The effect of LEDGINs present during virus production on the transcriptional state of the residual virus was also investigated [113]. Infection of cells with viruses produced in the presence of LEDGINs resulted in a residual reservoir that proved refractory to

reactivation. Integration of residual provirus was less favored near epigenetic markers associated with active transcription. However, integration near H3K36me3 targeted by LEDGF/p75 was not affected. In conclusion, LEDGIN treatment during virus production altered integration of residual provirus, resulting in a reservoir that is refractory to activation, even though this retargeting may not be solely due to abrogation of the LEDGF/p75–In interaction in this case. Moreover, it was shown that LEDGIN treatment of primary cells induced a reservoir resistant to reactivation due to a combined early and late effect during multiple rounds of infection with replicating HIV [113].

While LEDGIN treatment partially shifts integration out of transcription units in cell culture, integration is not random. Still, the transcription of residual provirus and reactivation of latent provirus can be suppressed at higher concentrations of inhibitor [109]. Fine-tuning of the optimal chromatin environment of the provirus by LEDGF/p75 and not only integration in or outside genes seems important. The direct link between integration sites and transcription was further investigated in a recent study that combined LEDGIN treatment with Barcoded HIV Ensembles (B-HIVE) [114]. B-HIVE allows to correlate the barcoded provirus, its integration site, and its barcoded mRNA levels. After LEDGIN-mediated inhibition of replication and retargeting integration, the effect on transcription was measured with B-HIVE. LEDGINs indeed retargeted integration out of actively transcribed and gene-dense regions. A clear increase of the distance to H3K36me3, the marker recognized by LEDGF/p75, was observed. Addition of LEDGINs during HIV infection resulted in provirus with reduced RNA expression boosting the proportion of silent provirus. Finally, silent proviruses obtained under LEDGIN treatment were found further distanced from epigenetic marks typical for active transcription. Of note, proximity to super-enhancers increased transcription irrespective of LEDGIN treatment, whereas the distance to H3K36me3 only changed after LEDGIN treatment. The experimental finding that proximity to these markers is associated with viral RNA expression corroborates the direct association between proviral integration site and viral RNA expression (Fig. 4.5). Of note, the role of super-enhancers was highlighted in a recent study on HIV integration hot spots; they are localized in the nuclear periphery and apparently in close contact to super-enhancers [115]. Apart from B-HIVE [111, 112, 114], branched DNA imaging (bDNA) [116] is used to investigate the relation between LEDGIN-mediated retargeting and transcriptional state of the provirus at the single-cell level. Moreover, DNase or MNase assays can elucidate the role of nucleosome positioning in translation [117]. Finally, the interaction between HIV capsid and cellular CPSF6 has been suggested to bypass heterochromatin in the nuclear periphery to allow integration in the nuclear interior [118]. These data contrast publications that show preferential integration in the nuclear periphery and thus request further investigation [44, 45, 48]. Another line of research addresses the possible role of a complex containing LEDGF/p75, Iws1, and Spt6 in the regulation of HIV latency [119]. We conclude that HIV may have evolved to employ LEDGF/p75 as a molecular anchor to ensure both a productive and a latent provirus population. In the absence of LEDGF/p75, the provirus may end up in a third, deep latent population refractory to reactivation.

4.5.2 Place Your Bets

What are the odds that LEDGINs can contribute to a functional cure? Intuitively one would answer negatively, since HIV reservoirs are formed in the first weeks after infection jeopardizing provirus retargeting approaches. Still, it has been documented that commencing cART early after infection is effective in reducing the size of the viral reservoir and therefore early treatment is now standard of care [120–122]. If a therapeutic window exists early after infection to diminish the size of the reservoir, addition of a LEDGIN to initial cART regimens to modify the functional reservoir would be a strong added value. When LEDGINs are tested as antivirals in

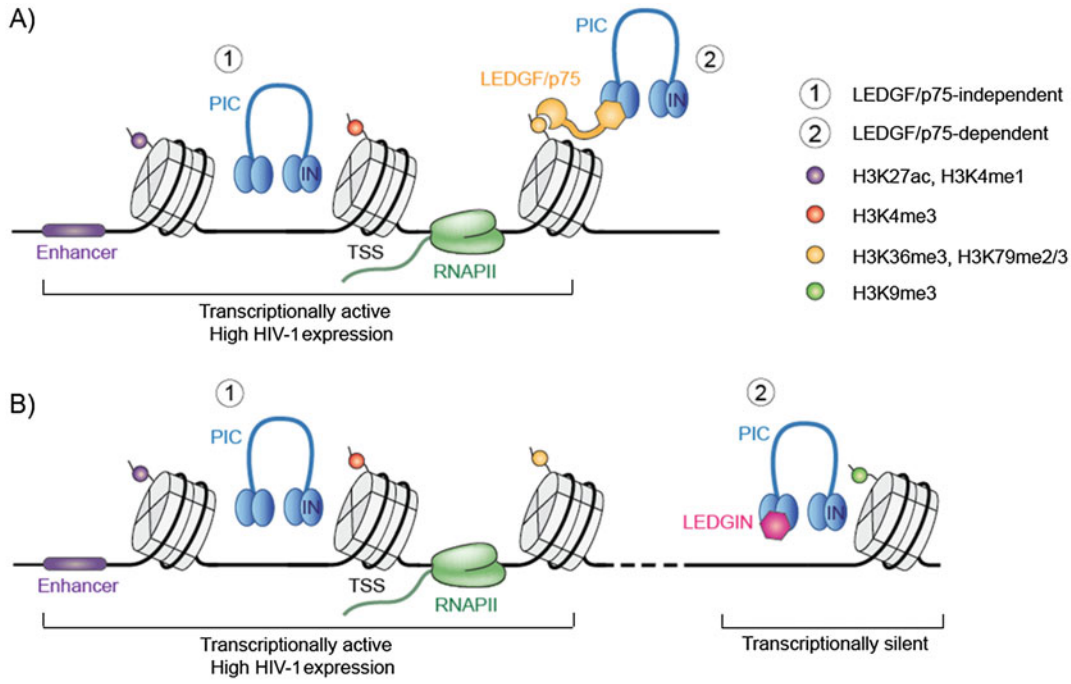

Fig. 4.5 Model that links HIV-1 integration with transcription. (**a**) HIV-1 integration sites are determined by the binding of LEDGF/p75 to the H3K36me3 mark associated with active genes. HIV-1 also integrates near enhancer regions characterized by H3K27ac and H3K4me1. Integration in these areas is associated with high RNA expression. (**b**) LEDGIN treatment retargets integration away from active transcription units to silent genes and intergenic regions. This results in proviruses with reduced RNA expression. Still, some LEDGF/p75-independent integrations might still occur near enhancer sites, explaining the observation of few high residual expressors even after treatment with LEDGINs (Figure adapted from [114] with permission)

future clinical trials for acute infection, the reservoirs in these patients ought to be followed up with proviral DNA measurements and quantitative viral outgrowth (qVOA) assays. This may provide supportive evidence for a block-and-lock strategy. LEDGINs may also be useful for HIV pre-exposure prophylaxis (PrEP), since any residual provirus under LEDGIN PrEP may result in a nonfunctional provirus characterized by deep latency.

In regard to chronic infection, recent research now suggests that the majority of the reservoir responsible for HIV rebound might only be established at the time cART is initiated [123, 124], meaning that LEDGINs may also benefit patients diagnosed years after infection. If no residual replication occurs once viral reservoirs are established, LEDGINs will fail to modulate the functional reservoir in patients on chronic cART. Yet, any residual integration at sanctuary sites with poor drug penetration could be retargeted by LEDGINs if their tissue penetration is optimal. Secondly, to date, the proportion of the functional reservoir that is mobilized after treatment interruption is not known. Possibly reinitiation of a cART regimen including LEDGINs after treatment interruption may modulate the new established functional residual reservoir if enough proviruses are mobilized and if replication is not fully blocked. Although this is currently a speculative model, it can be experimentally verified once LEDGINs are tested in a clinical setting.

In conclusion, LEDGINs provide us with an interesting research tool to decipher the link between integration and transcription, an essential

question in retrovirology. Addition of diagnostic readouts to monitor the impact on the functional reservoir during clinical trials with LEDGINs would provide the answers on the likelihood of success of the proposed ''block-and-lock'' strategy.

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Moving Fast Toward Hepatitis B Virus Elimination

5

Leda Bassit, Suzane Kioko Ono, and Raymond F. Schinazi

Abstract

Currently, there are two safe and effective therapeutic strategies for chronic hepatitis B treatment, namely, nucleoside analogs and interferon alpha (pegylated or non-pegylated). These treatments can control viral replication and improve survival; however, they do not eliminate the virus and therefore require long-term continued therapy. In addition, there are significant concerns about virus rebound on discontinuation of therapy and the development of fibrosis and hepatocellular carcinoma despite therapy. Therefore, the search for new, more effective, and safer antiviral agents that can cure hepatitis B virus (HBV) continues. Anti-HBV drug discovery and development is fundamentally impacted by our current understanding of HBV replication, disease physiopathology, and persistence of HBV covalently closed circular DNA (cccDNA). Several HBV replication targets are the basis for novel anti-HBV drug development strategies. Many of them are already in clinical trial phase 1 or 2, while others with promising results are still in preclinical stages. As research intensifies, potential HBV curative therapies and modalities in the pipeline are now on the horizon.

Keywords

Hepatitis B · Core inhibitor · cccDNA · DAA—directly acting antiviral · Immune therapy · Hepatocellular carcinoma

Abbreviations

Ad	Adenovirus
ALT	Alanine aminotransferase
APOBEC	Apolipoprotein B mRNA editing catalytic polypeptide-like
ARCUS	Gene-editing platform
ASO	Antisense oligonucleotide
CAM	Capsid assembly effectors or modulators
CAS	CRISPR associated
cccDNA	Covalently closed circular DNA
CES	Carboxylesterase
cIAPS	Cellular inhibitor of apoptosis proteins
CpAM	Core protein allosteric modulators
CRISPR	Clustered regularly interspaced short palindrome repeats

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DAAs	Direct-acting agents
DS	Double stranded
DSBs	Double-strand breaks
EC_{50}	Median effective concentration to inhibit HBV DNA replication
ENV	Envelope
FXR	Farnesoid X receptor
GalNAc	N-acetylgalactosamine
GLS4	Morphothiadin
HAP	Heteroarylpyrimidines
HBcAg	HBV core antigen
HBeAg	HBV e antigen
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HID	N-hydroxyisoquinolinediones
HPD	N-hydroxypyridinediones
IAPs	Inhibitors of apoptosis proteins
ID	Intradermal
IM	Intramuscular
IV	Intravenous
L	HBV large surface protein
LNA	Locked nucleic acid
M	HBV middle surface protein
MHC	Major histocompatibility complex
mRNA	Messenger RNA
n/a	Not applicable
NAPs	Nucleic acid polymers
nM	Nanomolar
NTCP	Sodium taurocholate cotransporting polypeptide
PD-1	Programed cell death protein 1
PD-L1	Programed death ligand protein 1
PEG-IFN	Peginterferon
pgRNA	Pregenomic RNA
PK	Pharmacokinetics
POL	Polymerase
PP	Phenylpropanamides
PS-ONs	Phosphorothioate oligonucleotides
rcDNA	Relaxed circular DNA
RIG-I	Retinoic acid-inducible gene-I
RISC	RNA-induced silence complex
RNAi	RNA interference
RT	Reverse transcriptase
S	HBV small surface protein
SBA	Sulfamoylbenzamides
SC	Subcutaneous
siRNA	Small interfering RNA
SMAC	Second mitochondria-derived activator of caspases
SSO	Single-stranded oligonucleotide
STOPs	s-Antigen transport inhibiting oligonucleotide polymers
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VLV	Virus-like vesicles
WHsAg	WHV surface antigen
WHV	Woodchuck hepatitis virus
YMDD RT motif	Tyrosine, methionine, aspartate, aspartate reverse transcriptase motif
αHT	α-hydroxytropolones
μM	Micromolar

Key Points

- Current treatments do not completely clear HBV from hepatocytes leading to the establishment of lifetime chronic infection.
- Novel anti-HBV therapies targeting different steps of HBV replication cycle with the potential of curing individuals chronically infected are needed.
- Elimination of cccDNA from the nuclei of hepatocytes and clearance of HBV surface antigen (HBsAg) from blood are crucial to achieving a functional and complete cure.
- Drug-drug combinations synergistically targeting key steps of HBV replication cycle and immunomodulators boosting the host immune response may lead to a functional cure.
- Novel strategies including CRISPR and siRNA technologies which can inactivate persistent HBV cccDNA and also target integrated viral DNA may eliminate HBV from chronically infected human hepatocytes.

5.1 Introduction

Chronic hepatitis B virus (HBV) infection affects approximately 300 million people worldwide [1], and while prophylactic vaccines and antiviral therapies are currently in use, they do not provide a cure. Therefore, safe antiviral agents that target the HBV replication cycle and sites of virus persistence are urgently needed to prevent the nearly one million human deaths annually due to liver diseases associated with hepatitis B. HBV is a hepadnavirus that replicates its DNA in the liver through two main steps: formation of covalently closed circular DNA (cccDNA) and the reverse transcription of a pregenomic RNA (pgRNA).

With current available antiviral therapies for chronic hepatitis B, it is possible to control HBV replication. However, treatment is non-curative and therefore requires long-term continued use which has resulted in concerns for the development of antiviral resistance and adverse events, such as renal impairment or gastrointestinal disorders (important issue when considering adherence to treatment) [2–4]. The clinical endpoints now are focused on suppressing viral replication and alanine aminotransferase (ALT) normalization. This desirable endpoint of a functional cure (loss of HBsAg) is unlikely with current nucleoside analogs or pegylated interferons [5]. This may be due to cccDNA that persists in the nuclei of infected hepatocytes where it forms the template for all viral transcripts and HBV integration. New HBV targets and immune therapies are being sought, and we aim to review them according to their stage in clinical development, focusing on medicinal chemistry and/or biochemistry/molecular biology [6]. In addition, this review focuses on the outcomes of antiviral drugs newly developed or in clinical evaluation, as well as novel experimental drugs.

5.2 HBV Pathogenicity (Immunological Background)

HBV is a hepatotropic virus and most of the time does not cause a cytopathic effect [7]. The host immune response determines whether the virus persists (chronic infection) or not (cleared infection). In the natural history of chronic hepatitis B infection, initially there is an immunotolerant phase characterized by the presence of HBeAg, high rates of HBV DNA replication, and absence of inflammatory liver disease progression [3]. In this phase, the innate immune system is poorly activated due to an intrinsic ability of the virus to escape recognition [8].

In contrast, a persistent immune response to HBV-infected hepatocytes is the determinant of chronic liver disease, with inflammation (with or without HBeAg) leading to progression of fibrosis and cirrhosis, and ultimately hepatocellular carcinoma [3, 9, 10]. Individuals who have resolved HBV infection, with HBsAg clearance with or without HBs antibody, undetectable HBV DNA, and normal levels of ALT, are in the so-called functional cure phase [11]. In this phase, HBV is not fully eliminated, with a few hepatocytes remaining with the cccDNA form under a repressed translational control by innate and adaptive immune mechanisms [9].

In this regard, several immune pathways with the potential to suppress HBV replication in infected hepatocytes are currently under consideration as targets for the development of new therapeutic strategies for chronic hepatitis B infection. For example, retinoic acid-inducible gene-I (RIG-I) and apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) [9] are among other pathways that will be discussed below.

5.3 HBV Replication

HBV is a 3200 bp partially double-stranded DNA (rcDNA, relaxed circular DNA) from *Hepadnaviridae* family. Difference greater than 8% in nucleotide sequence across the complete HBV genotype determines ten major genotypes (A to J) with differences in replication, natural history, pathogenesis, and treatment response [12, 13]. HBV genome encodes four overlapping genes. The HBV RNA transcripts are translated into seven proteins: HBsAg (surface large

[preS1+preS2+S domains], middle [preS2+S domain], small [S domain]), HBeAg, HBcAg (core), RT-polymerase, and X protein. The HBV virion particles have an outside envelope composed with three forms (large, middle, and small) of surface proteins that encloses the capsid with the double-stranded DNA genome (Fig. 5.1). An important intermediate form (occurring in the nucleus of infected cells) is the covalently closed circular DNA (cccDNA) that is the template for pregenomic RNA (pgRNA) transcription and produces the template for reverse transcription and viral genome replication [14].

5.3.1 Replication Cycle

HBV binds to the hepatocyte at the sodium taurocholate cotransporting polypeptide (NTCP) receptor and enters into the cells. HBV attachment is believed to be mediated through the preS1 domain [15]. After entry, the viral particles containing the relaxed circular DNA (rcDNA) are uncoated, and the nucleocapsid particle must be directed into the cellular nucleus. HBV rcDNA is converted to an episomal cccDNA (see detailed information below). HBV cccDNA is the transcription template for all four viral RNAs (Fig. 5.1):

1. A 2.4-kb mRNA for the large (L) envelope protein, a 2.1-kb mRNA for the middle (M), and major surface (S) proteins
2. A 0.7-kb mRNA for the X protein
3. A 3.5-kb pre-core mRNA that encodes the pre-core protein
4. A 3.5-kb pregenomic RNA (pgRNA) that encodes the core and the polymerase

The pgRNA, upon being exported to the cytoplasm, is encapsidated together with viral polymerase and subsequently reverse-transcribed into viral minus strand DNA. Then, the plus-stranded DNA is synthesized to form the partially double-stranded relaxed circular DNA. The mature nucleocapsid can either be recycled back to the nucleus to maintain the pool of cccDNA or packed with envelope proteins and exported as infectious virions to infect other cells [14, 16] (Fig. 5.1).

5.3.2 Role of cccDNA

Intrahepatic cccDNA is the episomal virus template in the nucleus of HBV-infected hepatocytes. It is considered an important cause of viral persistence and a key obstacle for a cure of chronic hepatitis B [17]. This is especially true because current antiviral therapies including nucleoside analogs do not eliminate HBV mini-chromosome (cccDNA) or integrated HBV; therefore, continued virus gene expression from these templates will drive pathogenesis toward hepatocellular carcinoma, one of the main complications of chronic hepatitis B. Another currently used treatment for chronic hepatitis B, interferon alpha, upregulates the expression of APOBEC3 nuclear deaminase resulting in a modest reduction in cccDNA copy number via deamination [18].

Because cccDNA elimination is a major goal for the future HBV antiviral agents for the treatment of chronic hepatitis B, it is important to monitor and study this particular HBV form. However, the amount of cccDNA compared to pgRNA is very low (median 1.5 copies and 6.5 per cell, respectively) [19]. Therefore, to detect HBV cccDNA unambiguously is a great challenge [17]. Southern blotting is the gold standard test for detection and quantification of HBV intermediates and cccDNA; however, few samples can be tested at a time, and it requires high amounts of infected cells to detect cccDNA. Because it is not a high-throughput system, other tests including cccDNA-specific PCR have been assessed using specific primers located at each side of the gap region of rcDNA together with the appropriate HBV DNA purification or nucleus enrichment and the use of appropriate enzymes to selectively remove HBV rcDNA without degrading cccDNA [20]. Because liver biopsy is required to quantify cccDNA in vivo, measurements of HBV RNA and HBcAg in the serum may serve as surrogate biomarkers for cccDNA [11].

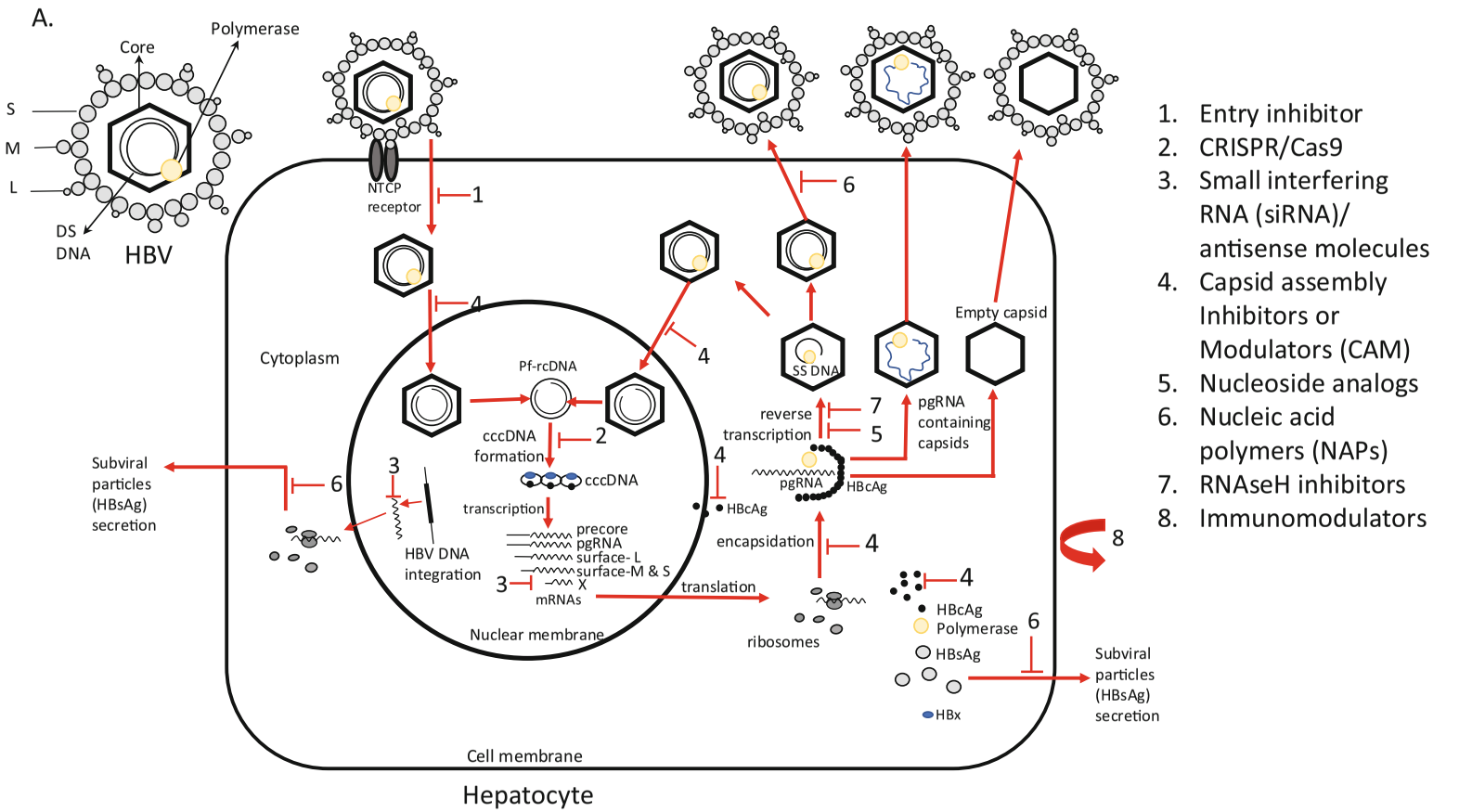

Fig. 5.1 (**a**, **b**) HBV replication mechanism, genome structure, and schematic representation of inhibition sites. (**a**) The HBV has an envelope composed with three forms (large, middle, and small) of surface proteins that encloses the capsid with the double-stranded DNA genome. (**b**) Replication starts with HBV binding to the hepatocyte at the NTCP receptor. After entry, the viral particles are uncoated, and the nucleocapsid particle goes to the cellular nucleus. HBV protein free rcDNA (Pf-rcDNA) is converted to an episomal cccDNA, which is the transcription template for all four viral RNAs. The pgRNA is encapsidated together with viral polymerase and subsequently reverse-transcribed into viral minus strand DNA, followed by degradation of the RNA by RNAseH. Then, the plus-stranded DNA is synthesized to form the partially double-stranded relaxed circular DNA. Mature nucleocapsid can either be recycled back to the nucleus to maintain the pool of cccDNA or packed with envelope proteins and exported as infectious virions to infect other cells. pgRNA containing nucleocapsid and empty nucleocapsids are also packed with envelope proteins and released. *S* small, *M* medium, *L* large, *DS* double stranded, *NTCP* sodium taurocholate cotransporting polypeptide, *CRISPR* clustered regularly interspaced short palindrome repeats; *CAS9* CRISPR associated 9

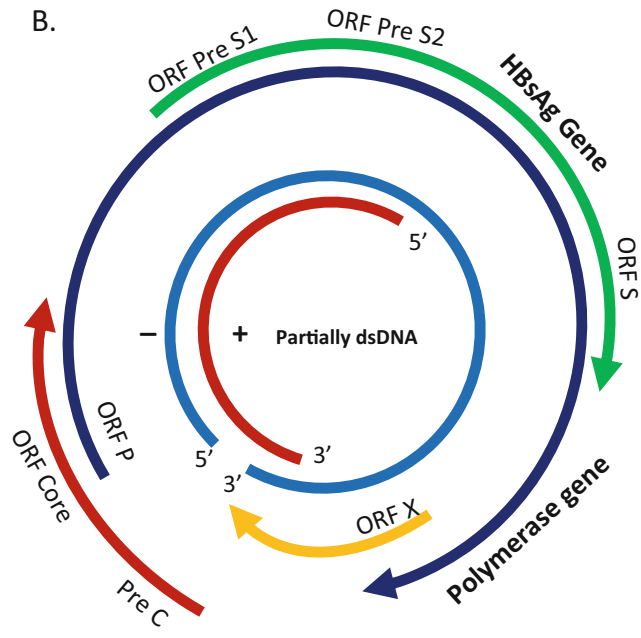

Fig. 5.1 (continued)

5.4 Overview of Current Therapies

Interferon alpha 2b (FDA approved in 1991) and peginterferon alpha-2a (approved in 2005) are immunomodulators administered subcutaneously, but due to adverse effects treatment duration varies up to 48 weeks (Table 5.1) [26, 27]. There are reports that HBV genotype A may present a higher response rate considering HBeAg seroconversion [28, 29].

Lamivudine (approved in 1998), adefovir (approved in 2002), entecavir (approved in 2005), telbivudine (approved in 2006), tenofovir (approved in 2008), and tenofovir alafenamide (approved in 2016) are nucleoside analogs used orally, with fewer adverse events compared to immunomodulators and very efficient to reduce viral load (Table 5.1 and Fig. 5.2) [27]. However, a functional cure (loss of HBsAg) is rarely seen with these therapies. Duration of treatment varies, most of the time lasting several years. Because of the long-term need for these medications, adhesion to treatment is a concern, together with the development of drug resistance [21, 30].

Other nucleoside analogs approved are clevudine (approved for HBV in South Korea and the Philippines) and besifovir (nucleotide approved in South Korea) [31]. Although clevudine was approved as an antiviral agent for HBV without significant toxicity during the six-month clinical trial, longer therapy (14 months) was found to cause reversible mitochondrial myopathy [32]. This nucleoside analog was one of the few drugs that seemed to have an impact on HBV cccDNA in a woodchuck model (Tennant, personal communication).

Thymosin alpha-1 (Zadaxin) is an immune modulator, administered subcutaneously with minimal side effects approved as monotherapy for chronic hepatitis B in Asian countries [33]. The activity is via an enhancement of T cell differentiation and maturation and is especially effective in settings where there is a reduction in T cell number and/or function [33].

Table 5.1 Antivirals approved for chronic hepatitis B

Drug	Route	Class	HBV DNA EC_{50}, μM	Company	FDA approval year
Lamivudine	Oral	Nucleoside analog	0.56 [21]	GlaxoSmithKline	1998
Adefovir dipivoxil	Oral	Nucleotide analog	0.58 [21]	Gilead Sciences	2002
Entecavir	Oral	Nucleoside analog	0.00036 [21]	BMS	2005
Tenofovir disoproxil fumarate	Oral	Nucleotide analog	0.1 [22]	Gilead Sciences	2008
Telbivudine	Oral	Nucleoside analog	1.3 [23]	Novartis	2006
Tenofovir alafenamide	Oral	Nucleotide analog	0.0347 [24]	Gilead Sciences	2016
Interferon alpha 2b	Parenteral	Immunomodulator		Merck	1991
Peginterferon alpha 2a	Parenteral	Immunomodulator		Genentech	2005
Clevudine	Oral	Nucleoside analog	0.053 [21]	Bukwang/Esai Pharmaceuticals	2009
Besifovir (LB80380)	Oral	Nucleotide analog	0.5 [25]	LG Chem Ltd.	n/a
Thymosin alpha-1	Parenteral	Immunomodulator		SciClone Pharmaceuticals	

EC_{50} median effective concentration to inhibit HBV DNA replication, *n/a* not applicable

5.5 Drugs in the Pipeline

There are several novel antiviral agents being developed for chronic hepatitis B. The drugs can be divided according to their strategies to eradicate chronic HBV infection (Table 5.2) [34]:

1. Virologic (direct-acting agents or DAAs)
2. Host immune approaches (indirect-acting agents or immune therapy)

5.5.1 Direct-Acting Antiviral Agents (DAAs)

Virologic antiviral agents or DAAs are new therapies that could directly target HBV replication steps without killing infected cells [35]. Nucleoside analogs target the viral reverse transcriptase enzyme, thus inhibiting HBV replication. Several nucleoside analogs are approved for chronic hepatitis B treatment as mentioned above, but because they require long-term use and do not completely clear HBV from hepatocytes, new DAAs are being developed, and next we will discuss different strategies used.

5.5.1.1 Capsid Assembly Effectors or Modulators (CAM)

The HBV nucleocapsid plays an essential role in the viral replication cycle that includes HBV genome packaging, reverse transcription, intracellular trafficking of relaxed circular DNA (rcDNA) into the nucleus, and maintenance of chronic infection. Capsid assembly modulators (CAM) are characterized by two types (Table 5.3 and Fig. 5.3): (1) class I or heteroarylpyrimidines (HAP) are core protein allosteric modulators (CpAM) that upon binding to HBV capsids promote their misassembled to aberrant non-capsid core polymers, and (2) class II or phenylpropanamides (PP), sulfamoylbenzamides (SBA), or derivatives are capsid assembly modulators that upon binding to the capsid form normal but empty nonfunctional capsids devoid of pgRNA/rcDNA.

Both classes of HBV capsid effectors can interfere with several steps of HBV replication cycle including pre- and post-capsid formation, prevention of capsid assembly, perturbation of capsid integrity of incoming virus particles, entry of HBV capsid and core particles into the cell nucleus, pregenomic RNA encapsidation, and consequently its reverse transcription. All

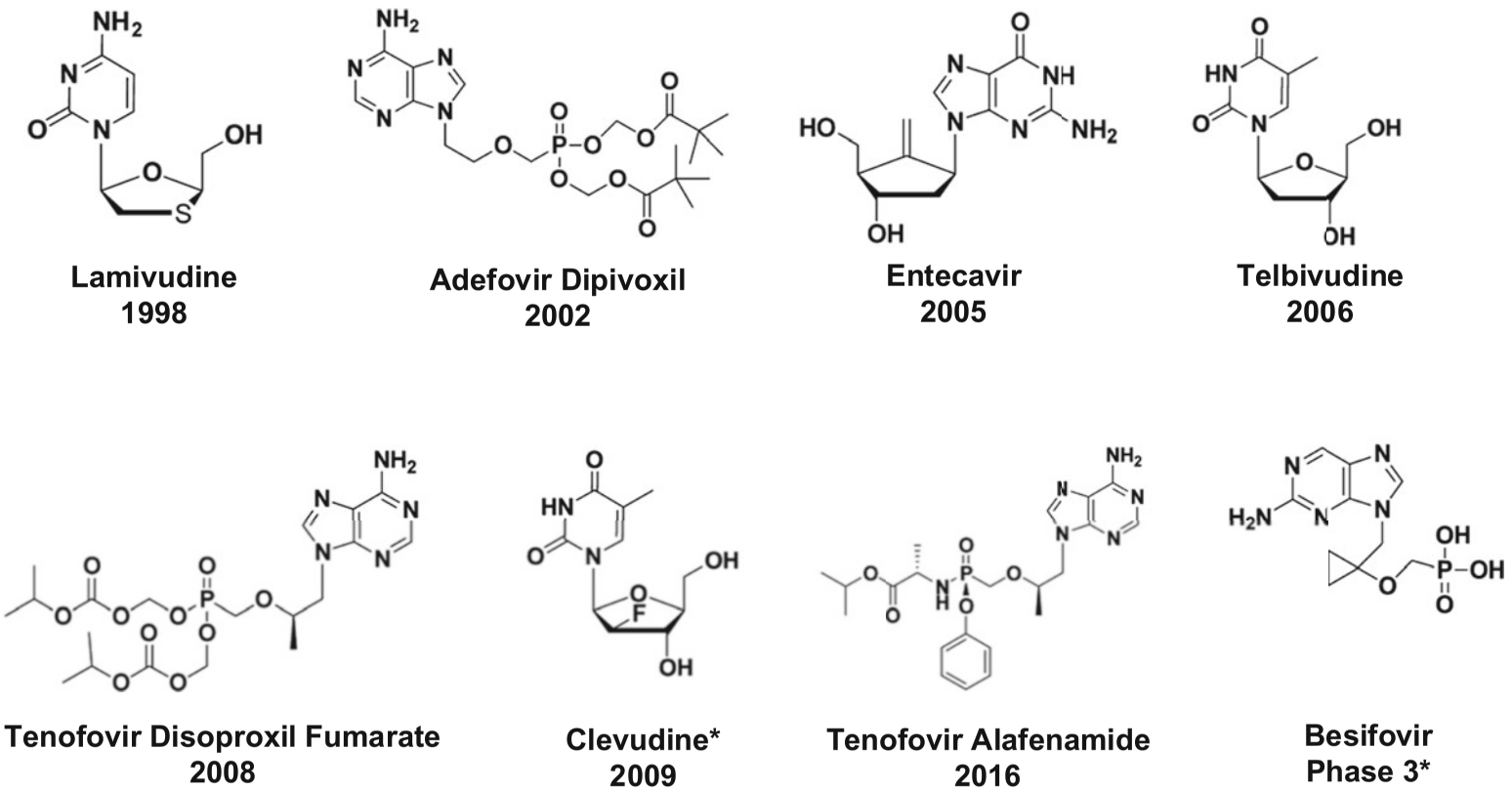

Fig. 5.2 Chemical structures of nucleoside/nucleotide analogs and year of FDA approval. *Approved in South Korea

Table 5.2 Classes of antiviral agents in the pipeline for HBV

Direct-acting antiviral agents (DAAs)
Capsid assembly inhibitors or modulators (CAM)
Entry inhibitor
Small interfering RNA (siRNA)
Nucleic acid polymers (NAPs)
HBsAg inhibitors
s-Antigen transport inhibiting oligonucleotide polymers (STOPs)
Antisense molecules
Nucleoside analogs
Indirect-acting agents (immune therapy)
Therapeutic vaccines
Innate immune stimulation
TLR-8 agonist
TLR-7 agonist
Host acting pathway
Apoptosis inducer
Cyclophilin inhibitor
Gene editing
Gene-editing CRISPR/Cas 9
Gene-editing ARCUS platform
Other mechanisms
Monoclonal antibody
FXR agonist
Host targeting antisense (LNA)
PD-L1
Cell immunotherapy
MicroRNA
Nucleic acid-directed HBV cell killing

these changes in the HBV replication cycle may ultimately prime inhibition of cccDNA formation and/or amplification (Fig. 5.1).

There are five capsid effectors in phase 2 clinical trials. Morphothiadin (GLS4) is a class I HAP compound developed from Bay41-4109 that has shown potent in vitro inhibition of HBV DNA replication; nevertheless, in vivo studies with health volunteers have shown that GLS4 needs an extra-booster (ritonavir) to increase its plasma concentration and achieve effective antiviral activity in humans [36]. Two CpAM ABI-H0731 and ABI-H2158 are in phase 2 clinical trials. ABI-H0731 has shown a decline in HBV RNA that correlated with HBV DNA decline in a 4-week therapy [37], and several phase 2 clinical trials are being conducted with this compound in combination with nucleoside analogs, including entecavir or tenofovir. ABI-H2158 has shown in vivo decline of HBV DNA and pgRNA by ~2 $\log_{10}$ IU/ml and is in phase 2 clinical trial in combination with entecavir [38]. JNJ56136379 is an inducer of empty nonfunctional HBV capsids (CAM-N) that was well tolerated by healthy volunteers in phase 1 and has shown reduced HBV DNA and RNA levels; in a 4-week phase 1b monotherapy study, baseline polymorphisms or enrichment of substitutions did not show an impact on virological response, though the emergence of resistance to longer treatments are underway in phase 2 studies [39]. QL-007 (Qilu, PR China) is in phase 2 clinical trials with entecavir or tenofovir for both safety and efficacy evaluation (Table 5.3).

Four capsid effectors are in phase 1 clinical trial including RG7907, EDP-514, ABI-H3733, and ZM-H1505R. RG7907 (RO7049389), a class I CpAM, reduced both HBV DNA and RNA

Table 5.3 Clinical status of capsid assembly effectors or modulators

Drug/class	Route	HBV DNA Log reduction[a]	Company	Clinical trial phase	ClinicalTrials.gov identifier
Morphothiadin (GLS4)/I	Oral	2.3	HEC Pharma, PR China	2	NCT03638076/NCT04147208/ NCT04147208
JNJ 56136379/ II	Oral	2.9	Janssen, Ireland	2	NCT02662712
ABI-H0731/II	Oral	3.9	Assembly Biosciences, USA	2	NCT03109730/NTC03780543/ NTC03577171/NTC03576066/ NTC04454567/NTC02908191
ABI-H2158/II	Oral	2.3	Assembly Biosciences, USA	2	NCT04398134
QL-007/	Oral	–	Qilu, PR China	2	NCT04157257/NCT04157699
RG7907 (RO7049389)/I	Oral	3–5	Roche, Switzerland	1	NCT02952924
EDP-514/II	Oral	> 4.0[b]	Enanta Pharma, USA	1	NCT04470388
ABI-H3733/II	Oral	5.0[c]	Assembly Biosciences, USA	1	NCT04271592
ZM-H1505R/ pyrazole	Oral	–	ZhiMeng Biopharma, PR China	1	NCT04220801
ALG-000184/ II	Oral	5.0[b]	Aligos Therapeutics, USA/Emory University	1	NCT04536337
GLP-26/II	Oral	1–3[b]	Emory University, Aligos Therapeutics	Preclinical	n/a
CB-HBV-001/ pyrazole	Oral	12[c]	ZhiMeng Biopharma, PR China	Preclinical	n/a

N/A not applicable
[a]HBV DNA Log_{10} IU/ml in vivo (data obtained from clinical trials)
[b]Log_{10} decrease in HBV DNA (data obtained from mice models)
[c]HBV DNA, EC_{50} (nM) in vitro

levels at the end of 28-days treatment, with favorable PK profiles [40]. EDP-514 is a class II core inhibitor that has shown to prevent de novo formation of cccDNA in human primary hepatocytes, and it is in phase 1a/1b study with healthy volunteers [41]. ABI-H3733 is a class II capsid inhibitor that has shown to be a potent inhibitor of HBV DNA (EC_{50} = 5 nM) and cccDNA formation (EC_{50} = 125 nM) in vitro [42]. ZM-H1505R is a new pyrazole compound that inhibits HBV DNA replication by inhibiting pgRNA encapsidation and cccDNA formation.

Three main capsid effectors are in preclinical studies: GLP-26, ALG-000184, and CB-HBV-001. GLP-26 (Emory University) is a novel class II CAM, with a unique glyoxamidopyrrolo backbone. It showed substantial in vitro effects in HBV DNA replication and HBe antigen with low nanomolar ranges (EC_{50} = 3 nM for both markers), with >1 log reduction in cccDNA, and no apparent cytotoxicity. Sustained decreases in HBeAg and HBsAg levels were also observed in HBV-infected humanized mouse model treated with GLP-26 in combination with entecavir up to 3 months after drug cessation [43–46]. ALG-000184 (Aligos Therapeutics/Emory University) is the prodrug of ALG-001075, another potent class II CAM that has shown picomolar activity in vitro and substantial effects in HBV DNA replication in mouse

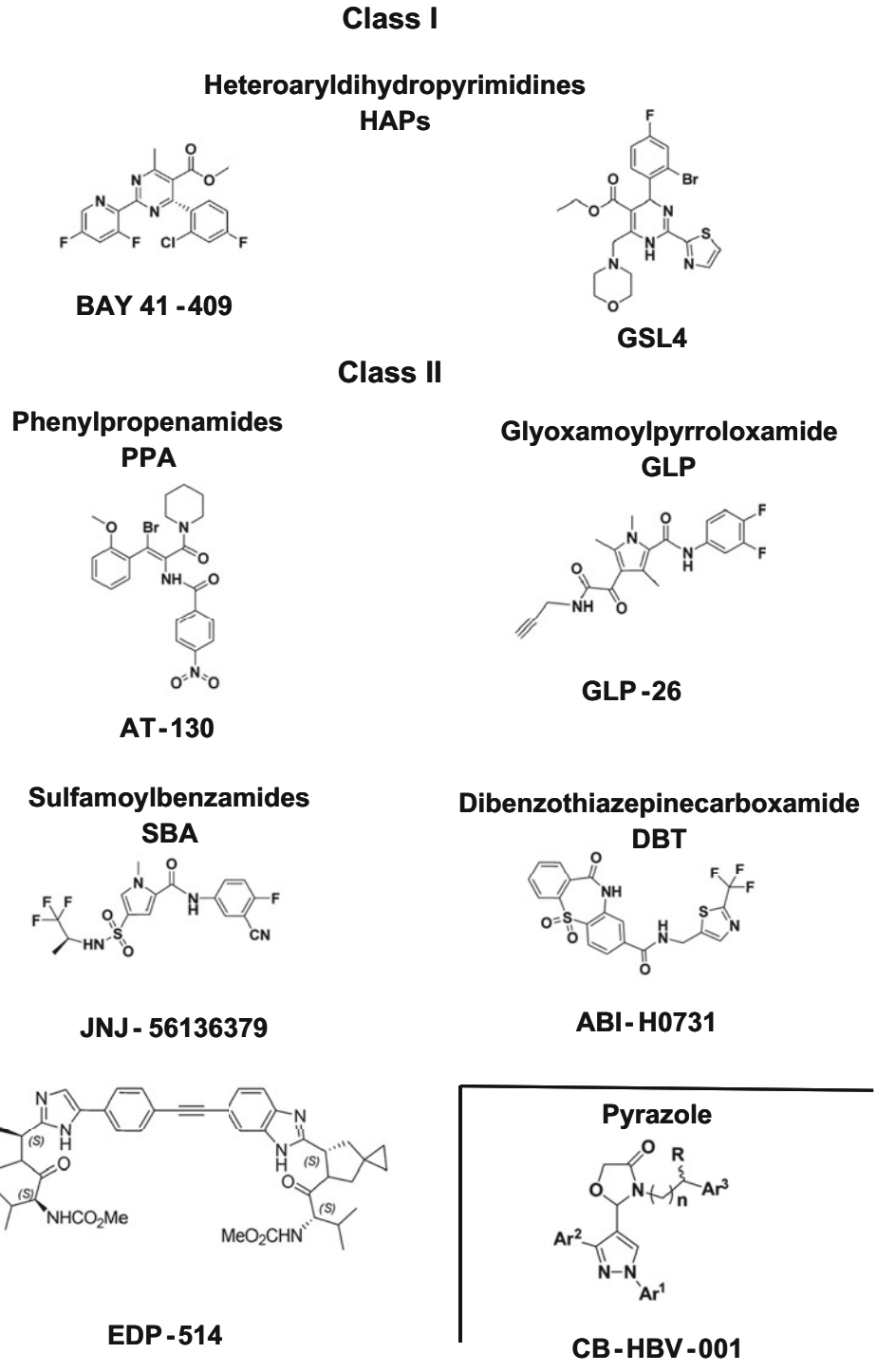

Fig. 5.3 Chemical structures of prototypical capsid assembly effectors or modulators (CAM)

Table 5.4 Entry Inhibitor in development

Drug	Route	Dose	Company	Clinical trial phase	ClinicalTrials.gov identifier
Bulevirtide (myrcludex B)	Parenteral	2–5 mg SC qd 48 weeks	Hepatera, Russia with MYR GmbH, Germany	2b	NCT02888106

model, with no apparent signs of toxicity and markedly improved solubility [47]. This drug is now entering phase 1a/1b clinical trial in New Zealand, Hong Kong and Republic of Moldova. CB-HBV-001 is a new oxazolidinone, pyrazole capsid inhibitor that is being evaluated in preclinical trials (AASLD 2018).

5.5.1.2 Entry Inhibitors

HBV enters the cell by attaching the receptor binding region of pre-S1 to the NTCP receptor at the membrane of the hepatocyte [48] (Fig. 5.1). Bulevirtide (Myrcludex B) binds irreversibly to NTCP inhibiting the HBV entry into the hepatocyte [49]. This drug is administered subcutaneously and is being studied for chronic hepatitis B and delta in phase 2b with or without peginterferon (PEG-IFN) alfa-2a (Table 5.4) [49]. Preliminary results showed that 12/30 (40%) of individuals treated with bulevirtide plus PEG-IFN for 48 weeks had alanine aminotransferase (ALT) normalization and HDV RNA negative. In the follow-up of 24 weeks of treatment, 4 out 15 individuals treated with 2 mg bulevirtide plus PEG-IFN had undetectable HBsAg, and three out four had HBsAg seroconversion [50]. Bulevirtide was well tolerated, with some drug-related adverse events primarily caused by an increase in total bile salts [50]. This is explained because the drug binding to NTCP prevents infection but also inhibits hepatic bile salt uptake leading to the transiently elevated bile salt level [51].

5.5.1.3 Small Interfering RNA (siRNA)

RNA interference (RNAi) is the mechanism through which double-stranded RNAs silence cognate genes (Fig. 5.1). It is characterized by the presence of RNAs about 22 nucleotides homologous to the gene that is being suppressed. Dicer is the cellular nuclease that cleaves double-stranded RNAs and can produce putative guide RNAs or small interfering RNA (siRNA) [52]. After the sense strand is removed and the antisense strand is loaded on the RNA-induced silence complex (RISC), it hybridizes to a complementary region of a target mRNA, which results in its degradation [53]. This phenomenon provides effective agents for inhibiting infectious, metabolic, cancer, and genetic diseases [53]. A critical issue in the development of siRNA-based drugs is to avoid toxicity such as (1) immunogenic reactions to dsRNA (2′-O-methyl base modifications have largely avoided this issue), (2) toxicity of excipients (work continues on developing potent and nontoxic nanoparticles), (3) unintended RNAi activity (avoided by detailed screening target sites against human genome sequences), and (4) on target RNAi activity in nontarget tissues (selection of highly diseased selective genes and delivery routes which reduce accumulation in nontarget tissues) [54]. Previous studies showed that siRNA could significantly inhibit HBV transcripts and cccDNA in vitro in HepG2 cells and in vivo in mice [53, 55, 56]. Currently, several siRNAs are being evaluated in preclinical and phases 1, 1/2, and 2 clinical trials shown in Table 5.5. VIR-2218 has shown dose-dependent HBsAg reductions (mean decline of 1.0 $\log_{10}$) in HBeAg negative or positive patients virally suppressed on nucleos(t)ide analogs without significant fibrosis [58]. Another siRNA drug, JNJ-3989 (ARO-HBV) that is in a phase 2a study, has demonstrated a $\geq$1.0 $\log_{10}$ reduction in HBsAg at nadir was achieved in 98% of patients [59]. In total, 15/38 (39%) of patients who were responders throughout the study were sustained responder at day 392 [59].

5.5.1.4 Nucleic Acid Polymers (NAPs)

NAPs are phosphorothioate oligonucleotides (PS-ONs) that inhibit HBV via a post-entry mechanism blocking the assembly/release of HBV

Table 5.5 Small interfering RNA (siRNA) drugs in development

Drug	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
VIR-2218 (ALN-HBV02)	Parenteral (SC)	Alnylam and Vir Biotech, USA	2	NCT03672188
JNJ-3989 (ARO-HBV)	Parenteral (SC)	Arrowhead Pharma with Janssen, USA	2a	NCT03365947
RG6346 (DCR HBVS)	Parenteral (SC)	Roche, Switzerland	2/1	NCT03772249
AB-729 [57]	Parenteral (SC)	Arbutus Biopharma, USA	1	NA

NA not available

Table 5.6 HBsAg inhibitors in development

Drug	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
REP 2139	Parenteral	Replicor, Canada	2	NCT02726789
REP 2165	Parenteral	Replicor, Canada	2	NCT02565719

subviral particles (Fig. 5.1). The universal model for NAP pharmacology is based on the interaction of the amphipathic protein domain and the hydrophobic side of NAPs, preventing the conformational changes in the target or its interaction with other amphipathic helices [60]. In this class of antivirals, there are the HBsAg inhibitors and the STOPs (s-antigen transport inhibiting oligonucleotide polymers).

HBsAg Inhibitors

Aside from the ability of HBsAg to sequester anti-HBs from the blood system, HBsAg has direct immunoinhibitory action against both innate and adaptive immune responses (Fig. 5.1). HBsAg loss is infrequently achieved with the current therapy; therefore, antivirals targeting the inhibition of HBsAg are being developed. NAPs have the ability to interact with hydrophobic surfaces of proteins and have emerged as the first therapy to be able to achieve rapid HBsAg loss [61].

REP 2139 is a phosphorothioate oligodeoxyribonucleotide (PS-ONs) with the sequence $(dAdC)_{20}$ (Table 5.6 and Fig. 5.4) [62]. Clinical studies of REP 2139 in combination with thymosin or PEG-IFN was well tolerated and resulted in liver flares (without liver dysfunctions) following initial reductions of serum HBsAg and HBV DNA [62]. REP 2165 is a version of REP 2139 which has been shown preclinically to retain antiviral activity with lower accumulation in the liver. The results of phase 2 randomized trial showed that addition of NAPs to tenofovir and PEG-IFN increased functional cure after therapy without altering tolerability [63].

STOPs (s-Antigen Transport Inhibiting Oligonucleotide Polymers)

STOPs are oligonucleotide aptamers (protein binding) comprised of a repeating poly AC sequence (Fig. 5.1). STOPs share the structural similarity with NAPs but contain several novel chemical features. STOPs can reduce HBsAg secretion by affecting protein trafficking from the infected cell resulting in its degradation [64]. In HepG2.2.15 cells, ALG-10133 reduced HBsAg secretion in nanomolar range and with synergistic effects when combined with class II CAMs [65]. ALG-10133 has been selected as the lead candidate, starting clinical trials on 2020 with projected human efficacious dose of 30–75 mg delivered SC weekly (Table 5.7) [66].

5.5.1.5 Antisense Molecules

Antisense oligonucleotides (ASO) (Table 5.8.) are small single-stranded nucleic acid sequences that bind with high selectivity to their target RNAs. This triggers degradation via an RNAse H-dependent pathway [68]. GSK 3228836 is a

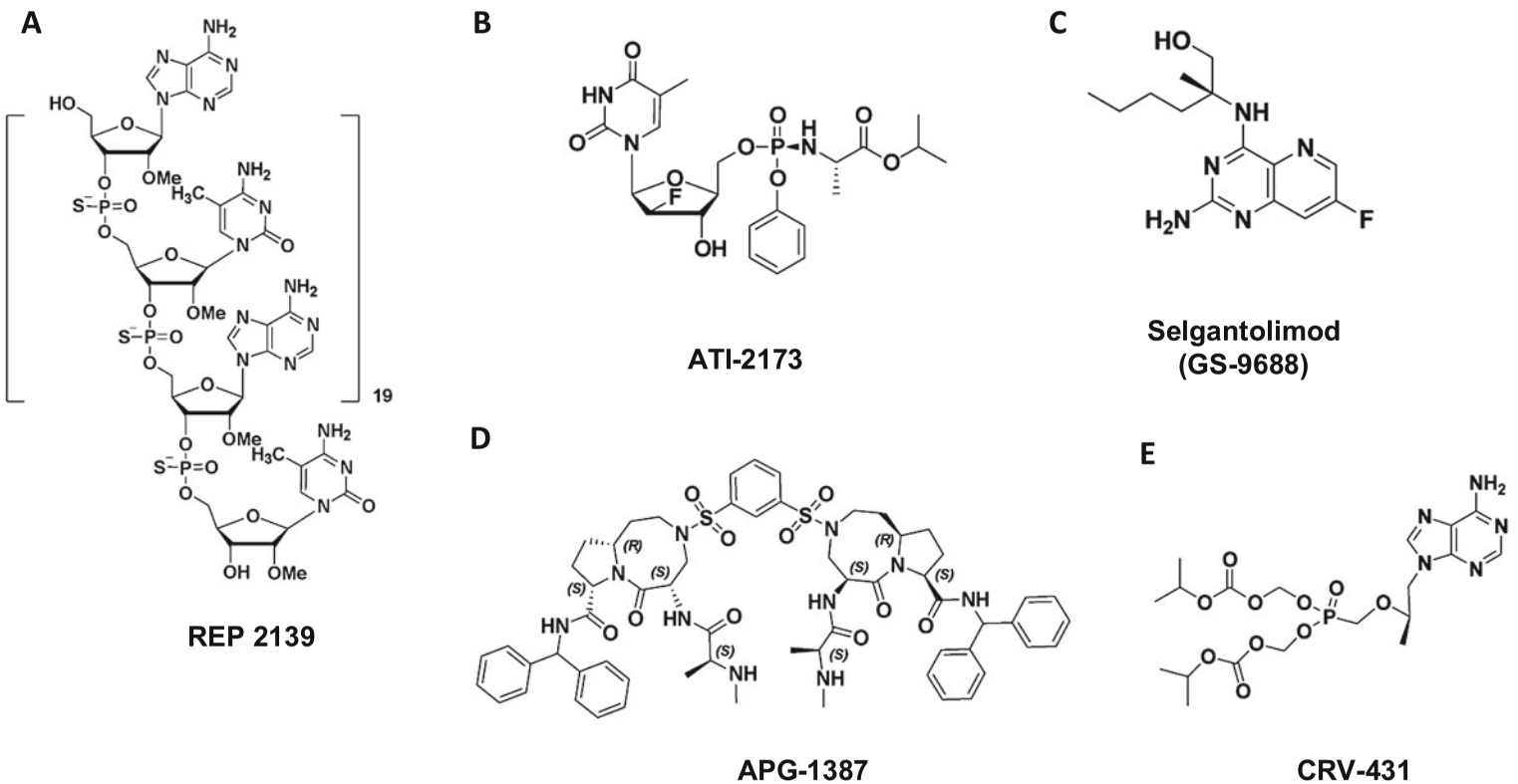

Fig. 5.4 Chemical structures of HBsAg inhibitor (**a**), nucleoside analog (**b**), innate immune stimulation (**c**), cellular inhibitor of apoptosis proteins (cIAPs) (**d**), and cyclophilin inhibitor (**e**)

Table 5.7 STOPs (s-antigen transport inhibiting oligonucleotide polymers) in development

Drug	EC_{50} μM	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
ALG-10133	0.0032	Parenteral (SC)	Aligos Therapeutics, USA	1	NCT04485663

EC_{50} median effective concentration to inhibit HBV DNA replication

Table 5.8. Antisense molecules in development

Drug	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
GSK 3228836 /IONIS-HBVRx/ ISIS505358	Parenteral (SC) [67]	Ionis with GSK, USA	2a	NCT04449029/ NCT02981602
GSK3389404/Ionis-HBV-LRx	Parenteral (SC)	Ionis with GSK	2a	NCT02647281
RO7062931	Parenteral (SC)	Roche	1a	NCT03505190/ NCT03038113

Table 5.9 Nucleoside analog in development

Drug	EC_{50} μM	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
ATI-2173	0.0013	Oral	Antios Therapeutics, USA	1	NCT04248426

EC_{50} median effective concentration to inhibit HBV DNA replication

2'-O-methoxyethyl free ASO currently in development for the treatment of chronic hepatitis B. It has been tested as a subcutaneous injection in doses up to 120 mg, and no safety concerns were identified [69]. GSK3389404 (GSK404) is a second-generation ASO that showed an acceptable safety profile [70]. GSK3389404 presented platelet dose-dependent declines that plateaued on treatment and started to recover after dose completion [70, 71]. RO7062931 is an N-acetylgalactosamine (GalNAc) conjugated single-stranded oligonucleotide (SSO) with locked nucleic acid (LNA) that is complementary to messenger RNAs (mRNAs) of the HBV genome [72, 73]. Gal-Nac conjugation should reduce ASO renal and platelet toxicities. It was well tolerated in healthy volunteers. Phase 1 studies showed a mean nadir of HBsAg of -0.5 $\log_{10}$ IU/mL, with treatment emergent ALT elevations with transient concurrent HBsAg decline (0.6–0.8 $\log_{10}$ IU/mL) with no changes in liver function [74].

5.5.1.6 Nucleoside Analogs

ATI-2173 is a novel phosphoramidate prodrug of clevudine in preclinical studies for chronic hepatitis B (Table 5.9 and Fig. 5.4) [67]. Long-term use of clevudine was found to exhibit reversible skeletal myopathy in a small group of individuals and therefore subsequently discontinued from development. ATI-2173 was designed by modifying clevudine that bypasses the first phosphorylation step where the 5-′-monophosphate is converted to the active 5-′-triphosphate in the liver [67]. ATI-2173 activity was decreased by 25 viral polymerase mutations associated with entecavir, lamivudine, and adefovir resistance, but not capsid inhibitor resistance mutations [67]. It has been claimed that this compound could behave as a non-nucleoside antiviral agent.

5.5.1.7 RNAseH Inhibitors

RNAseH is one of the two enzymatically active domains on HBV polymerase and destroys the HBV RNA after it has been copied into DNA by the reverse transcriptase [75]. RNAseH is a potential target for antiviral drugs, and over 150 RNAseH inhibitors are divided in four compound classes: (1) α-hydroxytropolones (αHT), (2) N-hydroxyisoquinolinediones (HID), (3) N-hydroxypyridinediones (HPD), and

(4) and N-hydroxynapthyridinones [76–81]. Novel amide αHT were studied with EC_{50} values from 0.31 to 54 μM [79]. Studies in chimeric mouse showed that an HPD and an αHT suppressed HBV replication to up to 1.4 log_{10} after two weeks of treatment followed by a rebound in the viral titers [82].

5.5.2 Indirectly Acting Antiviral Agents (Immune Therapy)

Specific immune therapy can maintain the HBV replication under control of a functional host antiviral response [9] (Fig. 5.1). An example of approved immune therapy for chronic hepatitis B is interferon alpha (pegylated or not). Pegylated interferon alpha alone or in combination therapy can achieve sustained off-treatment control but in only a small portion of individuals [26].

Therapeutic restoration of protective immunity is a strategy that can be considered to achieve the functional cure of HBV [83]. Several approaches are being considered such as therapeutic vaccines, innate immune stimulation (TLR-8 and TLR-7 agonists), host acting pathway (apoptosis inducer and cyclophilin inhibitor), gene editing, and many other mechanisms.

5.5.2.1 Therapeutic Vaccines

There is a renewed interest in therapeutic vaccines with the development of novel formulations, suitable immunization routes for designed adequate antigens, and adjuvant strategies (Table 5.10). In addition, it is important to consider adequate strategies, including combination therapy with other antivirals, either concomitant or sequential strategies.

5.5.2.2 Innate Immune Stimulation

The host immune responses to HBV determine if the individuals will clear (functional cure) or fail to clear the virus (chronic hepatitis B). Toll-like receptor (TLR) family and its functions are one way to modulate the immunological host responses [96]. TLR8 and TLR7 are endosomal TLRs members with a high degree of sequence and function similarity. They recognize pathogen-associated molecular patterns (viral single-stranded RNA fragments) and trigger innate and adaptive immune responses [96, 97]. Agonist ligands of Toll-like receptors 7 and 8 have immune-stimulating activity allowing to intervene several diseases and to be valuable vaccine adjuvant candidates [96].

Selgantolimod (formerly GS-9688) is a small molecular agonist of Toll-like receptor 8 (TLR8) [98]. It sustained reduced intrahepatic RNA and DNA of woodchuck hepatitis virus (WHV) in animal model. With a finite, short duration treatment, the serum WHsAg level reduced with half of animals with levels below the limit of detection [97]. Selgantolimod is an oral drug under phase 2 clinical trial (Table 5.11 and Fig. 5.4). RO7020531 (RG7854) is an oral prodrug of a TLR-7 agonist in phase 2 clinical trial (Table 5.11). Carboxylesterase (mainly CES2) and oxidation by aldehyde oxidase converts RO702053 into the active metabolite RO7011785 [99]. Preclinical data showed that a combination of HBV locked nucleic acid antisense oligonucleotide (HBV-LNA ASO) with RO7020531 reduced HBsAg and HBV DNA with delayed rebound off-treatment in mice [100].

5.5.2.3 Host Acting Pathway

Cellular inhibitor of apoptosis proteins (cIAPs) impairs clearance of hepatitis B virus (HBV) infection by preventing TNF-mediated killing/death of infected cells. Animal studies showed that drug inhibitors of cIAPs were able to reduce serum HBV DNA, hepatitis B surface, and core antigens [101]. APG-1387 is an apoptosis inducer; it is a second mitochondria-derived activator of caspase (SMAC) mimetic, and it targets inhibitors of apoptosis proteins (IAPs) [102]. Currently, APG-1387 is under clinical trial phase 1 study for chronic hepatitis B (Table 5.9 and Fig. 5.4).

CRV-431 is an oral cyclophilin inhibitor, non-immunosuppressive analog of cyclosporine A. CRV 431 is a small molecule under clinical development for the treatment of liver diseases including fibrosis and hepatocellular carcinoma [103]. Preclinical studies showed antiviral activity against hepatitis B reducing HBV DNA and

Table 5.10 Therapeutic vaccines in development

Drug	Platform	Route	Company	Clinical trial phase	ClinicalTrials.gov Identifier
NASVAC (ABX203)	HBs and HBc antigen mixed with carboxyl vinyl polymer [84]	Nasal	CIGB, Cuba	3	NCT01374308/ NCT02249988
HepTcell (FP-02.2)	Synthetic HBV-derived peptides formulated with IC31®, a TLR9-based adjuvant [85]	IM [86]	Altimmune, USA	2	NCT02496897
AIC 649	Parapoxvirus (iPPVO) [87]	IV	AiCuris, Germany	1	
JNJ 64300535 (HB-110)	Plasmids encoding HBsAg, HBcAg, and IL12 [88]	IM Electroporation	Janssen, Ireland/Ichor Medical Genexine, USA	1	NCT03463369
TG1050	Ad5 which encodes truncated HBV core, POL, and two small domains from the ENV [89]	SC	Transgene, France	1	NCT04168333/ NCT02909023/ NCT04168333
MVA-VLP-HBV	Modified vaccinia Ankara-virus-like particle-hepatitis B virus [90]		GeoVax, USA	Preclinical	n/a
Chimigen HBV	Recombinant chimeric fusion protein comprising hepatitis B virus (HBV) S1 and S2 surface antigen fragments, core antigen, and a murine monoclonal antibody heavy chain fragment (Fc) [91]		Akshaya, Canada	Preclinical	n/a
TherVacB	HBsAg, HBcAg, and a boost using a modified vaccinia virus Ankara (MVA) vector [92]		Helmholtz Zentrum Muenchen, Germany	Preclinical	n/a
3xT2A and Mix2A	VLVs expressing polymerase (Pol), core (HBcAg), and MHBs [93]		CaroGen, USA	Preclinical	n/a
HBV	TheraT® and VaxWave® investigational arenavirus-based immunization technologies [94]		HOOKIPA Pharma, Austria, with Gilead	Preclinical	n/a
VBI-2601 (BRII-179)	Recombinant, protein-based immunotherapeutic [95]		VBI Vaccines, USA	1b/2a	

ID intradermal, *SC* subcutaneous, *Ad* adenovirus, *POL* polymerase, *ENV* envelope, *VLV* virus-like vesicles, *N/A* not applicable

Table 5.11 Innate immune stimulation in development

Drug	Class	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
GS9688 (Selgantolimod)	TLR-8 agonist	Oral	Gilead Sciences, USA	2	NCT03491553/NCT03615066
RO7020531 (RG7854)	TLR-7 agonist	Oral	Roche, Switzerland	2	NCT02956850/NCT03530917/ NCT04225715

Table 5.12 Host-acting pathway

Drug	Class	Route	Company	Clinical trial phase	ClinicalTrials.gov identifier
APG-1387	Apoptosis inducer	IV	Ascentage Pharma, PR China	1	NCT03585322
CRV-431	Cyclophilin inhibitor	Oral [103]	Hepion, USA (formerly ContraVir)	1	NCT03596697

IV intravenous

Table 5.13 Gene editing

Drug	Class	Company	Clinical trial phase
EBT107	Gene-editing CRISPR/Cas 9	Excision Bio, USA	Preclinical
ARCUS nucleases	Gene-editing ARCUS platform	Precision Biosciences, USA	Preclinical

IV intravenous

HBsAg levels in transgenic mice and a phase 1 is ongoing (Table 5.12 and Fig. 5.4) [104].

5.5.2.4 Gene Editing

Clustered regularly interspaced short palindrome repeats (CRISPR)/Cas9-based antiviral strategy is one of the most versatile gene-editing tools, discovered as a bacterial adaptive immune system [105]. The CRISPR/Cas9 system can specifically destruct HBV genomes in vitro and in vivo, mediating specific cleavage of cccDNA [106–108] (Fig. 5.1). Several optimal targets in HBV genome have been described, such as the surface and polymerase overlap region; the YMDD RT motif and the HBV enhancer I, II, X protein; and pre-core regions with high efficacy [109]. However, CRISPR/Cas system inevitably targets integrated HBV DNA and induces double-strand breaks (DSBs) of host genome, raising concerns of genome instability and carcinogenicity [108, 110]. To avoid DSBs of the host genome, recently it was described a permanently Cas9-mediated base editors that effectively introduced nonsense mutations that generated premature stop codons of surface gene in both integrated and cccDNA reducing HBsAg secretion [110]. EBT107 is a gene-editing CRISPR/Cas 9 drug that uses a duplex gRNA excision knockout as a candidate for HBV in preclinical studies (Table 5.13) [111].

ARCUS genome-editing technology is another platform of gene editing being developed for chronic hepatitis B [112]. The ARCUS technology is based on the properties of a naturally occurring gene-editing enzyme – the homing endonuclease I-CreI—and reduces the risk of additional off-target DNA edits [113].

5.5.2.5 Other Mechanisms

Recombinant hepatitis B human monoclonal antibody: Lenvervimab (GC1102) is a recombinant hepatitis B human monoclonal antibody expected to improve sustained virological response reducing HBsAg levels in individuals with chronic hepatitis B infection [114]. It is under study for HBV-related liver transplant recipients (Table 5.14).

Farnesoid X receptor (FXR) agonist: HBV enters the hepatocyte by binding to NTCP, the genome of which contains two active farnesoid X receptor (FXR)α response elements that participate in HBV transcriptional activity [115]. In vitro studies showed that FXR agonists inhibited viral mRNA, DNA, and protein production and reduced the cccDNA pool size [115]. Vonafexor (EYP001) is a farnesoid X receptor (FXR) agonist with anti-HBV effects [116, 117]. It is under study in combination with PEG-IFN, nucleoside analogs in double or triple therapy (Table 5.14).

PD-L1 pathway: The programmed cell death protein 1 (PD-1)/programmed death-ligand 1(PD-L1) pathway is a key immune checkpoint regulator that controls the induction and maintenance of

Table 5.14 Drugs with other mechanisms in development

Drug	Route	Mechanisms	Company	Clinical trial phase	ClinicalTrials.gov identifier
Lenvervimab (GC1102)	IV	Monoclonal antibody	Green Cross, South Korea	2a	NCT03801798/ NCT02304315
Vonafexor (EYP001)	Oral	FXR agonist	Enyo Pharma, France	1	NCT04365933
ASC22 (KN035)	SC	PD-L1 pathway	Ascletis Pharma, PR China	2a	NCT04465890
LTCR-H2-1	IV	T cell immunotherapy	Lion TCR, Singapore	1	NCT04745403

IV intravenous, *SC* subcutaneous, *N/A* not applicable

immune tolerance in chronic hepatitis B infection [118]. ASC22 (KN035) is a novel fusion anti-PD-L1 antibody being studied for the treatment of solid tumors and in clinical trials for chronic hepatitis B phase 2a (Table 5.14).

T cell immunotherapy: LTCR-H2-1 (Table 5.14) is a preclinical drug that boosts adaptive immune response through T cell receptor (TCR) gene transfer [119]. It is engineered to target virus-derived peptides presented on MHC class I on the surface of virus-infected cells. This technology is based on leukapheresis to isolate white blood cells, followed by T cell expansion; HBV targeting TCR are introduced into the activated T cells by viral transduction or electroporation, and then after phenotypic and functional validation, the TCR-engineered T cells are infused back into the individual [120].

5.6 Conclusions

Currently, nucleoside analogs and peginterferon are available for chronic hepatitis B treatment and are quite effective and safe. They can prevent progression of disease, but even persons treated with these drugs can develop hepatocellular carcinoma. The treatments can achieve inhibition of HBV replication; however, few individuals achieve "functional cure" status (HBsAg clearance with or without surface antibody). Several novel drugs are in the pipeline for treatment and elimination of chronic hepatitis B. The drugs are at different stages of development from preclinical to phase 2 clinical trials, and some of them are considered for combination strategies. These drugs will be instrumental for a sustained HBV DNA undetectability with sustained clearance of HBsAg and for preventing liver cancer. Elimination of cccDNA and integrated HBV DNA will be key to eradicate chronic hepatitis B infection. Currently, there are numerous drugs that have the potential to cure HBV, but most do not have the necessary potency to clear all cccDNA. We now know that the half-life of cccDNA (several months and not decades) is shorter than was previously reported [121]. Thus, it may be possible to eliminate cccDNA in approximately 1 year with more potent agents or more likely a combined modality (e.g., capsid effector plus STOPs). As expounded above, a great number of approaches are being tried to eliminate HBV, and it is clear that we are beginning to turn the tide.

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Declaration of Competing Interest Drs. Schinazi and Bassit along with Emory University are entitled to equity and royalties related to anti-HBV products licensed to Aligos Therapeutics, Inc., being further evaluated in the

research described in this review. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

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Discovery and Development of Antiviral Therapies for Chronic Hepatitis C Virus Infection

6

Miguel Angel Martinez and Sandra Franco

Abstract

At the beginning of this decade, an estimated 71 million people were living with chronic hepatitis C virus (HCV) infection worldwide. After the acute stage of HCV infection, 18–34% of individuals exhibit spontaneous clearance. However, the remaining 66–82% of infected individuals progress to chronic HCV infection and are at subsequent risk of progression to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Chronic hepatitis C progression is generally slow during the first two decades of infection, but can be accelerated during this time in association with advancing age and cofactors, such as heavy alcohol intake and human immunodeficiency virus (HIV) co-infection. Since acute HCV infection is generally asymptomatic, HCV goes undiagnosed in a significant percentage of infected individuals. In 2014, direct-acting antiviral (DAA) therapy for chronic HCV was developed, which has increased the cure rates to nearly 100%. DAA therapy is among the best examples of success in the fight against viral infections. DAAs have transformed HCV management and have opened the door for the global eradication of HCV.

Keywords

HCV · Direct-acting antiviral (DAA) · Therapy

Abbreviations

ALT	Alanine aminotransferase
BOC	Boceprevir
CD	Cluster of differentiation
CLDN	Claudin
COVID	Coronavirus disease
DAA	Direct-acting antiviral
ERAP	Endoplasmic reticulum aminopeptidase
FDA	Food and Drug Administration
GPR	G-protein-coupled receptor
HCC	Hepatocellular carcinoma
HCV	Hepatitis c virus
HIV	Human immunodeficiency virus
IDU	Intravenous drug user
IFNG	Interferon-gamma
IFNG-AS	IFNG antisense RNA
IFNL	Interferon-lambda
IMP	Inosine-5′-monophosphate
IRES	Internal ribosome entry site
IU	International unit
KIR	Killer-cell immunoglobulin-like receptor

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LC	Light chain
LDV	Ledipasvir
MSM	Men who have sex with men
NS	Nonstructural
OCLN	Occludin
PCR	Polymerase chain reaction
RAS	Drug resistance-associated substitution
RdRP	RNA-dependent RNA polymerase
SMV	Simeprevir
SOF	Sofosbuvir
SR-B	Scavenger receptor class B
STAT	Signal transducer and activator of transcription
SVR	Sustained virological response
TVR	Telaprevir
USA	United States of America
UTR	Untranslated region
VEL	Velpatasvir
VLDL	Very-low-density lipoprotein

6.1 Introduction

The hepatitis C virus (HCV) is an enveloped positive-sense single-stranded RNA virus of the family *Flaviviridae*. It is predominantly a blood-borne virus, with very low risk of sexual or vertical transmission. Individuals at risk of infection predominantly include intravenous drug users (IDUs), recipients of contaminated blood products, and persons attending clinics where hygiene and sterilization processes are not correctly followed. HCV infection can also be acquired through certain sexual practices that may lead to blood exchange, particularly among some men who have sex with men (MSM).

The origin of HCV infection in humans remains unclear [1]. Until recently, HCV was the only known member of the genus *Hepacivirus*. Several years ago, other *Hepacivirus* RNA genomes were isolated from domestic dogs and horses [2, 3]. Additional hepaciviruses have also been found in small mammals, such as bats and rodents [4, 5]. It may be speculated that arthropod-borne HCV precursors could have been passed to insectivorous small mammals via the blood-borne route or through ingestion of insects. Then these HCV precursors might have diversified and eventually been transmitted to humans directly or via intermediate hosts [6].

HCV is hepatotropic and mainly replicates in liver hepatocytes; however, the initial manifestation of liver disease is primarily observed in hepatic stellate cells, causing liver inflammation and fibrosis. The interaction between HCV and hepatocytes leads to viral entry, which largely depends on the interaction of viral envelope glycoproteins with host lipoprotein components expressed at the hepatocyte surface. With an incubation period of 2–12 weeks, HCV infection begins with an acute phase that usually goes undiagnosed. After the acute phase, HCV infection is spontaneously cleared in 18–34% of infected individuals [7]. Infection eradication requires a robust broad HCV-specific CD8+ T-cell response [8]. Acute resolution of HCV is not associated with any long-term sequelae and almost never causes fulminant hepatic failure. The likelihood of spontaneous HCV resolution is associated with several genetic factors, including variations in interferon-lambda 3/4 protein (IFNL3/4) [9], the genes for major histocompatibility complex (MHC) and killer-cell immunoglobulin-like receptor (KIR) [10], the G-protein-coupled receptor 158 (GPR158) [11], and genes involved in immune responses, such as IFNG (interferon-gamma) antisense RNA 1 (IFNG-AS1), signal transducer and activator of transcription 1 (STAT1), and endoplasmic reticulum aminopeptidase 1 (ERAP1) [12].

Chronic infection is generally a slowly progressive disease, characterized by persistent hepatic inflammation leading to liver fibrosis and cirrhosis development in approximately 10–20% of infected individuals over 20–30 years of HCV infection [13] (Fig. 6.1). Once cirrhosis is established, disease progression is unpredictable. Cirrhosis can remain indolent for many years in some individuals while progressing to hepatocellular carcinoma (HCC), hepatic decompensation, and death in others. For

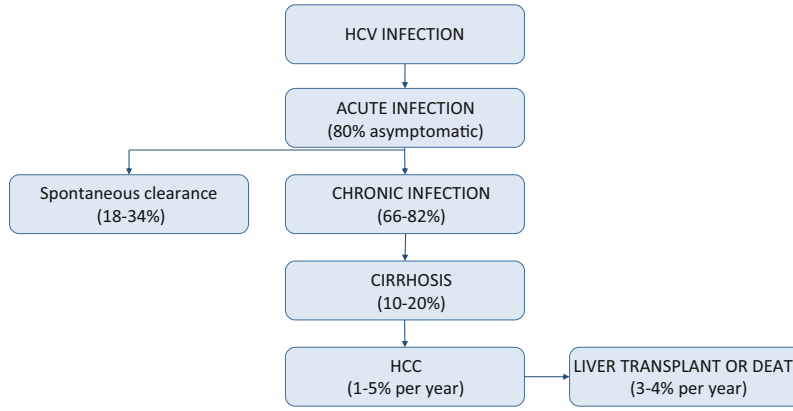

Fig. 6.1 Natural history of HCV infection. After acute HCV infection, the virus is spontaneously cleared in 18–34% of infected individuals. In cases of chronic HCV infection, after 20–30 years of infection, 10–20% of individuals will develop cirrhosis. After cirrhosis development, a patient carries a 1–5% annual risk of hepatocellular carcinoma (HCC)

individuals with decompensated cirrhosis and HCC, liver transplantation is the only potential salvage treatment option. Overall, after cirrhosis development, patients carry a 1–5% annual risk of HCC and a 3–6% annual risk of hepatic decompensation. After an episode of decompensation, the risk of death during the following year ranges from 15 to 20%. Fibrosis progression rates are not linear and are extremely variable, heavily influenced by age and years of infection. After 30 years of chronic hepatitis C infection, cirrhosis rates can reach 41%, almost three times higher than the rates predicted at 20 years of infection. Fibrosis progression can be influenced by host, viral, and environmental factors (e.g., alcohol intake).

According to the Global Hepatitis Report [14], in 2015, 71 million people were chronically infected with HCV, and there were an estimated 1.75 million new infections (global incidence: 23.7 per 100,000). The Global Burden of Disease Study estimated that 580,000 people died from HCV in 2017 [15]. The leading causes of HCV-related mortality were cirrhosis (342,000 deaths) and HCC (234,300 deaths). Decreased use of unsafe healthcare procedures has led to a reduced HCV incidence in the twenty-first century compared to in the second half of the twentieth century. Nevertheless, substantial regional variation exists, with persistently high HCV infection incidence in Europe (61.8 per 100,000) and in the eastern Mediterranean region (62.5 per 100,000). Country-specific data reveal that in the vast majority of countries, the annual HCV incidence peaked between 1970 and 2005. Injection drug users (IDUs) are predominantly affected in Europe, particularly in Eastern Europe. Egypt shows the highest prevalence, with 6.3% of the population affected in 2015 [16]. This is attributed to the use of nonsterile injections during a national campaign to treat schistosomiasis during the 1950s to 1980s—the largest-ever recorded iatrogenic transmission of an infectious disease [17, 18]. China has the greatest number of people living with HCV (10 million), followed by Pakistan (7.2 million) and India (6.2 million) [19].

HCV was first identified in 1989, thanks to the development of the polymerase chain reaction (PCR), which enabled the construction of a random-primed complementary DNA library from plasma, containing what was at that time called a non-A, non-B hepatitis agent [20]. Although acute HCV infection may be associated with jaundice and elevated alanine aminotransferase (ALT) levels, it is generally asymptomatic. Only one in five HCV-infected individuals is aware of their status [19], and thus a high proportion of HCV-attributable deaths remain unreported. Moreover, the global HCV-related health burden may also be underestimated because many infected individuals die of disorders that are caused by HCV infection but are not liver-related. The reduction of HCV transmission will require improved healthcare models and procedures, as well as improved screening and investment in HCV diagnostics, especially in high-risk populations.

6.2 HCV Replication

A single HCV particle has a diameter of approximately 68 nm (range: 45–86 nm) [21] and contains a linear positive-sense single-stranded RNA genome encoding ten viral proteins (Fig. 6.2) [22]. The HCV RNA genome is ~9.6 kb in length, with a single open reading frame and flanking 5′ and 3′ untranslated regions (UTRs), with a specific structure for viral RNA replication and translation. The 5′-UTR contains the internal ribosome entry site (IRES), from which begins the translation of an approximately 3000-amino acid polyprotein. This polyprotein is cleaved by cellular and viral proteases.

Cellular proteases cleave the HCV polyprotein into three structural proteins: core, E1, and E2 [23]. The HCV core protein encapsidates the positive strand viral genome, while the envelope glycoproteins E1 and E2 bind to the host proteins (designated as binding factors, co-receptors, or receptors) and promote virus entry into cells. The four main host factors mediating HCV entry are cluster of differentiation 81 (CD81), scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1), and occludin (OCLN). Expression of one or several of these host factors can confer cell susceptibility to HCV infection [24].

The HCV polyprotein is also cleaved into seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by the virus-encoded proteases NS2 and NS3/4A [23]. P7 is a membrane viroporin involved in HCV assembly and the release of virus from infected cells. The HCV nonstructural proteins NS2–NS5B play roles in virus replication and assembly [23]. During HCV polyprotein processing, the NS2 protease auto-cleaves *in cis* at the target site between NS2 and NS3. The NS3 protease, along with its cofactor NS4A, cleaves *in trans* at four targets in the polyprotein (NS3–NS4A, NS4A–NS4B, NS4B–NS5A, and NS5A–NS5B), thus releasing NS3, NS4A, NS4B, NS5A, and NS5B for their maturation. NS4B induces the formation of a membranous web that serves as the site of viral replication and assembly. The NS5A phosphoprotein is a replicase factor that interacts with many other viral and host proteins to regulate HCV replication. Finally, the NS5B polymerase synthesizes and replicates viral RNA to produce new viral genomes that are incorporated into the virus particles. Several host factors are involved in the virus replication and assembly processes. As for other positive-strand RNA viruses, HCV replication is exclusively cytoplasmic and involves no stable DNA intermediate.

HCV directly and indirectly induces autophagy through various mechanisms and temporally controls the autophagic flux, enabling the virus to maximize its replication and attenuate the innate immune responses that it activates [25]. The lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3) is a key event in autophagosome formation, which is induced upon HCV infection. Autophagosomes also serve as a platform for HCV RNA synthesis in the cell cytoplasm. Matured HCV particles are released from hepatocytes, primarily via the secretory pathways used by very-low-density lipoproteins (VLDL). Notably, exosomes, a component of microvesicles, carry the viral genome and assist in spreading the virus to naïve hepatocytes [26].

The complexity of the HCV replication cycle in hepatocytes may explain why infected sera has not exhibited efficient viral replication in experiments using cultured human hepatoma cells and primary hepatocytes or animal models, apart from chimpanzees. Following the identification of HCV in 1989, the development of HCV therapies was hindered by the absence of a virus replication system. In 1999, the first functional HCV replicon was generated [27]; it was a replicon of genotype 1b, which lacked virus structural proteins, included a selectable marker, and replicated in human hepatoma cells (Huh7). In 2005, the genotype 2a isolate JFH-1 was developed, which could grow in cell culture [28]—finally enabling recapitulation of the entire HCV life cycle in cell culture. The HCV replicon was essential for testing and refining candidate drugs against HCV enzymes and as a platform for conducting unbiased screens to identify new classes of HCV inhibitors.

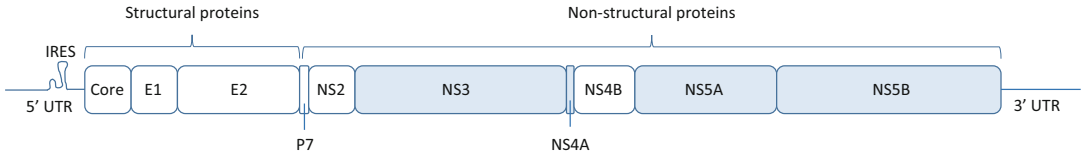

Fig. 6.2 HCV genome structure. The HCV genome has a length of approximately 10000 nucleotides and encodes 3 structural proteins (core, E1, and E2) and 7 nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). In the figure, the amino acid positions of these proteins are mapped, and the 5′ untranslated region (5′-UTR) and the 3′ untranslated region (3′-UTR) are indicated. Approved antiviral agents directly target NS3/4A, NS5A, and NS5B for effective inhibition of HCV replication

The enormous amount of data generated to understand the HCV life cycle has greatly improved our knowledge of virus biology, as well as enabled the production of HCV-specific direct-acting antiviral (DAAs) drugs. The successful identification of specific HIV antiretrovirals paved the road for targeting HCV proteins. HCV DAAs include NS3/4A protease inhibitors; NS5A phosphoprotein inhibitors and non-nucleoside polymerase inhibitors, which inhibit membranous web biogenesis and assembly; and nucleoside and nucleotide NS5B polymerase inhibitors, which block viral RNA synthesis (Fig. 6.2). Overall, four classes of drug actions have been discovered and translated to clinical use.

6.3 HCV Variability

Similar to other RNA viruses, genetic variability is a prominent feature of HCV [29, 30]. Experimental evidence demonstrates that HCV populations comprise a distribution of mutant genomes, termed quasispecies [31, 32]. This high genetic heterogeneity of HCV is supported by its high replication kinetics (10^{12} virions per day) and by the low fidelity of its RNA-dependent RNA polymerase (RdRP), which lacks the 3′-to-5′ exonuclease domain that proofreads RNA copies in replicative cellular DNA polymerases. These factors contribute to a high rate of viral mutation, with 10^{-4} to 10^{-5} misincorporations per copied nucleotide and replicative cycle. This high mutation rate, together with the evolutionary forces acting over time (selective pressure, recombination, and genetic drift), has yielded the generation of two levels of genetic heterogeneity: intra-host, i.e., the virus variant swarm (quasispecies) present in an infected individual, and inter-host, i.e., the worldwide circulating viruses that give rise to different virus genotypes and subtypes.

The extensive genetic inter-host heterogeneity of HCV in sub-Saharan Africa strongly suggests that this virus was endemic to this geographical area long before its global spread over the last 100–200 years. HCV has been classified into 8 genotypes and 105 subtypes based on genomic sequence variations [33, 34]. The different genotypes, named from 1 to 8 in order of their discovery, differ from each other by 30–35% of their nucleotide sequence. These genotypes are further divided into subtypes, defined by letters (1a, 1b, 2a, 2b, 3a, etc.), which differ from each other by 20–25% of their nucleotide sequence. Although the different genotypes and subtypes share basic biological and pathogenic characteristics, they differ in their epidemiology and responses to treatment.

Moreover, the prevalence rates and distributions of the different genotypes and subtypes vary according to geographical area [35]. Genotype 1 is the most prevalent genotype worldwide, with a higher prevalence of subtype 1a in the United States (USA) and Canada and of subtype 1b in Europe. Genotype 2 is predominant in West Africa, while genotype 3 is endemic to Southeast Asia. Genotype 4 is mainly found in the Middle East, Egypt, and Central Africa. Genotype 5 occurs almost exclusively in South Africa, while genotype 6 is predominantly distributed

throughout Asia. The recently discovered genotype 7 was identified in seven infected individuals in the Democratic Republic of the Congo, and genotype 8 was found in four infected individuals from Punjab (India). Worldwide, the most prevalent genotypes are 1 and 3, representing 44% and 25% of HCV infections, respectively. HCV genotype distribution also varies in accordance with the epidemiology of infected individuals (e.g., IDUs, hemophiliacs, MSM). For instance, genotype 4 is mainly prevalent in the Middle East, Egypt, and Central Africa, but now also shows high prevalence among IDUs in Southern Europe. As will be described in the next chapter, HCV genotype and subtype diversity affect the clinical efficacy of several DAAs. The natural virus variability includes mutations that confer resistance to different drugs, suggesting the possible necessity of virus genotyping before starting therapy.

Intra-host HCV genetic variability and quasispecies dynamics are also intimately related to both viral disease and antiviral treatment strategies [36, 37]. Intra-host genetic diversity is estimated to be over 10%. Quasispecies diversity presents challenges to host immune virus surveillance, antiviral therapies, and effective vaccine development. Nevertheless, genetic diversity is not equally distributed throughout the HCV genome. The structural proteins, which are most exposed to immune surveillance and pressure, show the highest diversity. Nonstructural virus proteins, which are the main targets of DAAs, are less heterogeneous, mainly because they must maintain their enzymatic activities. For instance, the NS3/4A protease, which is the target of several DAAs (Table 6.1), displays a nucleotide intra-host heterogeneity of $\leq$1% [36]. However, the error-prone nature of HCV (similar to other RNA viruses), along with its enormous capacity to adapt, renders HCV able to develop drug resistance-associated substitutions (RASs) upon replication in the presence of an inhibitor [38]. Depending on the fitness cost of an RAS, it may lead to a specific DAA becoming more or less potent for inhibiting HCV replication and thus more or less clinically effective.

6.4 HCV Therapy

In contrast to other chronic infectious diseases (e.g., HIV), HCV can be treated and cured. A chronically infected individual is considered cured of HCV infection when a sustained virological response (SVR) is achieved. SVR is defined as undetectable HCV RNA in serum or plasma at 12 or 24 weeks after completing treatment, based on a sensitive assay with a lower limit of detection of $\leq$15 international units (IU)/mL [39]. The rate of late relapse after achieving SVR is very low. In individuals without cirrhosis, SVR is generally associated with liver enzyme normalization and improvement or disappearance of liver inflammation and fibrosis. In contrast, individuals with advanced fibrosis (METAVIR F3 in individuals without cirrhosis) or cirrhosis (F4) remain at risk of life-threatening complications even with SVR. The METAVIR scoring system is used to assess the extent of inflammation and fibrosis in individuals with hepatitis C through histopathological evaluation of a liver biopsy. The METAVIR stage represents the amount of liver fibrosis: F0, no fibrosis; F1–3, mild to high fibrosis; and F4, cirrhosis. At any METAVIR stage, SVR may result in fibrosis regression, reduction of portal hypertension, and reduced risks of HCC and mortality. The benefits of SVR are even greater in the presence of risk cofactors, such as metabolic syndrome, alcohol consumption, or HIV co-infection [40]. Overall, curative HCV treatment is highly recommended in all acute or chronically infected individuals.

In 1997, interferon alfacon-1 (Infergen®) became the first US Food and Drug Administration (FDA)-approved drug for use against HCV infections (Fig. 6.3). However, this drug was discontinued in 2013 due to severe adverse events. The arsenal to combat HCV infections grew to include ribavirin (Copegus®, Rebetol®, Virazole®) in 1998, pegylated interferon 2b alfa (PegIntron®, Intron®-A) in 2001, and pegylated interferon 2a alfa (Pegasys®, Roferon®-A) in 2002 [41] (Fig. 6.3). Before 2011, interferon and ribavirin were the standard-of-care therapies; however, they exhibited rather low clinical

Table 6.1 Therapies for HCV infection approved by the FDA

Brand name	DAA	Drug target	Genotype	FDA-approved therapy	Date
Victrelis®	Boceprevir	NS3/4A	1	PegIFN[a]α/RBV for 4 weeks + 200 mg boceprevir, plus PegIFNα/RBV[b] for 44 weeks	May 2011
Incivek®	Telaprevir	NS3/4A	1	1125 mg telaprevir + PegIFNα/RBV for 12 weeks, plus PegIFNα/RBV for 12 or 36 weeks	May 2011
Olysio®	Simeprevir	NS3/4A	1, 4	150 mg simeprevir + 400 mg sofosbuvir for 12 or 24 weeks or 150 mg simeprevir for 12 weeks plus PegIFNα/RBV for 24 or 48 weeks	Nov 2013
Sovaldi®	Sofosbuvir	NS5Ba[c]	1–4	400 mg sofosbuvir + RBV for 12 or 24 weeks or 400 mg sofosbuvir + PegIFNα/RBV for 12 weeks	Dec 2013
Harvoni®	Sofosbuvir Ledivaspir	NS5Ba NS5A	1, 4–6	90 mg ledipasvir + 400 mg sofosbuvir, with or without RBV, for 12 or 24 weeks	Oct 2014
Viekira Pak™	Ombitasvir Paritaprevir Dasabuvir	NS5A NS3/4A NS5Bb[d]	1	12.5 mg ombitasvir + 75 mg paritaprevir + 50 mg ritonavir, plus 250 mg dasabuvir, with or without RBV, for 12 or 24 weeks	Dec 2014
Technivie™	Ombitasvir Paritaprevir	NS5A NS3/4A	4	12.5 mg ombitasvir + 75 mg paritaprevir + 50 mg ritonavir, with or without RBV, for 12 weeks	Jul 2015
Daklinza™ + Sovaldi®	Daclatasvir Sofosbuvir	NS5A NS5Ba	1, 3	60 mg daclatasvir + 400 mg sofosbuvir, with or without RBV, for 12 weeks	Jul 2015
Zepatier™	Elbasvir Grazoprevir	NS5A NS3/4A	1, 4	100 mg grazoprevir + 50 mg elbasvir for 12 weeks, or with RBV for 12 or 16 weeks	Jan 2016
Epclusa®	Sofosbuvir Velpatasvir	NS5Ba NS5A	1–6	400 mg sofosbuvir + 100 mg velpatasvir, with or without RBV, for 12 weeks.	Jan 2016
Vosevi™	Sofosbuvir Velpatasvir Voxilaprevir	NS5Ba NS5A NS3/4A	1–6	400 mg sofosbuvir + 100 mg velpatasvir + 100 mg voxilaprevir for 12 weeks	Jul 2017
Mavyret™	Glecaprevir Pibrentasvir	NS3/4A NS5A	1–6	300 mg glecaprevir + 120 mg pibrentasvir for 8 or 16 weeks	Aug 2017

[a]*IFN* interferon
[b]*RBV* ribavirin
[c]*NS5Ba* NS5B nucleoside inhibitor
[d]*NS5B* non-nucleoside inhibitor

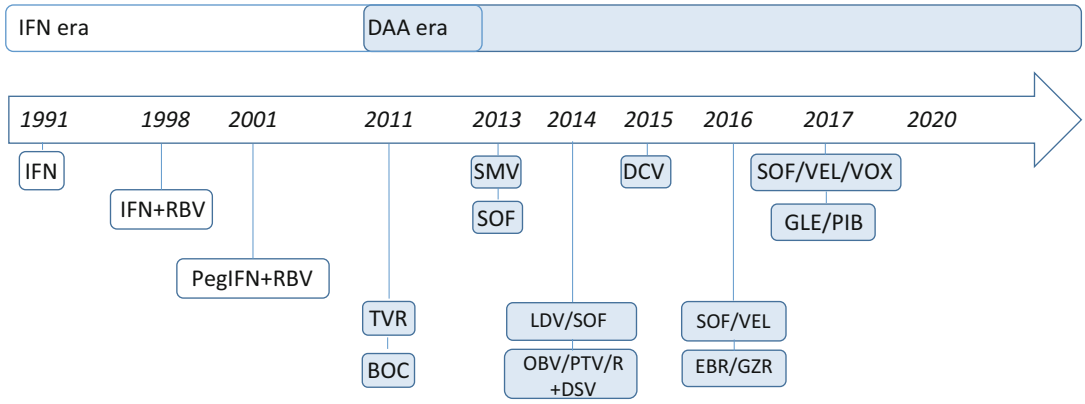

Fig. 6.3 The evolution of HCV treatment. *DAA* direct-acting antiviral, *IFN* interferon, *RBV* ribavirin, *Peg* pegylated, *TVR* telaprevir, *BOC* boceprevir, *SMV* simeprevir, *SOF* sofosbuvir, *LDV* ledipasvir, *OBV* ombitasvir, *PTV* paritaprevir, *R* ritonavir, *DSV* dasabuvir, *DCV* daclatasvir, *VEL* velpatasvir, *EBR* elbasvir, *GZR* grazoprevir, *VOX* voxilaprevir, *G* glecaprevir, *P* pibrentasvir

effectiveness and caused serious side effects. Neither interferon nor ribavirin target any specific viral protein. In principle, both represent the repurposing of drugs that have shown efficacy against other viruses. As we are currently seeing with coronavirus disease (COVID)-19, drug repurposing may not be the best strategy for fighting and controlling pandemic virus infections [42, 43].

Interferons are released by host cells in response to the presence of several viruses; however, many viruses, including HCV, have evolved mechanisms to resist interferon activity [44]. Viruses can circumvent the interferon response by blocking downstream signaling events that occur after interferon binds to its receptor, by preventing further interferon production, and by inhibiting the functions of interferon-induced proteins. Ribavirin was the first synthetic nucleoside analogue reported to be active against several RNA viruses—including HCV, respiratory syncytial virus, and influenza virus [45]. Ribavirin's principal mechanism of action is through the inhibition of inosine-5-′-monophosphate (IMP) dehydrogenase, which converts IMP to xanthosine monophosphate and, thus, impacts the de novo biosynthesis of GTP. The inhibitory activity of ribavirin on IMP dehydrogenase may contribute to its immunosuppressive effects and its partial success for HCV treatment when used in combination with pegylated interferon 2a alfa. Ribavirin is also used as a treatment for some hemorrhagic fever virus infections (e.g., Lassa fever) and for emerging virus infectious diseases, such as dengue virus, norovirus, Marburg virus, and Hendra and Nipah viruses [45]. However, its clinical efficacy remains controversial and further confirmation is required.

The efficacy of HCV interferon-based therapies is highly dependent on the virus genotype. SVR rates are reportedly high (up to 80%) for genotypes 2 and 3, but relatively low (∼50–60%) for genotypes 1 and 4 [46]. Moreover, these cure rates are reduced and difficult to achieve in individuals with advanced liver disease (e.g., cirrhosis) and with comorbidities (e.g., HIV infection). Additionally, interferon-based therapies have a long duration, ranging from 24 to 48 weeks, and require a complex dosage regimen. Adverse events are common, and large numbers of individuals have to abandon treatment, further reducing the cure rate of such therapy. Finally, many individuals remained untreated due to the high costs and interactions between interferon and other drugs. Globally, the treatment coverage with interferon-based treatment was very low, including only 1–5% of individuals with HCV chronic infection.

Advances in our knowledge about the HCV life cycle, and the generation of recombinant tissue culture infectious viruses, have enabled the development of DAAs. DAAs opened the door to cure HCV in most individuals [47]. Between May 2011 and August 2017, twelve therapies were approved by the FDA (Table 6.1) and another two in Japan (asunaprevir plus daclatasvir and vaniprevir plus ribavirin plus pegylated interferon alfa) [41]. Chemical structures of approved NS3/4A inhibitors are shown in Fig. 6.4, while NS5A and NS5B inhibitors are presented in Fig. 6.5. The first approved DAAs were the first-generation NS3/4A protease inhibitors, telaprevir (TVR) and boceprevir (BOC). These were approved in 2011, for use in combination with pegylated interferon and ribavirin, for the treatment of genotype 1-infected individuals. The SVR rates in genotype 1 infections were between 65 and 75% [48, 49]. However, both TVR and BOC have been discontinued due to severe side effects and commercial reasons. In 2013, another NS3/4A protease inhibitor, simeprevir (SMV), was approved for use in combination with pegylated interferon and ribavirin for the treatment of genotype 1 infection. Compared to its predecessors, SMV achieved comparable SVR rates but with better tolerability [50].

A milestone in the current therapies for HCV infection was the development of the NS5B polymerase inhibitor sofosbuvir (SOF). SOF is a nucleotide analogue that produces early chain termination after being incorporated into newly synthesized viral RNA [41]. SOF targets the conserved active site of NS5B, is active against all HCV genotypes, and has a high barrier to resistance. Although SOF resistance can be

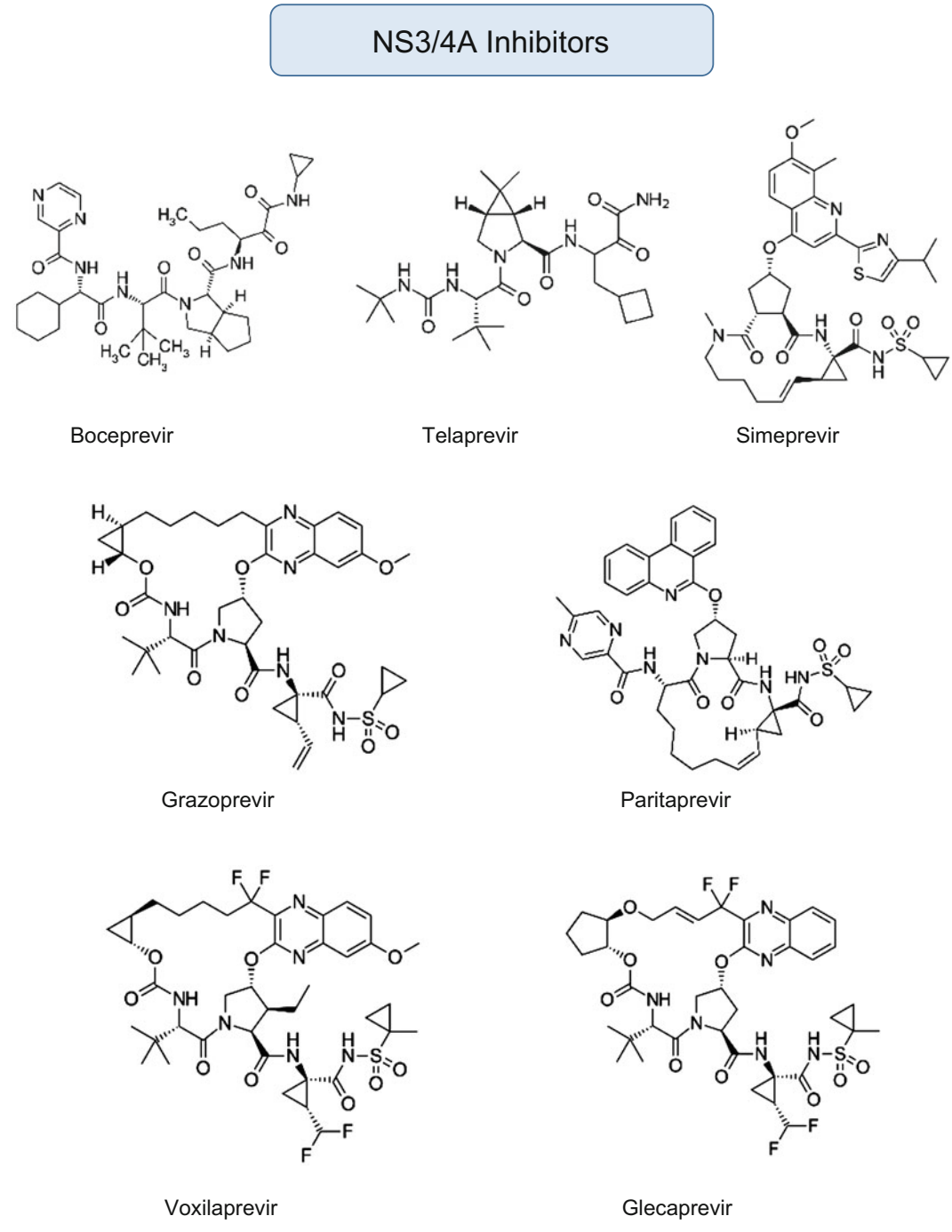

Fig. 6.4 Chemical formulas of FDA-approved HCV direct-acting antivirals targeting NS3/4A protease

produced in tissue culture, it has rarely been observed in vivo. Moreover, viruses containing the SOF resistance mutation, NS5B position S282T, exhibit very low replication capacity. SOF used in combination with pegylated interferon 2a alfa and ribavirin achieved SVR rates of

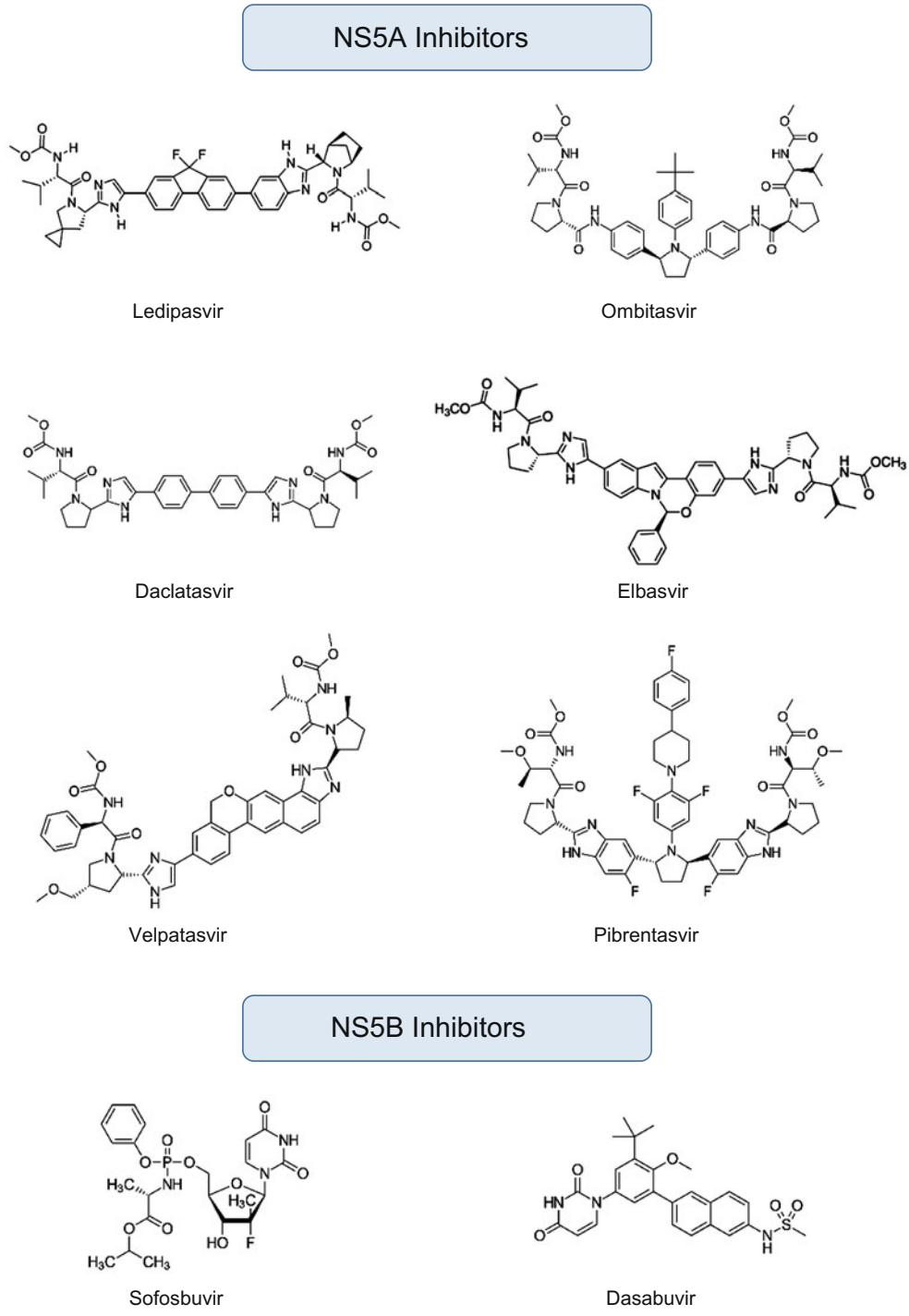

Fig. 6.5 Chemical formulas of FDA-approved HCV direct-acting antivirals targeting NS5A phosphoprotein and NS5B polymerase

90% after 12 weeks of therapy in individuals infected with genotypes 1 and 4 [51]. Similarly, an oral regimen of SOF plus ribavirin achieved SVR rates of 95% and 82% after 12 weeks of therapy in naive and treatment-experienced subjected infected with genotypes 2 and 3 [52]. For this reason, in 2013, the FDA approved SOF for use in combined therapy with pegylated interferon 2a alfa for genotypes 1 and 4 and with ribavirin alone for genotypes 2 and 3 (Fig. 6.3).

Notably, the combination of SOF with ribavirin introduced the era of interferon-free regimens. In 2014, the combination of SOF and the NS5A inhibitor ledipasvir (LDV) was approved as a once-daily co-formulation for the treatment of HCV genotype 1. This combination aimed for rapid suppression of viral replication, as well as prevention of resistant variant selection. The SOF/LDV combination was evaluated with and without ribavirin and achieved SVR rates of 94–99% after 12 weeks of therapy [53–55]. In 2014, the SOF/SMV combination was also approved for the treatment of HCV genotype 1 [56]. At the end of 2014, combinations of ombitasvir, ritonavir-boosted paritaprevir, and dasabuvir ribavirin were approved, which achieved SVR rates of >90% after 12 or 24 weeks of treatment in HCV genotype 1-infected individuals with or without cirrhosis [57, 58]. Paritaprevir is an NS3/4A protease inhibitor, ombitasvir is a NS5A inhibitor, and dasabuvir is a non-nucleoside NS5B polymerase inhibitor. Ritonavir is an HIV protease inhibitor that is used to boost other protease inhibitors of different families. More recently, different combinations of DAAs have been approved, which exhibit increased potency and pan-genotypic efficacy—including SOF plus velpatasvir (VEL; NS5A inhibitor), elbasvir (NS5A inhibitor) plus grazoprevir (NS3/4A inhibitor), glecaprevir (NS3/4A inhibitor) plus pibrentasvir (NS5A inhibitor), and SOF/VEL plus voxilaprevir (NS3/4A inhibitor) (Fig. 6.3).

Recently published data regarding real-world clinical experience confirm the results published in various DAA clinical trials. These studies have included individuals infected with all HCV genotypes and individuals with advanced liver disease. Even regimens including only 8 weeks of treatment have achieved SVR rates of over 95% [47, 59]. Since the new DAA therapies can achieve SVR in >95% of subjects, irrespective of HCV genotype, it is no longer required to perform genotype testing before starting treatment. This supports wider prescription of DAAs, especially in resource-limited environments where genotype testing can be challenging and costs prohibitive. Another important feature of therapy with new DAAs is that they are highly effective among special populations, including individuals with chronic kidney disease [60] or hemoglobinopathies [61], HIV/HCV-co-infected individuals [62], injected drug users [63], older individuals [64], and patients with advanced liver disease [65].

Although the successful rate of current and last generation DAAs almost reach 100%, the pipeline for the development of new compounds is not stopped. New NS5B nucleotide analogues have been described. Examples are guanosine [66] and uridine analogues [67] and non-nucleoside inhibitors [68, 69]. New pan-genotypic NS5A inhibitors have been also described [70–72]. The aim for developing new DAAs is to obtain compounds with a broader capacity to inhibit the different genotypes and HCV variants and with a better metabolic profile [73]. Nevertheless, the high cost of human clinical trials may preclude the arrival to the clinic of new HCV DAAs.

6.5 DAA Resistance

As mentioned earlier in this chapter, the extremely high rates of HCV replication and mutation allow this virus to rapidly explore the available sequence space. Similar to anti-HIV cocktail therapies, combination therapies that target different stages of the HCV life cycle have been developed to avoid DAA resistance (Table 6.2). Previous experience with HIV antiretroviral resistance has been useful for overcoming the problem of drug resistance with the new DAA-based therapies. Another factor that contributes to resistant variant development is

Table 6.2 Observed substitutions in treated individuals associated with resistance to DAAs

NS3	Telaprevir	Boceprevir	Simeprevir	Paritaprevir	Grazoprevir	Voxilaprevir	Glecaprevir
Genotype 1a	V36I/L/M	V36I/L/M	V36M	V36A/M	V36L/M	V36L	V36M
	T54A/S	T54A/S	Q80K/R	Y56H	Y56H/F	Q80K	R155T
	R155K	V55A/I	S122N/R		Q80K	A156/T/V	A156G/T/V
	D168E	R155K	R155G/K/T	Q80K/R	R155/K/I/Q/S/T		D/Q168A
		D168E	D168A/E/H/N/V	R155G/K/T	A156G/M/T/V		
		I/V70A/T	I170T	D168A/E/N/V/Y	V158A		
					D168A/C/E/G/K/N/T/V/Y		
Genotype 1b	V36I/L/M	V36I/L/M	Q41/R	Y56H	Y56F	F43S	NO
	T54A/S	T54A/S	F43S	Q80K/R	Q80L	A156T/V	
	A156S/T	V55A/I	Q80K/R	D168A/E/N/V	R155L		
	D168E	A156S	S122R/T		A156T		
		D168E	R155Q				
		I/V70A/T	A156S/T/V				
			D168A/E/F/H/N/T/V				
Genotype 2	NR	NR	NR	NR	NR	NO	NO
Genotype 3	NR	NR	NR	NR	NR	Q80R	Y56H/N
							Q80K/R
							A156G
							D/Q168K/L/R
Genotype 4	NR	NR	Q80R	Y56H	Y56H	NO	NO
			A156G	A156K	A156T		
			D168A/E/V				
Genotype 5	NR	NR	NR	NR	NO	NO	ND
Genotype 6	NR	NR	NR	NR	D168E	NO	NO
NS5A	Ledivaspir	Ombitasvir	Daclatasvir	Elbasvir	Velpatasvir	Pibrentasvir	
Genotype 1a	K24R	K24R	M28A/T	M28A/G/S/T	K24R	K24R	
	M28A/T/V	M28T/V	Q30E/H/K/R/Y	Q30E/H/R/Y	M28T/V	M28A/G	
	Q30E/H/K/L/R/Y	Q30E/H/K/L/R/Y	L31I/M/V	L31F/I/M/V	Q30E/H/K/L/R	Q30K/R	
	L31F/I/M/V	L31V	H58D	H58D	L31I/M/V	H58D	
	S38F	H58D	Y93C/F/H/N	Y93C/H/N/S	P32L	Y93H	
	H58D	Y93C/F/H/L/N/S			H58D		
	A92T				Y93H/N/R/S/W		
	Y93C/F/H/N						
Genotype 1b	L28M	L28M	L28M	L28M	L31M/V	NO	
	R30H	R30Q	R30G/Q	L31F/M/V	Y93C/H/N/S/T		
	L31F/I/M/V	L31F/V	L31F/I/M/V	Y93H			
	A92T	P58S	P58A/L/S				

(continued)

Table 6.2 (continued)

NS3	Telaprevir	Boceprevir	Simeprevir	Paritaprevir	Grazoprevir	Voxilaprevir	Glecaprevir
	Y93C/H	A92T	A92K				
		Y93H/S	Y93C/H/I/R				
Genotype 2	NR	NR	M28I	NR	L/M31I/M/V	ND	
			A30K		Y93H		
Genotype 3	NR	NR	L/V31I	NR	A30K/V	S24F	
			A62L		L/V31M/P/V	M28G/K	
			Y93H		Y93H/N/R	A30G/K	
						L/V31F/I/M	
						P58T	
						Y93H	
Genotype 4	L/I28M/V	K24Q	L/I28M/V	L/I28I/M/S	L/I28V	ND	
	L/R30H/R	L/I28A/C/I/S/V	L/R30H/R/S	L/R30H/R	L/R30R		
	M/L31I/L/V	L/R30R	M/L31V	M/L31M	L31M		
	P/T58L	M/L31I/L	Y93H/W	P/T58D	Y93H		
	Y93C/H/S	Y93C/H/S		Y93C/S			
Genotype 5	ND	NR	NO	NR	ND	ND	
Genotype 6	ND	NR	NO	NR	NO	ND	
NS5B	Sofosbuvir	Dasabuvir					
Genotype 1a	L159F	C316Y					
	E237G	M414T/V					
	S282R/T	Y448C/H					
	C316F	A553T/V					
	L320F	G554S					
	V321A	S556G/N/R					
		G558R					
		D559G					
Genotype 1b	L159F	C316H/N/Y					
	S282G/T	M414I					
	C316F/H/N	S556G					
	V321I	D559G/N					
Genotype 2	NO	NR					
Genotype 3	E237G	NR					
	S282T						
	V321A						
Genotype 4	E237G	NR					
	S282T/C						
	V321A/I						

(continued)

Table 6.2 (continued)

NS3	Telaprevir	Boceprevir	Simeprevir	Paritaprevir	Grazoprevir	Voxilaprevir	Glecaprevir
Genotype 5	NO	NR					
Genotype 6	NO	NR					

Only substitutions observed in DAA-treated infected individuals are shown here [38, 74, 75]
NO not observed in infected individuals, although some RASs have been detected in vitro, *NR* not recommended for use in individuals infected with this genotype, *ND* not described in infected individuals nor in vitro

the genetic barrier to drug resistance, which is based on the numbers and types of mutations needed for RAS emergence. Additionally, the fitness of the resistant variant populations is critical, as it determines the likelihood that a resistant variant will persist within the larger viral population.

In contrast to the nucleotide/nucleoside analogues used for HIV therapy, the leading nucleotide analogue employed in HCV DAA therapies, SOF, presents a higher barrier to drug resistance. Additionally, SOF RAS (e.g., SOF RAS S282T) (Table 6.2) have a higher impact on HCV fitness. These factors have greatly minimized the general problem of resistance with the new DAA therapies. The emergence of viral resistance is also determined by the level of drug exposure, in that exposure to suboptimal concentrations of antiviral agents will lead to RAS selection by allowing maintenance of a viral load in the presence of a mild selective pressure. Since HCV can be eradicated from infected individuals, HCV therapies are administered for a limited period of time, in contrast to the lifelong administration of HIV antiretroviral therapies. Therefore, the drug toxicity of HCV therapies may be less relevant, as higher drug doses can be used for a limited period of time (e.g., 8 weeks).

Since the initial use of DAA-based therapies, there has been growing interest in identifying pre-existing RASs that are present within HCV populations (Table 6.2). Several methods have been used to perform sequencing to varying depths, with the aim of identifying population bulk viral variants, as well as minority low frequency variants present in HCV quasispecies [36, 37, 76, 77]. In most studies, pre-existing RASs have been identified by performing population sequencing via the traditional Sanger method. However, this bulk sequence strategy lacks sensitivity and generally cannot detect viral populations that are present at proportions lower than 10–25% of the total population.

The development of high-throughput next-generation sequencing technologies (e.g., Illumina, 454, Ion Torrent) has rapidly improved our ability to detect viral subpopulations present in ever-smaller proportions within HCV populations. These new sequencing techniques can identify variants with frequencies of 0.1–1%. Exhaustive lists of DAA resistance-associated substitutions have been previously published [74, 75]. In general, RASs that are present in low proportions (<15%) do not significantly affect treatment outcomes, whereas RASs existing in proportions of greater than 15% of the overall population are more often associated with treatment failure. It has been agreed that a 15% cut-off should be used in all clinical trials and in studies of real-world infected individuals for the reporting of RASs by population and next-generation sequencing [59].

The pan-genotype potency of new DAA therapies is expected to reduce the incidence of treatment failure due to the existence of baseline RASs. However, it is notable that the two newer pan-genotype regimens approved for the treatment of HCV genotypes 1–6 (glecaprevir/pibrentasvir and SOF/VEL/voxilaprevir) each include an NS3/4A protease inhibitor (glecaprevir or voxilaprevir) (Fig. 6.3). It was recently demonstrated that NS3 position 156 is a hotspot for RASs among genotypes 1–4 (but not for genotypes 5 and 6) and confers resistance to glecaprevir or voxilaprevir (Table 6.2)

[78]. Individuals who fail to respond to DAA therapies have exhibited 156-RASs, which might pose a threat to the clinical effectiveness of these new combination treatments. In contrast to 156-RASs, the NS5B substitution S282T that confers resistance to SOF is rarely observed in treated individuals.

6.6 HCV Eradication

Based on the breakthroughs achieved using the current DAAs, it is possible to anticipate that the next decade will bring about a substantial decrease of global HCV infections and even that HCV eradication may be a conceivable objective in the near future [79]. Nevertheless, several remaining obstacles must be overcome to achieve this ambitious goal. The two main challenges for HCV eradication are the high proportion of undiagnosed infected individuals and the inequities in therapy coverage. In 2017, it was estimated that only 20% of people living with HCV worldwide had been diagnosed, with vast disparities among regions. Among people living with HCV, 57% remained undiagnosed in high-income countries, compared to 92% in low- and middle-income countries [19]. HCV infection is often asymptomatic for years, and HCV testing requires resources, which can be challenging in some settings. Early diagnosis prevents HCV complications and, importantly, transmission. HCV diagnosis usually requires serological testing for HCV antibodies, followed by a confirmatory HCV RNA test.

Therapy price is also an important issue, particularly in low-income settings. Although DAAs were initially expensive, the overall costs have substantially decreased, largely due to the issuing of voluntary licenses that enable generic DAA production. Current prices wide vary among countries, with the total cost of a complete DAA treatment regimen ranging from less than US $100 to US $40,000 [80]. Since HCV is the leading cause of HCC and liver transplantation, early diagnosis and treatment is clearly a cost-effective strategy. Ideally, all subjects with chronic hepatitis C should be treated before the development of advanced fibrosis or cirrhosis, since this is the best means of preventing further progression of liver disease and adverse outcomes, including hepatic deaths. It is especially important to target diagnosis and therapy interventions to specific groups of individuals at greater risk of HCV infection (e.g., IDUs and MSM).

In conclusion, the increased availability of DAA therapy for HCV infection is starting to have impacts on morbidity and mortality. In western countries, there has been a decrease of HCV-infected individuals on the liver transplantation waiting list [81]. DAA treatment is also correlated with reductions of HCC, liver-related mortality, and overall mortality in patients with cirrhosis [82]. Curative treatment with DAAs is important because cured people do not transmit the infection. Eliminating hepatitis C requires the provision of DAA treatment coverage to all people living with HCV. There remains a need for comprehensive strategies for testing and diagnosis, as well as for treatment initiation and care follow-up. Epidemiological data should be expanded to more accurately characterize high-risk populations. Finally, the price of DAA treatment should be made more affordable in some settings.

Conflicts of Interest The authors declare no conflict of interest.

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Phytoconstituents as Lead Compounds for Anti-Dengue Drug Discovery

7

Kamatchi Sundara Saravanan, Selvam Arjunan, Selvaraj Kunjiappan, Parasuraman Pavadai, and Lakshmi M. Sundar

Abstract

Dengue is an arthropod-borne viral disease common in subtropical and tropical regions. The widespread use of traditional medicines in these regions for dengue fever (DF) has encouraged researchers to explore the therapeutic effect of herbs and their phytochemicals in dengue infection. Phytochemicals such as quercetin, baicalein, luteolin, oxindole alkaloids, celastrol and geraniin have shown significant inhibition of dengue virus in vitro. Many phytoconstituents have better selectivity index supporting their safety profile for future development. However, in vivo studies supporting therapeutic potency for these active phytoconstituents are limited. There is a need for studies translating anti-dengue profile of active phytoconstituents to find successful anti-dengue compounds.

Keywords

Dengue · Phytoconstituents · Flavonoids · Alkaloids · Terpenoids

Abbreviations

BHK-21	Baby Hamster Kidney cells
CC_{50}	50% cytotoxic concentration
COS	Monkey kidney cells of Simian origin with SV40 genes
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
dsRNA	Double-stranded ribonucleic acid
DSS	Dengue shock syndrome
E protein	Envelope protein
EC_{50}	Half maximal effective concentration
FFURA h	Foci Forming Unit Reduction Assay hour
HepG2	Hepatocellular carcinoma cells
HMEC	Human dermal microvascular endothelial cells
HMGB1	High mobility group box 1
HPLC	High-performance liquid chromatography
Huh	Human liver cancer cell line

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IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IHH	Immortalized human hepatocytes
IL	Interleukin
ISG	Interferon-stimulated genes
LC_{50}	Median lethal dose
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LLC-MK	Rhesus monkey kidney epithelial cells
miRNA	MicroRNA
MMP	Matrix metalloproteinases
MNTD	Maximum non-toxic dose
NS	Non-structural protein
prM	Precursor membrane
QSAR	Quantitative structure-activity relationship
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse transcription-polymerase chain reaction
SI	Selectivity index
TCM	Traditional Chinese medicine
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor

7.1 Introduction

Dengue is an arthropod-borne viral infectious disease of significant public health concern across subtropical and tropical regions [1, 2]. In recent decades, there has been a dramatic increase in the incidence of dengue around the world. Currently, dengue is endemic in over 100 countries [3]. A modelling study reported in 2019 on current and future global distribution of dengue estimated that roughly 53% of global population (between 3.45 and 4.09 billion people) are at risk of being exposed to dengue virus. A vast majority of them live in Asia, Africa and Latin America [4].

Dengue epidemics are explosive in urban areas and affect large portions of the population [5, 6]. They start during the rainy season, when there is usually plenty of the vector mosquito, *Aedes aegypti*. By feeding on a viraemic human, the female mosquito acquires the dengue virus (DENV) and transmits it to other humans. Mosquitoes are infective after an 8–14-day cycle and potentially remain so for life (1–3 months). In the tropics, continuous mosquito breeding preserves the disease throughout the year.

Though a vast majority of dengue infections are asymptomatic or mild and are self-managed, detecting the disease progression associated with severe dengue infection becomes pertinent [7]. If detected early and provided with appropriate medical care, the case fatality rate of dengue infection is lower than 1%. However, if not recognized early or left untreated, the case fatality rate in severe dengue infection could be as high as 5–10% [7, 8].

7.2 Structural Composition and Replication of Dengue Virus

Dengue virus is a single-stranded enveloped RNA genome and belongs to the genus *Flavivirus* (Fig. 7.1a). Worldwide, there are four distinct serotypes of DENV—viz. DENV-1, DENV-2, DENV-3 and DENV-4. The RNA genome of DENV comprises about 10,700 nucleotides, encoding 3411 amino acids (Fig. 7.1c). During dengue infection, DENV enters the host cells by receptor-mediated endocytosis and releases RNA into the cytoplasm. The viral RNA undergoes replication and translation to form polyprotein in the endoplasmic reticulum (ER) of the host cell (Fig. 7.1b). The polyprotein is converted by post-translation process to form the virus with three structural proteins, namely, capsid, precursor membrane (prM) and envelope (E) proteins, and seven non-structural (NS) proteins. The NS proteins present in DENV are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Fig. 7.2). The peptidases of host cell ER are involved in the processing of prM, E, NS1, NS4A and NS4B proteins. The capsid protein and other NS proteins are processed by NS2B-NS3 protease enzyme. The furin protease enzyme of host cell Golgi complex converts prM to mature

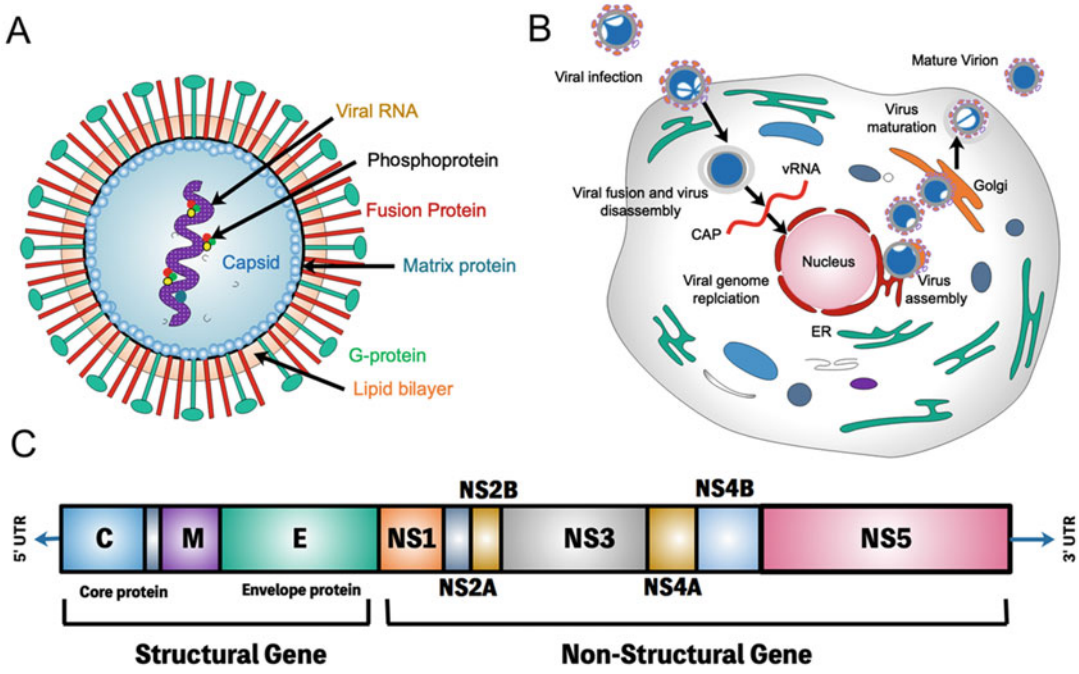


Fig. 7.1 Structure of dengue virus and its replication in host cell. (**A**) Dengue virus with single-stranded RNA and structural protein capsid. (**B**) Entry of virus into host cell by endocytosis, followed by virus fusion and disassembly. Replication of viral RNA in ER, maturation in Golgi complex and release of mature virus from host cell. (**C**) Structure of viral RNA genome from 5′ terminal for structural proteins, capsid, membrane and envelope proteins, and non-structural proteins (NS)—NS1, NS2A, NS2A, NS3, NS4A, NS4B and NS5. The figure was kindly provided by Dr. Vasanthanathan Poongavanam

membrane protein and releases the mature virus (Fig. 7.1b) [9, 10].

These structural proteins are part of the mature virus particle and do not participate in viral genome replication. The NS proteins play an important role in the replication of DENV and are expressed only in the infected cell (Fig. 7.1b). NS1 is a glycoprotein (PDB ID: 4O6B) which mediates fusion of the viral and cellular membranes (Fig. 7.2a). NS1 is identified as an important target for anti-dengue drug design due to its association with vascular leakage and thrombocytopenia during dengue infection [11]. NS2A is involved in the formation of viral RNA, viral assembly and formation of NS1. NS2B is an activation factor for NS3 protease enzyme (NS2B-NS3pro). NS3 (PDB ID: 5XC7) is a multifunctional enzyme with serine protease domain in the N-terminal region (NS3 protease). The C-terminal region of NS3 contains domains for 5' RNA triphosphatase, nucleoside triphosphatase and helicase functions (NS3 helicase). NS3 helicase is the vital enzyme for DENV replication and survival in infected cells (Fig. 7.2c). Both NS4A and NS4B do not have enzymatic function, but play indirect roles in viral replication. NS4A is involved in the stabilization of replication complex, while NS4B participates in the dissociation of NS3 helicase from viral RNA. NS5 (PDB ID: 5ZQK) is the largest NS protein in DENV, with two enzymatic functions, namely, methyltransferase and RNA-dependent RNA polymerase (RdRp) (Fig. 7.2d). NS proteins, being essential components of viral replication and infection, serve as important targets in the discovery of anti-dengue molecules [12, 13]. In addition to these NS proteins, the E protein (PBD ID: 1TG8) is an important target for

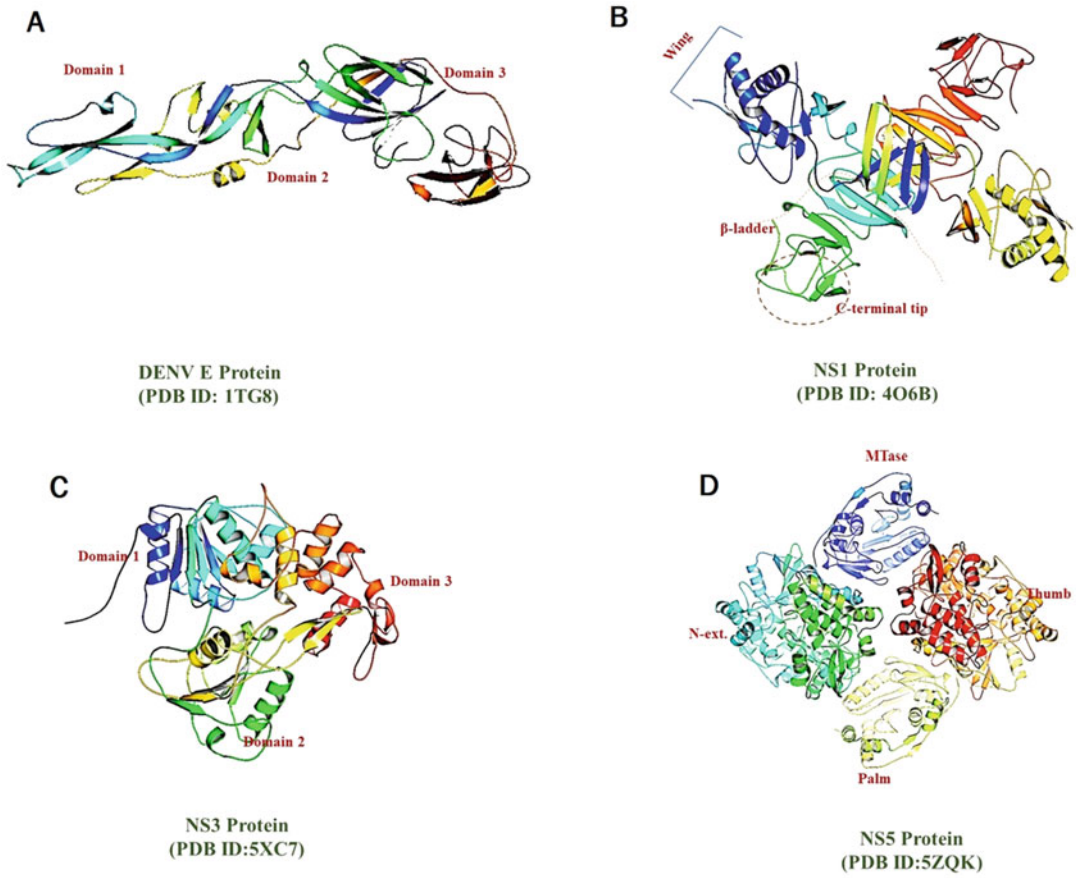


Fig. 7.2 Dengue envelope (DENV E) and non-structural (NS) proteins. (**A**) DENV E protein: The structural protein involved in the attachment of virus to the host cell membrane. (**B**) NS1 protein: A glycoprotein involved in fusion of virus and cellular membrane, also associated with vascular leakage and thrombocytopenia in severe dengue infections. (**C**) NS3 protein: Multifunction protein with protease, 5' RNA triphosphatase, nucleoside triphosphatase and helicase functions, vital for viral RNA replication process. (**D**) NS5 protein: Largest NS protein with two enzymatic functions—methyltransferase and RNA-dependent RNA polymerase (RdRp)

anti-dengue molecules, which acts at an early stage of DENV life cycle. E protein helps DENV virus to bind with host cell membrane and enter the host cell (Fig. 7.2a) [14].

7.3 Clinical Manifestations and Clinical Features

Clinical manifestations of dengue infection are varied and range from asymptomatic infection to critical illness such as dengue haemorrhagic fever with shock syndrome [7]. In symptomatic cases, it could manifest as undifferentiated fever (indistinguishable from other viral infections), dengue fever, dengue haemorrhagic fever or expanded dengue syndrome/isolated organopathy.

Clinical illness of dengue fever starts 4–7 days after an infectious mosquito bite and may include various non-specific, constitutional symptoms such as headache, backache and general malaise. Following this, there is usually a sudden rise in temperature which can fall by about the third day and rise again by 5–8 days ("saddleback"). As per the comprehensive guidelines by WHO, other common symptoms include flushed face,

headache, retro-orbital pain, photophobia, myalgia, arthralgia, anorexia, altered taste sensation, constipation, colicky pain, dragging pains in the inguinal region, sore throat and general depression [7]. Though dengue fever can present with haemorrhagic symptoms such as petechiae, epistaxis, hypermenorrhea and gastrointestinal bleeding in rare cases, dengue fever with haemorrhagic manifestations must be differentiated from dengue haemorrhagic fever.

Dengue haemorrhagic fever (DHF), in its early phase, presents with signs and symptoms similar to dengue fever. However, the disease is more severe, and often there is a tendency to progress to hypovolemic shock. This is commonly referred to as dengue shock syndrome (DSS) [15]. Thrombocytopenia and rising haematocrit/haemoconcentration usually precede reduction in temperature and onset of shock [16]. Abnormal haemostasis and plasma leakage into interstitial spaces are the main pathophysiological hallmarks of DHF and often result in serious complications such as DSS. DHF is more common in children $<$15 years of age. However, the incidence of DHF in adults is increasing, showing higher rates in hyperendemic areas. Emerging evidence indicates that a second dengue infection is a specific risk factor for serious illness. In DHF, plasma leakage determines the severity of the disease and is the primary difference between DHF and DF. Based on severity of the disease and clinical symptoms, DHF is classified into four grades I to IV, with grade IV being the most severe. DSS arises in severe cases with critical plasma loss and can be life-threatening if not properly treated. DSS features a quick, weak pulse with decreasing pulse pressure ($<$20 mmHg), cold, clammy skin and restlessness. The patient can die in 12–24 h after experiencing shock or recover quickly by fluid-based therapy.

7.4 Treatment of Dengue Infection

The common mode of treatment for dengue infection largely depends on symptomatic treatment with the existing therapeutics. Currently available medicines can be classified as fluid-based therapy, blood products, immunomodulators and vaccines. Fluid-based therapy consisting of saline solution, dextran or Ringer's lactate is the immediate choice of treatment for a person with DSS to recover from increased vascular permeability and subsequent hypovolemic shock. For the treatment of thrombocytopenia, a common symptom of DENV infection, fresh plasma transfusion is highly recommended to improve platelet count. As dengue infection is characterized by release of various inflammatory cytokines, treatment with immunomodulators like chloroquine or statins is also recommended. Chloroquine and statins are drugs which are repurposed for treating dengue infection. Though dengue vaccine (Dengvaxia) is available, it was noted to have a high risk of causing severe dengue infection especially in children after vaccination. Medicinal compounds that target DENV directly by the inhibition of viral replication would be the preferred choice of therapy for dengue infection, but are currently unavailable.

Extensive research is being carried out worldwide to develop specific anti-dengue candidate drugs. Anti-dengue drug discovery process focuses on the design and development of small molecules inhibiting target proteins such as NS2B-NS3 protease, NS3 helicase, NS4A/B and NS5 RdRp of DENV. Direct-acting anti-dengue molecules like balapiravir, an inhibitor of NS5, were promising and reached to the stage of clinical trial, but failed to control the dengue infection in clinical populations [17]. Despite several research towards the development of direct-acting anti-dengue molecules, the success rate is very poor, and till date, there are no approved direct-acting anti-dengue medicines [14, 18].

In regions where dengue is endemic, local population has been known to extensively use traditional medicines for the control of dengue infection. The widespread use of traditional medicines for DF in different parts of the world has encouraged researchers to explore and provide scientific evidence for the therapeutic effect of herbs and their phytoconstituents in dengue infection. These studies led to the identification of new active phytoconstituents and new use for

the existing herbs. There are numerous publications on various herbal extracts for treating dengue infection, more so in the last 5 years [19, 20]. This chapter, using a structured literature review process, aims to provide the readers with the most recent update on the potential role of phytoconstituents in the treatment of dengue. It also puts forth the existing gaps in knowledge and highlighting the need for exploring them towards the development of anti-dengue therapeutics from plant sources.

7.5 Phytoconstituents with Potential Anti-Dengue Activity

The most common phytoconstituents present in plant sources are flavonoids, alkaloids, terpenoids, phenolic compounds, tannins and coumarins. Publications on the anti-dengue profiles of various plants highlight the important role of flavonoids in the inhibition and control of dengue infection. Nevertheless, other categories of phytoconstituents such as alkaloids and terpenoids have also shown good potential against dengue infection. The activity profiles of phytoconstituents could provide potential lead towards their development as anti-dengue molecules. This review summarizes the anti-dengue research profiles of various plant-based molecules under the following categories: flavonoids, alkaloids, terpenoids, other categories of phytoconstituents and herbal extracts.

7.5.1 Flavonoids

Flavonoids are one of the largest classes of naturally occurring plant products consisting of chromane ring with phenyl ring attached at either the second or third position. Flavonoids are further classified into different types as flavones, flavonones, flavonol, isoflavones, biflavones, anthocyanidins and xanthones. Their distribution in natural sources may be either in free form or as glycosides. Hitherto, numerous herbals enriched with flavonoids have been extensively studied for their activity against dengue infection. The details pertaining to the anti-dengue activity of flavonoids are reviewed in this section (Tables 7.1 and 7.2).

7.5.1.1 Baicalein

Zandi et al. reported anti-dengue activity for aqueous extract of *Scutellaria baicalensis* (Labiatae) root, an official traditional Chinese medicine (TCM). Analysis of the root extract by mass spectrometry confirmed the presence of active flavonoid baicalein (Fig. 7.3, 1.03 μg/gm dried extract). The anti-dengue activity was evaluated against dengue serotypes (DENV-1 to DENV-4) in Vero cells by Foci Forming Unit Reduction Assay (FFURA). The CC_{50} value of the extract was 912.6 μg/mL in Vero cells. An IC_{50} of 86.59 μg/mL (DENV-1), 93.66 μg/mL (DENV-2), 89.39 μg/mL (DENV-3) and 95.19 μg/mL (DENV-4) and selectivity index (SI) of 10.5 (DENV-1), 9.7 (DENV-2), 10.2 (DENV-3) and 9.6 (DENV-4) were observed in Vero cells when the extract was added after DENV adsorption. When the extract was added during the time of adsorption, the IC_{50} values declined to 69.14 μg/mL (DENV-1), 56.02 μg/mL (DENV-2), 77.41 μg/mL (DENV-3) and 73.59 μg/mL (DENV-4), and the SIs improved to 13.2 (DENV-1), 16.3 (DENV-2), 11.8 (DENV-3) and 12.4 (DENV-4), respectively. Treatment of Vero cells with *S. baicalensis* extract 2 h before viral inoculation indicated a frail prophylactic property as the IC_{50} values were >250 μg/mL against all four serotypes compared to other two treatment modes [21].

In another study, pure flavonoid baicalein exhibited CC_{50} at 109 μg/mL in Vero cells. The replication of DENV-2 was inhibited at an IC_{50} of 6.46 μg/mL and SI of 17.8 (post-cellular adsorption). Treatment of cells with baicalein 5 h before viral infection and continuously up to 4 days after infection established an IC_{50} of 5.39 μg/mL and SI of 21.3. Baicalein showed direct virucidal effect at an IC_{50} of 1.55 μg/mL and anti-adsorption effect at an IC_{50} of 7.14 μg/mL. The anti-viral efficacy of baicalein can be attributed to its direct virucidal activity (SI, 74.3) along with its potency against viral adsorption as well as

Table 7.1 List of flavonoids from plants with anti-dengue activity

Phytoconstituents	Plant species	In vitro study	In vivo study
Baicalein	*Scutellaria baicalensis*	Anti-dengue activity in Vero cells against all 4 types of DENV [21, 22]	NR
Quercetin, catechin and gallic acid	*Psidium guajava*	Anti-DENV-2 activity in Huh 7 cells [23]	NR
Fisetin	–	Anti-dengue activity in Vero cells [24]	NR
5-Hydroxy-7-methoxy-6-methylflavone	*Syzygium samarangense*	Inhibition of virus-virus cell fusion in LLC-MK2 cells [25]	NR
Luteolin	*Viola yedoensis*	Anti-dengue activity in DENV infected Huh 7 cells, Vero cells, BHK-21 and HEK-293T No inhibition of DENV NS3, NS5 and E proteins Inhibition of furin protease enzyme [26, 27]	No anti-dengue activity, slight reduction in virus particles in DENV infected Sv/129 mice [27]
Sophoroflavenone G	*Sophora flavescens*	Inhibition of RdRp enzyme in infected A549 cells [28]	NR
Biflavonoids: Amentoflavone, podocarpusflavone A, isoginkgetin, hinokiflavone	*Dacrydium balansae*	Inhibition of DENV NS5 RdRp enzyme [29]	NR
Chartaceones A−F, Pinocembrin	*Cryptocarya chartacea*	Inhibition of DENV NS5 RdRp enzyme [30]	NR

NR Not Reported

intracellular replication [22]. In silico analysis of baicalein showed potent binding affinity with E, NS3–NS2B and NS5 proteins. Hydroxy and keto groups in baicalein exhibited hydrogen bonding interactions, and aromatic ring showed π-π interactions with NS2B-NS3 protease. The binding score was better for NS2B-NS3 protease (−7.5 kcal/mol) as compared to other two proteins [41].

7.5.1.2 Quercetin and Catechin

Anti-dengue activity of pure flavonoids quercetin (Fig. 7.3), daidzein, naringin and hesperetin was also evaluated at various stages of DENV-2-infection and replication cycle. DENV replication was measured by FFURA and quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The CC_{50} of quercetin, daidzein, naringin and hesperetin in Vero cells by MTT assay were 252.6 ± 0.17, 147.8 ± 0.31, 230.3 ± 0.19 and 110.3 ± 0.32 μg/mL, respectively.

Quercetin exhibited significant anti-dengue activity by affecting intracellular DENV virus replication with an IC_{50} of 35.7 μg/mL (post-viral adsorption). The IC_{50} value was 28.9 μg/mL when Vero cells were subjected to continuous treatment with quercetin for 5 h prior to viral infection and 4-day post-infection. At the concentration of 50 μg/mL, the DENV-2 RNA level was reduced by 67%. The SIs of quercetin during post-infection and continuous treatment were 7.07 and 8.74, respectively. The differences between SI values of quercetin could be due to its intracellular accumulation in continuous treatment. A weak prophylactic activity, significant effect during continuous treatment and post-infection suggested that quercetin acted against different stages of intracellular replication of DENV-2 rather than at the early stages such as viral attachment or entry. Other flavonoids such as daidzein, naringin and hesperetin are not good candidates for anti-dengue drug development as the first two displayed a very weak SI of 1.03 and

Table 7.2 List of flavonoid glycosides from plants with anti-dengue activity

Phytoconstituents	Plant species	In vitro study	In vivo study
Pectolinarin, acacetin-7-*O*-rutinoside	*Distictella elongata*	Anti-DENV-2 activity in LLC-MK2 cells [31]	NR
Flavanone apiofuranoside	*Faramea bahiensis, Faramea hyacinthine, Faramea truncata*	Anti-DENV-2 activity in HepG2 cells [32–34]	NR
Glycosides of quercetin and kaempferol	*Bauhinia holophylla*	Anti-dengue activity in Vero cells infected with DENV-2 [35]	NR
Quercetin glucoside, quercetin rhamnoside gallic acid, galloylglucopyronside, corilagen, geraniin, rutin, syringin, syringin dimer, digalloylglucopyronside, trigalloylglucopyronside, apigenin rhamnoside	*Phyllanthus watsonii*, *Phyllanthus amarus*, *Phyllanthus niruri*, *Phyllanthus urinaria*	Anti-dengue activity in DENV-2 infected Vero cells [36]	NR
Glycosides of quercetin and kaempferol	*Euphorbia hirta*	Anti-dengue activity in Vero cells infected with DENV-1 and -2 [37, 38]	NR
Glycosides of quercetin, gallic acid and kaempferol	*Psiloxylon mauritianum*	Decreased DENV E proteins in Huh 7.5 cells infected with all four types of DENV [39]	NR
Hyperoside	*Norantea brasiliensis*	Decreased secretion of DENV NS1 protein and immunomodulatory effect in monocytes [40]	NR

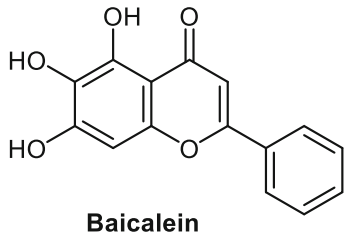
NR Not Reported

1.3, while hesperetin lacked anti-dengue activity [42].

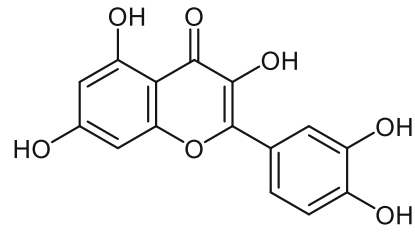
The bioactive fraction from ethanol extract of *Psidium guajava* (Myrtaceae) bark showed antiviral activity in DENV-2 infected Vero cells. This active fraction was found to consist of gallic acid, quercetin, catechin (Fig. 7.3), naringin and hesperidin. In vitro study of these isolated compounds in Vero cells revealed that quercetin had highest anti-dengue activity ($EC_{50} = 19.2$ μg/mL) with better SI (CC_{50} = 659.8 μg/mL, SI = 34.3). The effect of these five flavonoids on DENV-2 before and after entry of virus was also examined by pre- and post-treatment methods. Inhibition of virus was highest by catechin (100%), followed by gallic acid (52.5%) and quercetin (50%) in pre-treated cells. However, quercetin exhibited 100% inhibition of virus infection in post-treatment, while the inhibition by catechin, gallic acid and naringin was 91.8%, 67.3% and 64.5%, respectively. Hesperidin did not show anti-viral effect in both pre- and post-treatment methods.

The outcome of this study for quercetin is similar to that reported by Zandi et al. in 2011. In both studies, quercetin displayed potential antiviral effect with favourable SI in cells post- infection. Interestingly, catechin is also noted to be a promising molecule with better SI (24.8) and antiviral effect (EC_{50} = 33.7 μg/mL, $CC_{50} = 833.3$ μg/mL) at an early stage of infection. Based on the findings reported for isolated flavonoids, a combination of catechin and quercetin can be proposed, anticipating its anti-dengue efficacy at both early and later stages of infections [23].

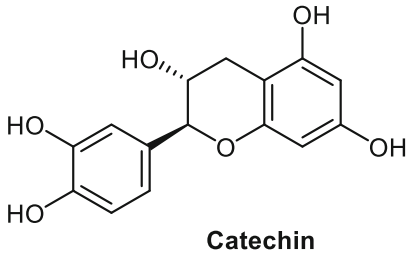
Molecular docking study for the binding efficacy of quercetin with DENV E protein indicated that quercetin binds by two ionic and one hydrogen bonds. Thr280 forms hydrogen bond interaction, while oxygen atoms of quercetin ionically bind to Ala50 and Gln200 of DENV E protein. It was proposed that quercetin could block the virus



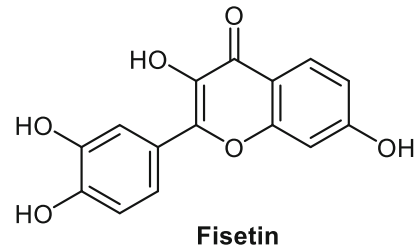
Baicalein



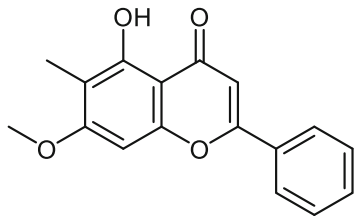
Quercetin



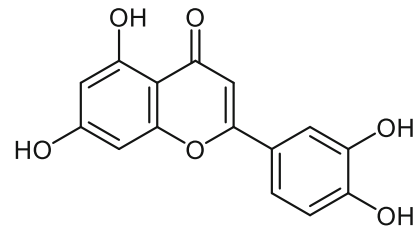
Catechin



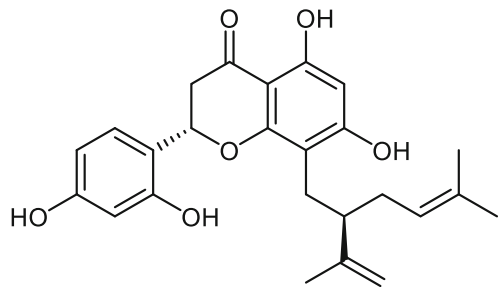
Fisetin



5-Hydroxy-7-methoxy-6-methylflavone



Luteolin



Sophoroflavenone G

Fig. 7.3 Structure of flavonoids showing anti-dengue activity. Baicalein: Inhibited DENV-2 replication, IC_{50} = 6.46 μg/mL. Quercetin: Inhibited DENV-2 replication, EC_{50} = 19.2 μg/mL. Catechin: Inhibited DENV-2 infection at an early stage, EC_{50} = 33.7 μg/mL. Fisetin: Inhibited DENV-2 replication, IC_{50} = 43.12 μg/mL. 5-Hydroxy-7-methoxy-6-methylflavone: Inhibited virus cell-host cell fusion, EC_{50} = 4.21 μM. Luteolin: Inhibited furin enzyme, Ki = 58.6 μM. Sophoroflavenone G: Inhibited DENV replication, IC_{50} = 14.5 μM

entry process through its interaction with DENV E protein and hence prevent fusion process [43]. However, in vitro studies showed that quercetin was effective at later stage of infection. In silico analysis of quercetin for other targets would provide further insight in understanding the mechanism of its activity.

7.5.1.3 Fisetin

Zandi et al. reported the anti-viral effects of fisetin (Fig. 7.3), rutin and naringenin against DENV-2 in vitro at different stages of infection using FFURA and qRT-PCR. Among the flavonoids evaluated, fisetin exhibited a significant activity on viral replication. Fisetin inhibited viral replication with an IC_{50} of 55 μg/mL and SI of 4.49 after viral adsorption in Vero cells. An IC_{50} of 43.12 μg/mL and SI of 5.72 were observed when Vero cells were continuously treated for 5 h before viral infection and continuously up to 4 days post-infection. Fisetin was reported not to possess prophylactic and virucidal activity. Although rutin and naringenin did not inhibit DENV-2 replication, naringenin presented virucidal activity with an IC_{50} of 52.64 μg/mL and a low SI <1. The inhibition mechanism of fisetin on DENV replication was not clear, but at the same time, absence of prophylactic activity suggested that it was unlikely to act at an early stage of virus life cycle. Probably, fisetin would have inhibited DENV replication by affecting the NS5 RdRp [24]. Fisetin could be tested further by in vitro NS5 RdRp assay or in silico analysis for its binding affinity with NS5 RdRp.

7.5.1.4 5-Hydroxy-7-Methoxy-6-Methylflavone

5-Hydroxy-7-methoxy-6-methylflavone (Fig. 7.3) was isolated from the hexane fraction of ethyl acetate extract of *Syzygium samarangense* leaves (Myrtaceae). In 2018, Pimsiri et al. reported 5-hydroxy-7-methoxy-6-methylflavone as a new compound exhibiting anti-dengue activity ($EC_{50} = 4.21$ μM, $CC_{50} > 100$ μM) in DENV-2 infected LLC-MK2 cells. Fusion inhibition, protease inhibition and replicon inhibition assays were applied to understand the mechanism of anti-dengue activity of 5-hydroxy-7-methoxy-6-methylflavone. This flavone inhibited virus cell-host cell fusion, but did not inhibit NS2B-NS3 protease and replication system. Molecular docking and dynamics studies showed that 5-hydroxy-7-methoxy-6-methylflavone has strong binding affinity with DENV E protein. This revealed that the flavone could prevent virus-host cell fusion by inhibiting DENV E protein [25].

7.5.1.5 Luteolin

Luteolin (Fig. 7.3) was identified as an active flavone present in *Viola yedoensis* (Violaceae). In the study reported by Peng et al., luteolin was tested for anti-dengue activity in in vitro and in vivo studies. Luteolin showed anti-dengue activity in DENV infected human liver cancer 7 (Huh) cells, Vero cells, Baby Hamster Kidney 21 (BHK) cells and Human Embryonic Kidney cells 293 T by plaque titration assay and time of addition assay. As there was no change in the level of DENV proteins NS3, NS5 and E in the time of addition assay, furin protease inhibition assay was performed in this study to know the mechanism of luteolin in the inhibition of DENV infection. Luteolin could inhibit furin enzyme in a dose-dependent manner (>95% at 200 μM, $Ki = 58.6$ μM). However, luteolin could not protect DENV infected Sv/129 mice from infection in vivo following oral administration (100 mg/kg, four times a day for 4 days) but reduced viral production by twofold in comparison to untreated mice. Lack of anti-dengue activity in vivo could be due to less efficacy in vivo or low bioavailability [26, 27]. Though in vivo results were not promising, this study demonstrated another possible target furin protease enzyme for exploring the anti-dengue activity of molecules.

7.5.1.6 Sophoroflavenone G

Sophoroflavenone G (Fig. 7.3) is an active constituent from the ethanol extract of *Sophora flavescens* (Fabaceae) root. Sophoroflavenone G was reported to inhibit NS5 RdRp enzyme ($IC_{50} = 14.5$ μM) in DENV infected human epithelial lung cancer cells (A549). However, cytotoxicity was observed for this molecule in A549

cells ($CC_{50} = 58.21$ μM) and immortalized human hepatocytes (IHH, $CC_{50} = 42.87$ μM) [28]. The anti-cancer activity of sophoroflavenone G [44] could be linked for its cytotoxicity in A549 and IHH cells. This indicates that derivatives of sophoroflavenone G could be explored as anti-dengue molecules with low cytotoxicity.

7.5.1.7 Biflavonoids

Biflavonoids from *Dacrydium balansae* (Podocarpaceae) leaf exhibited effective inhibitory potential on DENV NS5 RdRp and full DENV NS5. The biflavonoids hinokiflavone, podocarpusflavone A (Fig. 7.4), amentoflavone and isoginkgetin displayed an IC_{50} of 0.26 ± 0.01, 0.75 ± 0.03, 1.40 ± 0.09 and 3.12 ± 0.09 μM, respectively, against DENV NS5 RdRp, in tandem with 0.75 ± 0.02, 1.40 ± 0.14, 3.10 ± 0.09 and 5.30 ± 0.25 μM, respectively, against full DENV NS5. A strong inhibition on DENV NS5 RdRp and full DENV NS5 was exhibited by hinokiflavone, followed by podocarpusflavone A. These flavonoids were claimed to be the strong non-nucleoside inhibitors of DENV NS5 RdRp, substantiated by their inhibitory potential on DENV polymerase and on full-length DENV NS5. The dimeric nature of these biflavonoids has been ascribed for their potency. Even though the IC_{50} of podocarpusflavone A is higher than that of hinokiflavone, the former was considered to be a preferred candidate for anti-dengue drug development as its LC_{50} (22.6 μM) was higher compared to hinokiflavone with LC_{50} (5 μM) in both COS and BHK cells [29].

7.5.1.8 Mono- and Dialkylated Flavanones (Chartaceones)

Mono- and dialkylated flavanones (chartaceones A−F) and pinocembrin, a dihydroxy flavanone from *Cryptocarya chartacea* (*Lauraceae*) bark, were screened for anti-dengue activity by DENV NS5 RdRp inhibition assay. An appreciable NS5 RdRp inhibitory property was demonstrated by chartaceones C, E and F evidenced by their low IC_{50} values (chartaceone C, 4.2 ± 0.1 μM; chartaceone E (Fig. 7.4), 2.9 ± 0.3 μM; chartaceone F (Fig. 7.4), 2.4 ± 0.3 μM) with no cytotoxicity in KB (nasopharynx human carcinoma) cell line at 10 μg/mL. The arylheptanoid side chains in these bioactive compounds were considered crucial for the significant inhibition of polymerase [30]. In silico analysis revealed that chartaceones C–F showed better binding affinity with NS5 RdRp protein of DENV [45]. Therefore, chartaceones may be considered as a new category of non-nucleoside inhibitors against DENV NS5 RdRp.

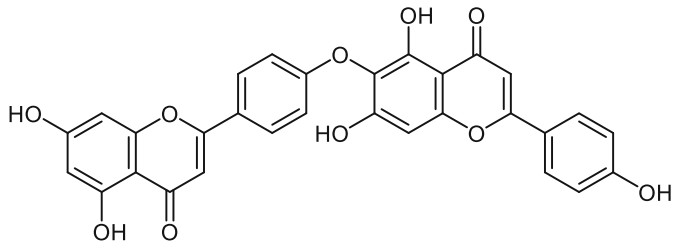
7.5.1.9 Flavone Glycosides

Pectolinarin and Acacetin-7-*O*-Rutinoside

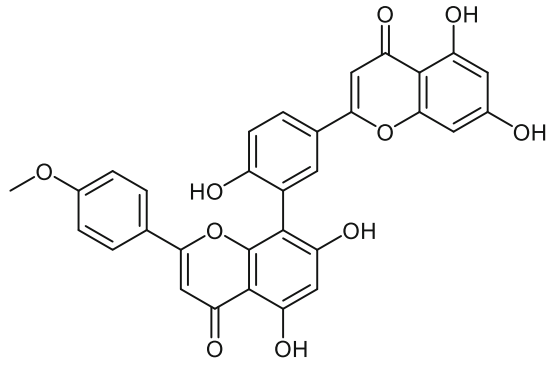
The ethanol extract of leaf, fruits and stem of *Distictella elongata* (Bignoniaceae) and its key phytoconstituents were evaluated in vitro against DENV-2 by MTT assay. Among these, the leaf extract showed anti-dengue activity in LLC-MK2 cells with EC_{50} 9.8 ± 1.3 μg/mL, CC_{50} 14.4 ± 0.4 μg/mL and SI of 1.5. The active compound pectolinarin (Fig. 7.5), isolated from leaf extract, was less effective against DENV-2 (EC_{50} 86.4 ± 3.8 μg/mL; CC_{50} 402.6 ± 9.8 μg/mL; SI 4.6); however, a blend of pectolinarin and acacetin-7-*O*-rutinoside (Fig. 7.5) isolated from fruit extract showed superior anti-dengue activity (EC_{50} 11.1 ± 1.6 μg/mL; CC_{50} > 500 μg/mL) with SI > 45. The pectolinarin and acacetin-7-*O*-rutinoside combination exhibited around eightfold increased potency than pectolinarin alone, besides a tenfold higher SI, suggestive of stronger putative effect of acacetin-7-*O*-rutinoside in exhibiting anti-dengue activity [31].

Flavanone Apiofuranoside

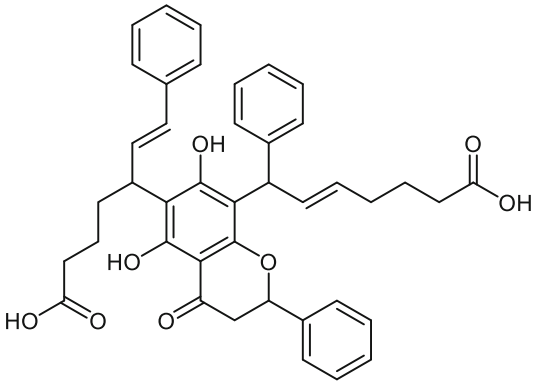
In 2017, a new flavanone-*O*-glycoside, flavanone apiofuranoside (Fig. 7.5), was isolated from the extract of *Faramea bahiensis* (Rubiaceae). It was found to be an active compound with IC_{50} of 13.1 μg/mL by plaque reduction assay in DENV-2 infected human hepatocarcinoma cells (HepG2) [32]. Cytotoxicity was not observed for flavanone apiofuranoside till 200 μg/mL. RT-PCR analysis revealed 67% reduction in DENV RNA indicating that flavanone apiofuranoside exhibited anti-dengue activity by the inhibition of DENV RNA replication.



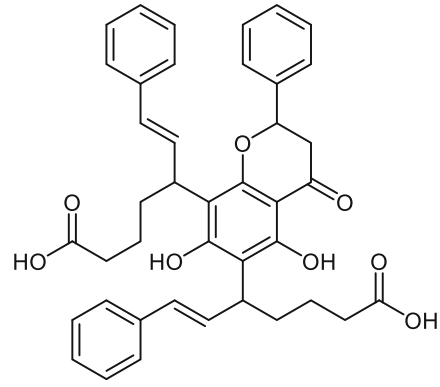
Hinokiflavone



Podocarpusflavone A



Chartaceone E



Chartaceone F

Fig. 7.4 Structure of biflavonoids and alkylated flavonoids showing anti-dengue activity. Hinokiflavone: Inhibited DENV NS5 RdRp, IC_{50} = 0.26 μM. Podocarpusflavone A: Inhibited DENV NS5 RdRp, IC_{50} = 0.75 μM. Chartaceone E: Inhibited DENV NS5 RdRp, IC_{50} = 2.9 μM. Chartaceone F: Inhibited DENV NS5 RdRp, IC_{50} = 2.4 μM

In another study reported by Barboza et al., methanol extracts from leaves of *F. hyacinthina* and *F. truncata* exhibited anti-dengue activity in DENV-2 infected HepG2 cells. Phytochemical

analysis by solid phase extraction methods led to the identification of flavanone apiofuranoside in these extracts too. Glycosides of quercetin, kaempferol and naringenin were also reported to be present in these methanol extracts [33]. LC-MS/MS analysis of methanol extracts from leaves of three *Faramea* species also showed that they are a rich source of different types of glycosides including flavone-*O*-glycosides, flavone-*C*-glycosides and flavonol-*O*-glycosides [34]. Bioassay-guided fractionation from methanol extracts of *Faramea* species could be considered further for exploring their anti-dengue activity.

Glycosides of Quercetin, Kaempferol and Other Flavonoids

Glycosides of quercetin and kaempferol are widely distributed in various plant sources. Herbal extracts consisting of these glycosides were reported to show anti-dengue activity in vitro. The hydro-ethanol extract prepared from leaves of *Bauhinia holophylla* (Fabaceae) inhibited DENV-2 infected Vero cells in a dose-dependent manner. One of the sub-fractions obtained from hydro-alcohol extract was found to exhibit potent anti-dengue activity in DENV infected Vero cells (IC_{50} = 4.6 μg/mL, CC_{50} > 100 μg/mL and SI of 24.4). Phytochemical analysis and NMR studies indicated the presence of quercetin-3-*O*-*α*-L-arabinofuranoside and quercetin-3-*O*-*α*-L-rhamnopyranoside in this active sub-fraction along with aglycone quercetin [35]. Quercetin and its derivatives have been identified to exhibit anti-dengue activity by inhibiting RNA polymerase complex [42]. Thus, the presence of quercetin and its glycosides in the active sub-fraction of leaf extract could be responsible for the anti-dengue activity of *Bauhinia* spp.

Lee et al. reported the effect of aqueous cocktail extract from aerial parts of *Phyllanthus* spp. (*P. watsonii*, *P. amarus*, *P. niruri* and *P. urinaria*; family Euphorbiaceae) in the ratio 2:2:1:1 against DENV-2 inoculated Vero cells. The HPLC and LC-MS/MS analysis of cocktail extract revealed the presence of polyphenolic compounds such as quercetin glucoside, quercetin rhamnoside, gallic acid, galloylglucopyronside, corilagen, geraniin, rutin, syringin, syringin dimer, digalloylglucopyronside, trigalloylglucopyronside and apigenin rhamnoside along with geraniin as the major component. Anti-viral activity was interpreted based on percentage inhibition of viral copy number in cells and supernatants following different modes of treatment (pre, simultaneous, post) and at regular intervals. With inhibition percentages of 94.69, 92.78 and 93.61 in cells and 82.85, 84.61 and 91.48 in supernatants at 24, 48 and 72 h, respectively, cocktail extract unveil its potential anti-viral activity during simultaneous treatment. Conversely, very low inhibition percentages of 6.38, 4.66 and 0 in cells and 46.07, 39.01 and 17.87 in supernatants were observed at 24, 48 and 72 h, respectively, indicating no viral reduction in post-treatment mode.

The time of addition studies postulated that cocktail of aqueous extracts from *Phyllanthus* spp. would have possibly hindered the viral entry into host cells by altering/blocking the viral or host factors and further by directly inactivating virus particles. Proteome analysis revealed significant alteration in expression of 14 proteins. These proteins are either part of host cell or virus, involved in host-viral adsorption, viral entry, production of viral polyprotein, viral RNA replication, viral assembly and maturation. The authors proposed *Phyllanthus* as an early inhibitor owing to its activity prior to or during DENV infection, which was further reinforced by proteomic analysis [36].

Euphorbia hirta (Euphorbiaceae) is commonly used in the Philippine islands for treating dengue infection [37, 38]. In vitro study of ethyl acetate extract of *E. hirta* exhibited 85% and 90% inhibition of DENV-1 and DENV-2 infection in Vero cells, respectively, by plaque titration assay. Glycosides of quercetin and kaempferol and lupane-type triterpenes were isolated from the active extract by chromatography [46]. Glycosides isolated from *E. hirta* were identified as kaempferol 3-*O*-arabinofuranoside, kaempferol 3-*O*-rhamnopyranoside and quercetin

3-*O*-rhamnopyranoside. Presence of these flavonoid glycosides along with the terpene lupeol could be responsible for the anti-dengue profile of *E. hirta* extract.

Psiloxylon mauritianum (Myrtaceae) is another medicinal plant containing glycosides of quercetin, kaempferol and gallic acid. In the study reported by Clain et al., the aqueous extract of aerial parts of *P. mauritianum* reduced virus particles in C6/36 cells when determined by FFURA. Analysis of DENV (1 to 4 serotypes) infected Huh 7.5 cells at 48 h post-treatment with *P. mauritianum* extract at 100 μg/mL showed 90% inhibition of DENV E protein. The extract was also found to be safe as there was no effect on cell viability at this concentration till 48 h [39].

Hyperoside (quercetin 3-*O*-*β*-D-galactopyranoside, Fig. 7.5) was identified as one of the compounds present in *Norantea brasiliensis* (Marcgraviaceae). The ethanol crude extract of *N. brasiliensis* leaf and various fractions (dichloromethane, ethyl acetate and butanol fractions) from that crude extract were tested in DENV infected monocytes in vitro. The extract and fractions of *N. brasiliensis* reduced the secretion of NS1 protein and also various inflammatory cytokines (TNF-*α*, IFN-*α*, IL-6 and IL-10) indicative of anti-dengue and immunomodulatory activities. However, the role of hyperoside in anti-dengue and immunomodulatory activities of *N. brasiliensis* was not explored in this study [40]. Further studies are needed to understand the mechanism of anti-dengue activity of hyperoside present in these active fractions of *N. brasiliensis.*

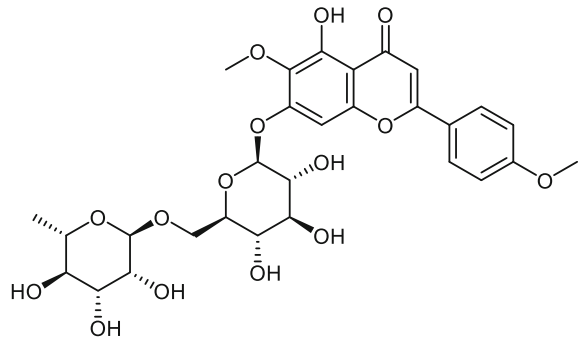
Flavonoids and their glycosides show variations in their efficacy and mechanism of anti-dengue activity. The biflavonoid hinokiflavone was found to be the most potent molecule ($IC_{50} = 0.26$ μM) among all of them. The anti-dengue activity of alkylated flavanones chartaceones C, E and F was comparable to that of 5-hydroxy-7-methoxy-6-methylflavone. Baicalein, quercetin and catechin were also found to be promising for their further development as anti-dengue molecules. The activity profile of the combination of flavone glycosides pectolinarin and acacetin-7-*O*-rutinoside indicates that combination approach could be explored based on their SIs and mechanism of action. An early-stage inhibitor 5-hydroxy-7-methoxy-6-methylflavone in combination with RNA replication inhibitor like baicalein or quercetin could be studied for their synergistic mechanism in controlling dengue infections.

7.5.2 Alkaloids

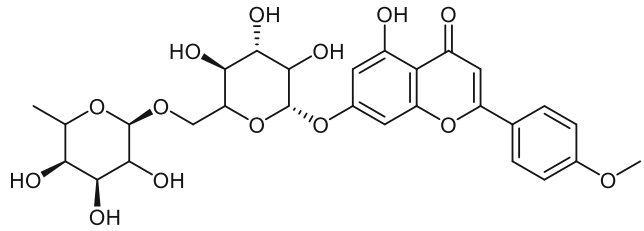
Alkaloids are nitrogen-containing secondary metabolites produced in plants in response to environmental stress. They are diverse group of compounds usually classified based on the heterocyclic ring present in their structure. They occur as free bases or as salts of organic acids like acetic acid, tartaric acid, tannic acid, etc. The pharmacological activity of alkaloids is quite broad, and many of them are available as approved therapeutic medicines [47]. In this section, the plant sources for various alkaloids showing anti-dengue activity are discussed (Table 7.3).

7.5.2.1 Carpaine

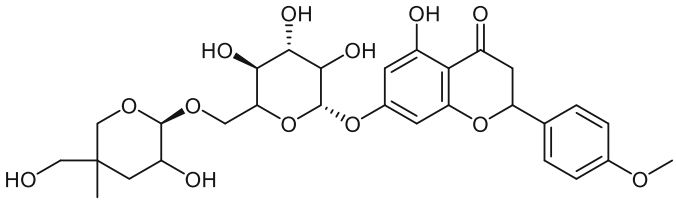
Among the natural products used for the treatment of DF, *Carica papaya* leaf extract is notably one of the most widely used herbal medicines in Asia. There are numerous clinical studies reported on papaya leaf juice for the treatment of patients with DF, especially for its role on controlling thrombocytopenia [57]. Carpaine (Fig. 7.6) is identified as one of the active constituents in papaya leaf juice or papaya leaf extract. Carpaine-rich extracts and isolated carpaine were evaluated for anti-thrombocytopenia effect in *Wistar* rats. This study provided evidence that the alkaloid carpaine present in bioassay-guided papaya leaf extract could be responsible for its therapeutic effect in improving the platelet count in vivo [58]. A clinical study conducted by Chinnappan et al. established the mechanism of papaya leaf juice on thrombocytopenia. This study demonstrated that incubation of plasma from dengue-infected patients with papaya leaf juice inhibited platelet aggregation, which could be the probable mechanism for controlling thrombocytopenia in dengue-infected patients [50].



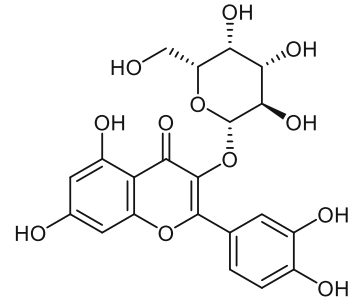
Pectolinarin



Acacetin-7-O-rutinoside



Flavanone apiofuranoside



Hyperoside

Fig. 7.5 Structure of flavonoid glycosides showing anti-dengue activity. Pectolinarin and acacetin-7-*O*-rutinoside combination: Inhibited DENV-2 infection, EC_{50} 11.1 μg/mL. Flavanone apiofuranoside: Inhibited DENV-2-replication, IC_{50} = 13.1 μg/mL. Hyperoside: Inhibited NS1 production

To explore the molecular mechanism of papaya leaf extract or juice in dengue virus infection, various in vitro and in vivo studies in animals such as rats and mice were reported. An in vitro study carried out in THP-1 cells infected with DENV showed that papaya leaf extract could exert anti-viral effect by its ability to inhibit the expression of DENV E. However, in an in vivo study conducted in DENV-2 infected AG129 mice model, treatment with freeze-dried preparation of papaya leaf juice for three consecutive days at two different doses of 500 and 1000 mg/kg/day did not reduce the level of structural protein NS1 of DENV and viral RNA level in mice plasma [48]. The in vivo study could not provide scientific evidence for the anti-dengue activity of papaya leaf juice.

Table 7.3 List of alkaloids from plants with anti-dengue activity

Phytoconstituents	Plant species	In vitro study	In vivo study
Carpaine	*Carica papaya*	Decreased DENV E proteins in THP-1 cells [48]	Immunomodulation effect in AG129 mice model, but no anti-viral activity [49] Thrombocytopenia activity in patients with dengue [50]
Bisbenzylisoquinoline alkaloids—Grisabine, grisabutine, panurensine, norpanurensine, krukovine, limacine, peinamine, 7-*O*-demethylpeinamine, N-methyl, 7-*O*-demethylpeinamine, macolidine and macoline	*Cissampelos pareira*	Decreased secretion of DENV NS1 protein in HepG2 and Vero cells infected with all four types of DENV [51]	Anti-viral activity in AG129 mouse [51]
Speciophylline, mitraphylline, uncarine F, pteropodine, isomitraphylline and isopteropodine (pentacyclic oxindole alkaloids)	*Uncaria tomentosa*	Decreased secretion of DENV-2 NS1 protein in HMEC1 cells [52]	NR
Hirsutine	*Uncaria rhynchophylla*	Inhibition of assembly of virus particles in DENV infected A549 cells [53]	NR
Oxindole alkaloid	*Uncaria guianensis*	Decreased secretion of DENV-2 NS1 protein and inflammatory cytokines IL-6, IL-8 and macrophage migration inhibitory factor in Huh 7 cells [54]	NR
Voacangine	*Tabernaemontana cymose*	Anti-dengue activity in DENV-2 infected Vero cells [55]	NR
Ficuseptine and antofine	*Ficus septica*	Anti-viral activity in HepG2 cells infected with DENV-1 and -2 [56]	NR

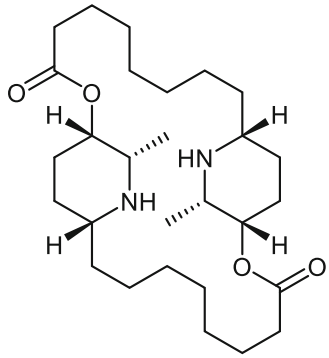
NR Not Reported

An increased formation of inflammatory cytokines, referred to as "cytokine storm", indicates the severity of dengue infection. Papaya leaf juice could control the cytokine storm by inhibiting the expression of genes for inflammatory cytokines in the liver of DENV infected AG129 mice, thereby exerting its immunomodulation effect in DENV infected mice rather than direct anti-viral activity [49].

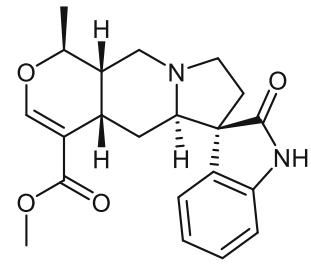
7.5.2.2 Bisbenzylisoquinoline Alkaloids

Cissampelos pareira (Menispermaceae), commonly known as velvet leaf, contains bisbenzylisoquinoline alkaloids. They include grisabine (Fig. 7.6), grisabutine, panurensine, norpanurensine, krukovine, limacine, peinamine, 7-*O*-demethylpeinamine, N-methyl,7-*O*-demethylpeinamine, macolidine and macoline [59, 60].

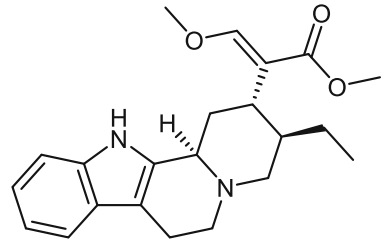
Sood et al. reported the alcohol extract of *C. pareira* to be a potent inhibitor of all four DENVs (DENV-1 to DENV-4) in cell-based assays. They evaluated viral NS1 antigen secretion using ELISA and viral replication using plaque assays. The inhibitory potential of *C. pareira* extract on viral antigen synthesis and viral titres in DENV-3 infected Vero cells was promising in the maximum concentration (100 μg/mL) used and was dose dependent. The extract conferred statistically significant protection against DENV infection in vivo in AG129 mouse model [51]. The authors also conducted an in vivo study in AG129 mice infected with DENV-2. The median survival time (MST) of



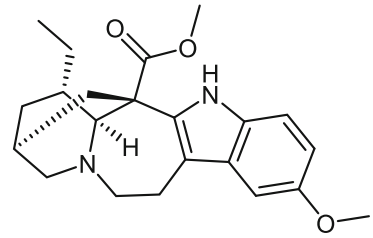
Carpaine



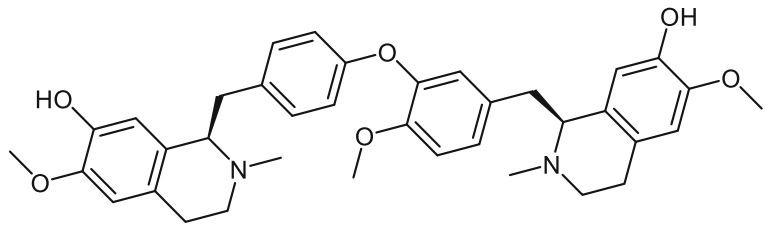
Speciophylline



Hirsutine



Voacangine



Grisabine

Fig. 7.6 Structure of alkaloids showing anti-dengue activity. Carpaine: Anti-thrombocytopenia effect in dengue-infected patients and immunomodulation activity in DENV-2 infected mice. Speciophylline: One of the constituents in bioactive fraction from *Uncaria tomentosa* bark; this fraction inhibited NS1 and IL-8 production. Hirsutine: Inhibited DENV-1 infection, $EC_{50} = 1.9$ μM. Voacangine: Inhibited DENV-2 infection, $EC_{50} = 10.1$ μg/mL. Grisabine: A key constituent in the extract of *Cissampelos pareira*; the extract inhibited DENV infection by all serotypes

infected mice was 19 days, whereas administration of 125 mg/kg extract twice daily for 5 days increased the MST to 28 days with 50% survival rate. The survival increased to ~67% at double the dose, suggestive of dose dependency. The study reported that co-treatment with paracetamol did not influence the anti-dengue profile of *C. pareira* extract in vitro. However, co-administration of the extract with paracetamol had synergistic effect in lowering the body temperature in brewer's yeast-induced pyrexia in *Wistar* rats.

Furthermore, it was reported that the extract appreciably suppressed the release of pro-inflammatory cytokines TNF-α and IL-1β with IC_{50} of 6.1 ± 1.3 and 5.7 ± 2.7 μg/mL, respectively. The cytotoxicity at the abovementioned concentration was indistinct with CC_{50} of 78.9 and >200 μg/mL in HepG2 and LLC-MK2 cell lines, respectively. *C. pareira* extract manifested anti-dengue activity against the most prevalent dengue serotypes. Hence, the extract could be formulated into a suitable dosage form to combat dengue. Future phytochemical studies could embark on isolation and identification of bioactives followed by drug discovery process towards anti-dengue therapeutics [51].

7.5.2.3 Indole Alkaloids

The alkaloid-enriched fraction from the bark of *Uncaria tomentosa* (Rubiaceae) was evaluated for anti-viral property in human dermal microvascular endothelial cells (HMEC-1) infected with DENV-2. The HPLC analysis of alkaloid fraction established the presence of six pentacyclic oxindole alkaloids (speciophylline (Fig. 7.6), mitraphylline, uncarine F, pteropodine, isomitraphylline and isopteropodine). The fraction presented no cytotoxicity towards HMEC-1 cell culture. A significant reduction of NS1 antigen secretion in supernatant was noticed at 24 and 48 h post-infection at concentrations from 1, 10 and 50 μg/mL. Moreover, a reduction of IL-8 concentration in supernatant was also determined by ELISA representing immunomodulatory effect. The most significant activity against NS1 antigen and IL-8 was exerted at 1 μg/mL. The paracellular permeability measurement by transendothelial electrical resistance demonstrated a reduction of endothelial permeability in DENV infected HMEC-1 and was time dependent [52].

Hirsutine (Fig. 7.6), an indole alkaloid, was reported to be present in *Uncaria rhynchophylla*. The anti-dengue activity of hirsutine was studied in DENV-1 infected A549 cells by plaque titration assay and found to exhibit potent anti-viral activity (EC_{50} = 1.9 μM, CC_{50} > 10 μM, SI > 5.3). Various other in vitro studies including time of addition, time of elimination and RNA replication assays were conducted on hirsutine to understand the mechanism of anti-dengue activity. All these assays indicated that hirsutine could act on DENV by inhibition of entry, replication, assembly and release of virus particles in host cells [53].

The hydro-alcohol extracts obtained from the leaf and bark of *U. guianensis* have also shown anti-dengue activity in DENV-2 infected Huh 7 cells causing reduction in NS1 protein. In addition to anti-dengue activity, these two extracts were also reported to have immunomodulatory effect as there was reduction in IL-6, IL-8 and macrophage migration inhibitory factor in Huh 7 cells. Preliminary phytochemical analysis of both leaf and bark extracts indicated the presence of oxindole alkaloids and flavonoid kaempferitrin. However, the role of these phytochemicals for anti-dengue activity of *U. guianensis* requires further investigation [54].

Voacangine (Fig. 7.6), another indole alkaloid, was reported with potential anti-dengue activity in DENV-2 infected Vero cells (EC_{50} = 10.1 μg/mL, CC_{50} = 1136.3 μg/mL). Voacangine was obtained from the ethanol extract of *Tabernaemontana cymosa* (Apocynaceae) seeds along with another active constituent lupeol acetate [55].

7.5.2.4 Ficuseptine and Antofine

The methanol leaf extract of *Ficus septica* (Moraceae) exhibited anti-dengue activity in HepG2 cells infected with DENV-1 (IC_{50} = 10.1 ± 2.4 μg/mL) and DENV-2- (IC_{50} = 12.2 ± 2.1 μg/mL). The extract was found to be safe with no cytotoxicity in A549 cells at the maximum tested concentration of 100 μg/mL [56]. In this study, the anti-viral effect of these extracts was also explored against Aichi

Table 7.4 List of terpenoids from plants with anti-dengue activity

Phytoconstituents	Plant species	In vitro study	In vivo study
Andrographolide	*Andrographis paniculata*	Anti-dengue activity in Vero cells infected with all four types of DENV [62] Decreased DENV E and NS3 proteins in HpG2 and HeLa cells infected with DENV-1 and -2 [63]	NR
Azadirachtin, nimbin, nimbidin, nimbolide, and limonoids	*Azadirachta indica*	Anti-dengue activity in C6/C36 cells infected with DENV-2 [64]	Anti-dengue activity in mice [64]
Celastrol	*Tripterygium wilfordii*	Inhibition of RNA replication in DENV-2 infected Huh 7 cells, Induction of the anti-viral interferon IFN-α [65]	Anti-dengue activity in DENV-2 infected mice [65]
Betulinic acid and betulinic aldehyde	*Diospyros glans* and *Diospyros carbonaria*	Inhibition of DENV NS5 RdRp enzyme [66, 67]	NR
Lupeol	*Maytenus gonoclada*	Anti-dengue activity in LLC-MK2 cells [68]	NR
Lupeol acetate	*Tabernaemontana cymose*	Anti-dengue activity in DENV-2 infected Vero cells [55]	NR
Swielimonoid B	*Swietenia macrophylla*	Anti-dengue activity in Huh 7 cells infected with DENV-2 [69]	NR
3-α-Tigloylmelianol and melianone	*Melia azedarach*	Anti-dengue activity in DENV-2 infected BHK-21 cells [70]	NR

NR Not Reported

virus, which has no envelope. Interestingly, extracts of *F. septica* did not show inhibition of Aichi virus, indicating that the possible mechanism of anti-viral activity against DENV could be due to the inhibition of viral membrane proteins. This herb contains alkaloids ficuseptine and antofine, which have been earlier reported to show anti-bacterial and anti-fungal activities. The possible role of these two alkaloids in anti-dengue activity of *F. septica* needs to be studied further.

Though studies are available on the anti-dengue activity of plants containing alkaloids, the information reported on isolated alkaloid is less in comparison to flavonoids. Indole alkaloids hirsutine and voacangine have the potential to be considered further for their anti-dengue activity. Future studies in alkaloids could focus on their isolation from extracts and bioactive fractions to develop lead molecules. Carpaine from papaya leaf extract or juice was effective as immunomodulatory rather as direct-acting anti-dengue molecule. Oxindole alkaloids from *Uncaria* species showed immunomodulatory property along with anti-dengue activity. Another important observation from the reports of oxindole alkaloids is they act on NS1 protein, which plays a role in vascular leakage and thrombocytopenia during dengue infections. This is in contrast to flavonoids, which act mainly on NS3 or NS5 proteins, inhibiting viral replication.

7.5.3 Terpenoids

Terpenes and terpenoids are another large group of compounds made of isoprene units. They are classified as monoterpenes, diterpenes, sesquiterpenes, triterpenes, tetraterpenes and polyterpenes (carotenoids) based on the number of isoprene units. Artemisinin (anti-malaria), ginkgolide (anti-inflammatory) and taxol (anti-cancer) are few examples of medicinally important terpenoids from plant sources [61]. Terpenoids are also reported to exhibit anti-dengue activity, and the following section provides an overview of their anti-dengue profile (Table 7.4).

7.5.3.1 Andrographolide

Andrographis paniculata (Acanthaceae) commonly known as "king of bitters" is native to South Asian countries such as India and Sri Lanka [71]. It is one of the widely used traditional medicines in India for dengue infection. *A. paniculata* extracts were found to contain diterpenoids and flavonoids; among them, andrographolide (Fig. 7.7) is considered as the major bioactive constituent. Ethanol extract exhibited anti-viral property in an in vitro study conducted in Vero cells infected with all four types of DENV [62]. Tang et al. reported that methanol extract of *A. paniculata* displayed 75% inhibition of cytopathic effects in DENV-1 infected Vero cells [72].

The virtual screening analysis reported by Vijayakumar et al. indicated that andrographolide has better binding affinity with active site of DENV E protein [73]. The in vitro study of andrographolide in DENV-2 and DENV-4 infected HepG2 and HeLa cells demonstrated anti-dengue activity at EC_{50} of 21.30 and 22.74 μM, respectively. Andrographolide did not show any cytotoxicity in both these cell lines till the concentration of 100 μM. Incubation with andrographolide reduced viral production and decreased the formation of DENV E and NS3 proteins in both pre-infection and post-infection experimental conditions [63]. Results of this in vitro study substantiate the findings reported by Vijayakumar et al. [73].

7.5.3.2 Azadirachtin

Neem (*Azadirachta indica*, Meliaceae) contains various bioactive components including azadirachtin, nimbin, nimbidin, nimbolide, and limonoids, which play a role in disease management through the modulation of various genetic pathways and other activities [74]. Parida et al. reported in vitro and in vivo inhibitory effects of neem leaves' aqueous extract and pure neem compound azadirachtin (Fig. 7.7) on the replication of DENV-2. The aqueous extract of neem leaves inhibited the virus in DENV-2 infected C6/C36 cells at 1.9 mg/mL without any cytopathic effects. In vivo, neem leaves' extract (120 mg/mL) inhibited virus replication in suckling mice as confirmed by RT-PCR [64].

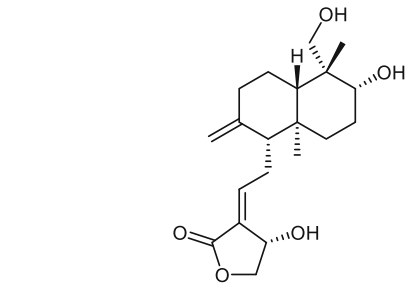
7.5.3.3 Celastrol

Celastrol, a pentacyclic triterpenoid (Fig. 7.7), is the major bioactive molecule found in the root extracts of *Tripterygium wilfordii* (Celastraceae). It has been probed for its anti-dengue activity both in vitro and in vivo [65]. In this study, celastrol had shown inhibition of RNA replication in vitro in DENV-2 infected Huh 7 cells ($EC_{50} = 0.12 \pm 0.01$ μM). It also exhibited dose-dependent inhibition of DENV NS2B protein till the concentration of 0.2 μM. Celastrol was found to be active in vivo also in DENV-2 infected mice.

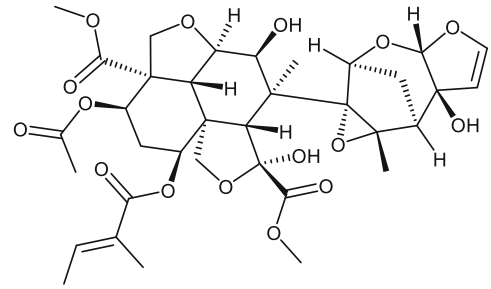
Gene expression studies in DENV-2 infected Huh 7 cells and mice proved that anti-viral activity of celastrol could be due to its ability to induce the anti-viral interferon IFN-α. However, earlier reports indicated challenges in the development of celastrol as a successful drug candidate because of its poor solubility (13.25 ± 0.83 mg/mL at 37 °C), poor bioavailability (17.06%), narrow therapeutic index and associated side effects (gastrointestinal effects and upper respiratory infection) [75, 76]. Development of suitable formulations for celastrol could improve its pharmacokinetic properties and make it as a safe therapeutic molecule against dengue infection.

7.5.3.4 Betulinic Acid and Betulinic Aldehyde

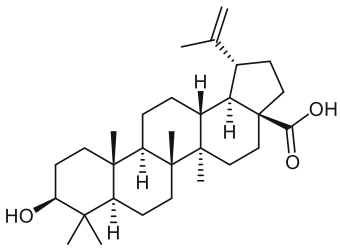
The ethyl acetate extract of *Diospyros glans* and *Diospyros carbonaria* (Ebenaceae) bark demonstrated anti-virus activity against DENV-2. This extract contains six active compounds; among them, two triterpenoids betulinic acid ($IC_{50} = 6.6 \pm 0.6$ μM, Fig. 7.7) and betulinic aldehyde ($IC_{50} = 7.0 \pm 1.4$ μM, Fig. 7.7) showed potent activity in DENV NS5 RdRp assay in vitro. Both betulinic acid (50% cell viability) and betulinic aldehyde (70% cell viability) were less cytotoxic at the concentration tested (10 μM) in COS cells. These two compounds have also been found to be present in endophyte *Phomopsis* spp., growing on *Diospyros* spp. [66, 67].



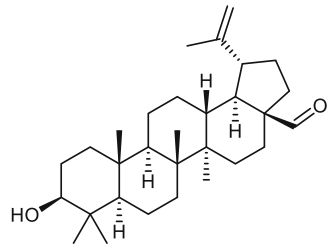
Andrographolide



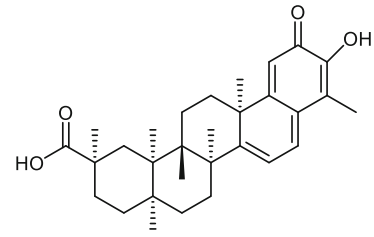
Azadirachtin



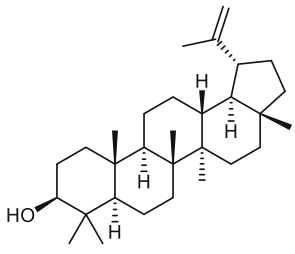
Betulinic acid



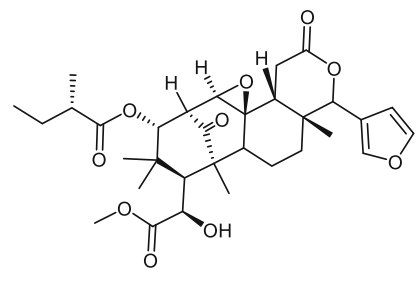
Betulinic aldehyde



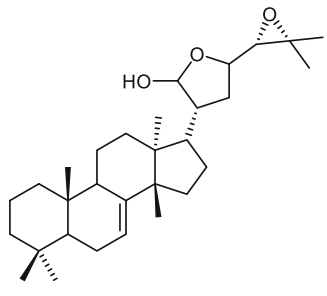
Celastrol



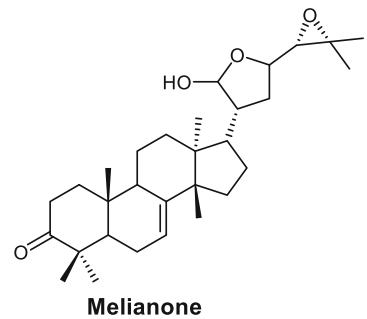
Lupeol



Swielimonoid B



3-alpha-tigloylmelianol



Melianone

Fig. 7.7 Structure of terpenoids showing anti-dengue activity. Andrographolide: Inhibited DENV-2 infection, EC_{50} = 21.30 μM, and DENV-4 infection, EC_{50} = 22.74 μM. Decreased the production of E and NS3 proteins. Azadirachtin: Inhibited DENV-2 replication in a dose-dependent manner. Betulinic acid and betulinic aldehyde: Inhibited NS5 RdRp enzyme at IC_{50} of 6.6 μM and IC_{50} of 7 μM, respectively. Celastrol: Inhibited NS2B protein, EC_{50} = 0.12 μM. Lupeol: Inhibited DENV-2-infection, EC_{50} = 9.77 μg/mL. Swielimonoid B: Inhibited DENV-2 infection, EC_{50} = 7.2 μM. 3-Alpha-tigloylmelianol: Inhibited DENV-2 infection, EC_{50} = 3 μM. Melianone: Inhibited DENV-2 infection, EC_{50} = 12 μM

7.5.3.5 Lupeol

Lupeol is a pentacyclic triterpenoid (Fig. 7.7) isolated from the ethyl acetate extract of branches and roots of *Maytenus gonoclada* (Celastraceae). Lupeol showed anti-dengue activity in vitro in DENV-2 infected LLC-MK2 cells (EC_{50} = 9.77 μg/mL, CC_{50} = 127.7 ± 21.43 μg/mL, SI = 13.07) with low cytotoxicity by plaque titration assay. As the solubility of lupeol is low, its anti-dengue activity was also checked for the complex formed between lupeol with *β*-cyclodextrin. However, this complex was not found to be active against DENV [68]. Another compound, lupeol acetate, was reported as one of the active constituents in fraction from ethanol extract of *Tabernaemontana cymosa* (Apocynaceae) seeds. The anti-dengue activity of lupeol acetate was demonstrated in DENV-2 infected Vero cells (EC_{50} = 37.5 μg/mL, CC_{50} = 4015.5 μg/mL). Lupeol was found to be more potent than lupeol acetate as evidenced by their EC_{50} values [55].

7.5.3.6 Limonoids

Limonoids isolated from the ethanol extract of *Swietenia macrophylla* (Meliaceae) seeds were evaluated for anti-dengue activity in Huh 7 cells infected with DENV-2. Among them, swielimonoid B (Fig. 7.7), swietenolide, swietenine acetate, 7-deacetoxy-7*α*-hydroxygedunin and methyl angolensate exhibited anti-dengue activity with EC_{50} values of 7.2 ± 1.33, 3.5 ± 0.34, 6.3 ± 1.12, 12.5 ± 2.35 and 4.3 ± 2.31 μM, respectively, while the CC_{50} values were > 200, 68 ± 1.21, 83 ± 3.45, 105 ± 3.89 and 116 ± 4.64 μM, respectively. Swielimonoid B was considered to exhibit significant anti-viral activity as it possessed weak cytotoxic effect, i.e. > 200 μM, with marked EC_{50} of 7.2 ± 1.33 μM and SI >27.7 [69].

Similarly, limonoids from *Melia azedarach* (Meliaceae) fruits were also evaluated against DENV-2 based on the inhibition of virus-induced cytopathogenicity in BHK-21 cells. Of the various limonoids screened, 3-*α*-tigloylmelianol (EC_{50} = 3 μM, CC_{50} = 20 μM, Fig. 7.8) and melianone (EC_{50} = 12 μM, CC_{50} = 50 μM, Fig. 7.8) exhibited remarkable activity against DENV-2. The probable mechanism could be the inhibition of the entry process or the early episode of life cycle during the first hour post-infection [70].

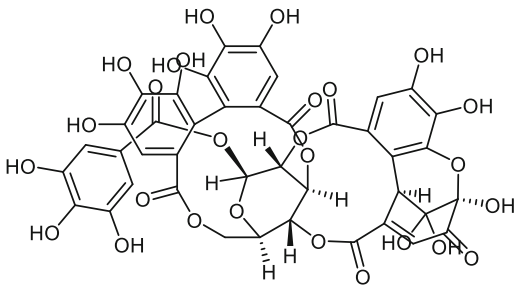
Anti-dengue profiles of terpenoids are similar to those of flavonoids as they also exert their activity by inhibiting viral replication targeting NS3 or NS5 proteins. Moreover, sufficient reports are available on the activity of terpenoids isolated from their plant sources. Among terpenoids, celastrol was found to be the most potent molecule, but it has poor SI. Other terpenoids with better efficacy against DENV include betulinic acid, betulinic aldehyde, lupeol and limonoids. The major challenge reported for terpenoids is their poor solubility. This highlights the requirement for suitable formulations of active phytoconstituents to improve their bioavailability.

7.5.4 Miscellaneous

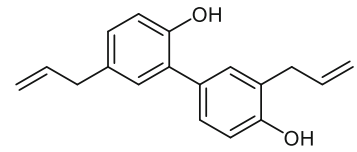
In addition to the three large categories of phytochemicals discussed in Sects. 7.5.1, 7.5.2, and 7.5.3, there are other categories of phytoconstituents effective against DENV infection (Fig. 7.9). This section provides insight on anti-dengue profiles of natural compounds other than flavonoids, alkaloids and terpenoids (Table 7.5).

7.5.4.1 Tannins: Geraniin

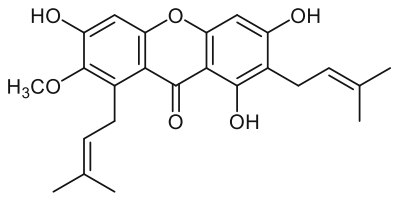
Geraniin, a hydrolysable tannin (Fig. 7.8), is an active phytochemical obtained from the fruit rind of *Nephelium lappaceum* (Sapindaceae). Geraniin is an official anti-diarrhoeal compound in Japan [91] and is distributed in various other natural sources, namely, *Phyllanthus urinaria*, *Phyllanthus amarus* and *Geranium carolinianum*. The anti-dengue activity of geraniin for DENV-2 was studied extensively by Siti Aisay et al. [78]. In this study, the anti-dengue activity of geraniin was determined by plaque reduction assay (IC_{50} = 1.75 μM) in Vero cells infected with DENV-2. Geraniin did not show cytotoxicity in Vero cells at 2 mM concentration.



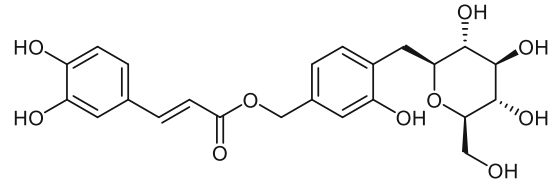
Geraniin



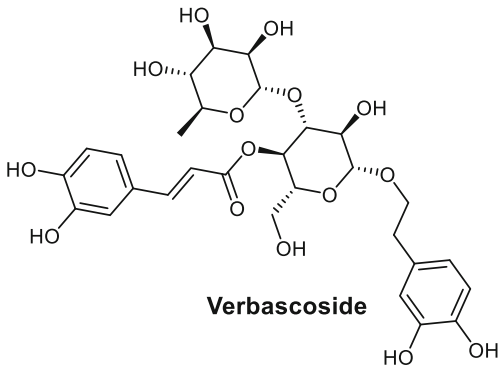
Honokiol



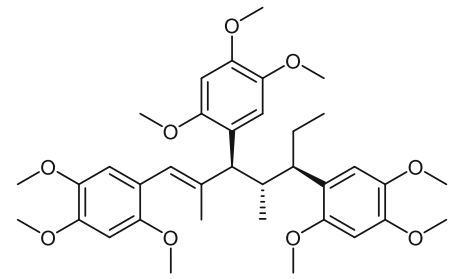
Alpha-Mangostin



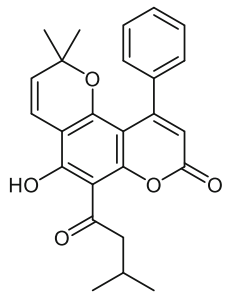
Caffeoylcalleryanin



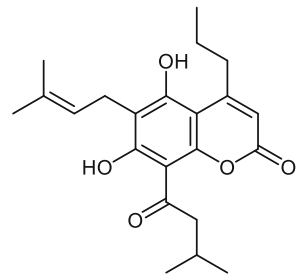
Verbascoside



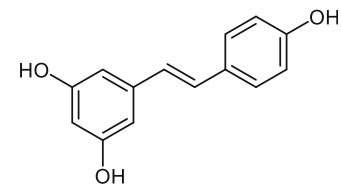
Tatanan A



Coumarin A



Coumarin B



Resveratrol

Fig. 7.8 Structure of different categories of phytoconstituents showing anti-dengue activity. Geraniin: Inhibited DENV-2 infection, IC_{50} = 1.75 μM, and expression of DENV E gene, IC_{50} = 1.78 μM. Honokiol: Inhibited DENV-2 infection and expression of NS1 and NS3 proteins at 20 μM. Alpha-mangostin: Inhibited dengue infection by four serotypes of DENV through inhibition of DENV E protein at 20 μM and suppressed cytokine/chemokine expressions also. Caffeoylcalleryanin: Inhibited DENV-2 infection, EC_{50} = 2.8 μg/mL. Verbascoside: Inhibited DENV-2 infection, EC50 = 3.4 μg/mL. Tatanan A: Inhibited DENV-2-infection, EC_{50} = 3.9 μg/mL, and decreased formation of E and NS1 proteins. Coumarins A and B: Inhibited DENV infection possibly through their effect on NS5 proteins at EC_{50} = 9.6 and 2.6 μg/mL, respectively. Resveratrol: Inhibited DENV infection through its effect on NS3 protein at 80 μM, also inhibited HMGB1 and induced ISG in host cells

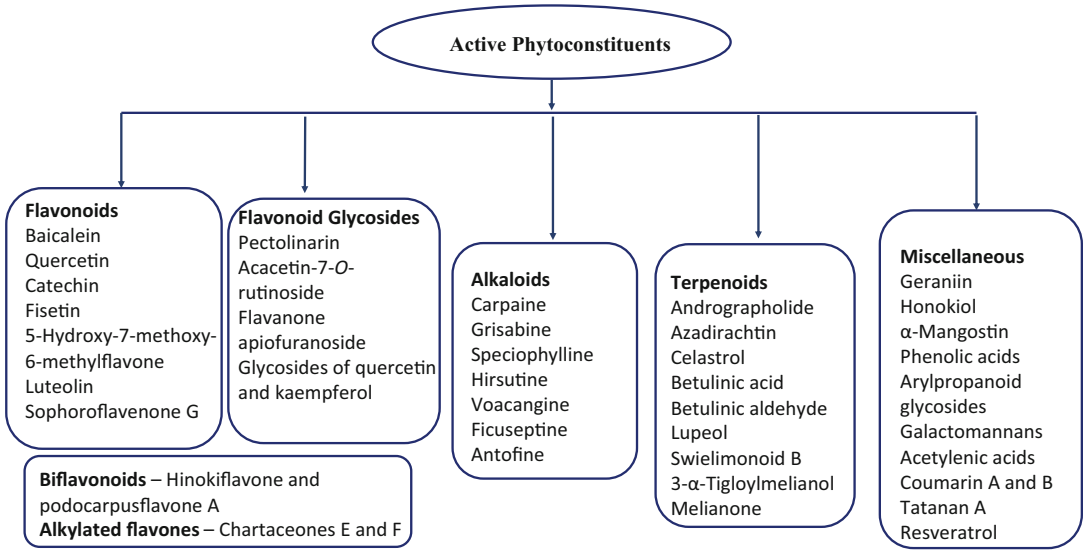


Fig. 7.9 List of different categories of phytoconstituents showing anti-dengue activity. Major class of active compounds includes flavonoids, alkaloids and terpenoids. Other classes of compounds are tannins (geraniin), lignans (honokiol), phenolic acids (gingerol), arylpropanoid glycosides, galactomannans, coumarins and stilbene derivative (resveratrol)

The time of addition assays at various stages of Vero cells infection by DENV-2 revealed that geraniin inhibited virus (90%) when added till 16 h post-infection. The activity decreased drastically (50%) when geraniin was added at 24 h post-infection. The attachment assay also showed that geraniin significantly reduced plaque formation in Vero cells at an early stage. Time of addition and attachment assays indicated that the mechanism of anti-dengue activity could be the prevention of virus binding with host cell receptors. So, the probable target for anti-dengue activity of geraniin is DENV E protein, which mediates the virus attachment to host cell. Docking experiments also proved that geraniin binds to DENV E protein through hydrogen bonding with Phe337 and hydrophobic interactions in the domain III region of DENV E protein.

In another study by Siti Aisay et al., the effect of geraniin on the synthesis of DENV-2 RNA was investigated by qRT-PCR. Geraniin inhibited the expression of DENV E gene completely at 26.3 μM (IC_{50} = 1.78 μM) [77]. In this study, the anti-dengue activity of geraniin in DENV-2 infected BABL/c mice was also studied after intravenous administration for 7 days. Geraniin inhibited the viral load, liver damage and splenomegaly in mice. All these results prove geraniin as another important phytoconstituent with anti-dengue activity. Similarly, anti-dengue activity was reported for another medicinal plant *Phyllanthus phillyreifolius*, which has geraniin as the major constituent, in Huh 7.5 cells infected with all DENV-1 to DENV-4 serotypes [92].

7.5.4.2 Honokiol

Honokiol, a lignan biphenol (Fig. 7.8) obtained from *Magnolia* tree, was tested for its anti-viral activity against DENV-2 serotype in transfected BHK cells harbouring luciferase-reporting subgenomic replicon. Honokiol exhibited a dose-dependent inhibitory effect on DENV replication by 23.3 ± 3.3% at 5 μM, 51.6 ± 2.5% at 10 μM and 65.1 ± 0.6% at 20 μM. Further, in vitro DENV yield reduction assay was also performed in BHK and Huh 7 cells to substantiate the observed effect on DENV replicon inhibition. Honokiol also inhibited intracellular DENV-2-replicon as well as suppressed the replication of

Table 7.5 List of different categories of phytoconstituents from plant with anti-dengue activity

Phytoconstituents	Plant species	In vitro study	In vivo study
Geraniin	*Nephelium lappaceum*	Inhibition of virus binding with receptors in DENV-2 infected Vero cells and reduction of DENV E protein [77, 78]	Anti-dengue activity in DENV-2 infected BABL/c mice [77]
Honokiol	*Magnolia* sps	Inhibition of DENV-2 replication in BHK and Huh7 cells [79]	NR
α-Mangostin	*Garcinia mangostanan*	Decreased DENV E proteins in HepG2 cells infected with all four types of DENV [80]	NR
Phenolic acids—Gingerols, shogaols, and paradols	*Zingiber officinale*	Inhibition of expression of MMP-2 and MMP-9, Upregulation of the expression of TIMP-1 and TIMP-2 in DENV infected Vero cells [81]	NR
Arylpropanoid glycosides—Verbascoside and caffeoylcalleryanin	*Arrabidaea pulchra*	Anti-dengue activity in DENV-2 infected Vero and LLC-MK2 cells [82]	NR
Sulfated galactomannans	*Caesalpinia ferrea, Dimorphandra gardneriana, Adenanthera pavonina*	Anti-dengue activity in DENV-2 infected Vero cells [83]	NR
Anacolosine ((*E*)-tridec-2-en-4-ynedioic acid), octadeca-9,11,13-triynoic acid, (13*E*)-octadec-13-en-9,11-diynoic acid and (13*E*)-octadec-13-en-11-ynoic acid	*Anacolosa pervilleana*	Inhibition of DENV-2 RdRp enzyme [84]	NR
Hexanedioic acid, bis (2-ethylhexyl) ester; 2,6,10,14,18,22-tetracosahexane; 2,6,10,15,19,23-hexamethyl-(ALL-E)-3,7,11,15-tetramethyl-2-hexadecen-1-oic acid, N-hexadecanoic acid, octadecanoic acid	*Pavetta tomentosa*	Anti-dengue activity in DENV infected C6/C36 cells [85]	NR
Coumarins A and B	*Mammea americana*	Inhibition of NS5 protein of DENV infected Vero cells [55, 86]	NR
Tatanan A	*Acorus calamus*	Anti-dengue activity in DENV infected BHK-21 cells—Reduction in the level of DENV E and NS1 proteins [87]	NR
Resveratrol[a]	–	Reduction in DENV viral particles and NS3 protein in DENV infected Huh 7 cells [88] Inhibits the translocation of HMGB1 from nucleus to cytoplasm Induction of ISG [89, 90]	NR

NR Not Reported
[a]Phytoalexin

DENV-2 in BHK and Huh 7 cells leading to a massive reduction in viral yield by more than 90% at the maximum non-toxic dose (MNTD) 10 μM and 20 μM, respectively. The CC_{50} of honokiol was 13.35 ± 1.13 μM for BHK cells and 31.19 ± 1.49 μM for Huh 7 cells.

The effect of honokiol on the expression levels of NS1, NS3 and the viral replicating intermediate, double-stranded RNA (dsRNA), was also reported. A sharp decline of viral particles was observed in BHK-DENV cells at 5 μM (about 50%) and 10 μM (> 90%). Conversely, the effect was less intense in Huh 7 cells; only the NS3 expression was sensitive to honokiol at 10 μM ($p < 0.001$). However, a significant reduction of NS1 ($p < 0.001$), NS3 ($p < 0.001$) and dsRNA ($p < 0.01$) was reported at 20 μM. Honokiol not only inhibited the expression of NS1 and NS3, but it also inhibited dsRNA expression as well [79].

Treatment of BHK cells with 10 μM honokiol followed by subsequent DENV infection after 24 and 48 h illustrated a distinct increase of fluorescence signal in flow cytometry analysis corroborating the attachment of DENV to BHK cells. Hence, honokiol neither altered the degree of DENV binding to the host cell surface nor affected the DENV receptor(s) expression on host cells. The increase in intracellular early endosomes and co-localization of DENV E protein after viral infection in Huh 7 cells were suppressed by honokiol at 10 and 20 μM representing the possible interference with endocytotic process during viral entry into the host cells. Further, it was also reported that honokiol did not suppress the DENV replication by altering the cell cycle progression in BHK and Huh 7 cells. Altogether it was summarized that honokiol inhibited in vitro viral replication and expression of NS1, NS3 and dsRNA and also interfered with the viral entry process. Based on all these grounds, honokiol gains the merit to be considered for further development as anti-dengue molecule [79].

7.5.4.3 α-Mangostin

α-Mangostin, a xanthone (Fig. 7.8), is an important active phytochemical isolated from the pericarp of *Garcinia mangostana* (Guttiferae). α-Mangostin (20 μM) was reported to reduce the viral infection by 55, 48, 50 and 47% in DENV-1 to DENV-4 infected HepG2 cells, respectively. The mechanism for the anti-dengue activity of α-mangostin was identified as an inhibitor of DENV E protein. RT-PCR analysis revealed that α-mangostin reduced the cytokine/chemokine expressions also in DENV infected HepG2 cells. The effect of α-mangostin on the expression of IL-6, TNF-α, macrophage inflammatory protein-1β, interferon-γ-inducible protein 10 transcription and RANTES transcription was significantly higher than those of standard molecule ribavirin. Thus, α-mangostin was identified as a promising phytoconstituent for severe dengue infection associated with "cytokine storm" due to its antiviral and anti-inflammatory activities [80].

7.5.4.4 Phenolic Acids

Ginger (*Zingiber officinale*, Zingiberaceae) is a common and widely used spice and herbal medicine for a long time [81]. The major active compounds found in ginger include phenolic acids such as gingerols, shogaols and paradols [93]. Sharma et al. reported that aqueous extract of ginger rhizome could reduce plasma leakage in dengue infections and subsequent complications of DHF and DSS. The ginger extract used in this study was standardized for the active constituent gingerol. In this study, the ginger extract inhibited the expression of matrix metalloproteinases (MMP-2 and MMP-9) in DENV-3 infected Vero cells in a dose-dependent manner till the concentration of 50 μg/mL. At the same time, the expression of tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2) was enhanced in Vero cells. MMPs are endopeptidases produced in DENV infected cells causing vascular leakage, whereas TIMP inhibits the activities of MMPs, thereby preventing vascular leakage. Thus, the ginger extract could play a major role in preventing severe dengue complications [81].

7.5.4.5 Arylpropanoid Glycosides

Ethanol leaf extract of *Arrabidaea pulchra* (Bignoniaceae) exhibited anti-DENV-2 property in LLC-MK2 cells ($EC_{50} = 46.8 \pm 1.6$ μg/mL, $CC_{50} = 124.4 \pm 0.8$ μg/mL) with SI 2.7. Bioassay-guided fractionation of leaf extract afforded two arylpropanoid glycosides, verbascoside and caffeoylcalleryanin (Fig. 7.8), along with a terpenoid, ursolic acid. Verbascoside ($EC_{50} = 3.4 \pm 0.4$ μg/mL, $CC_{50} = 12.9 \pm 1.1$ μg/

mL, SI = 3.8) and caffeoylcalleryanin (EC_{50} = 2.8 ± 0.4 μg/mL, CC_{50} = 56.1 ± 2.4 μg/mL, SI = 20) demonstrated significant anti-DENV-2 profile. These bioactive compounds were reported to act by interfering viral attachment and adsorption [82].

7.5.4.6 Galactomannans

The galactomannans from seeds of *Adenanthera pavonina*, *Caesalpinia ferrea* and *Dimorphandra gardneriana* were chemically sulphated and evaluated for their anti-dengue activity. The sulphated galactomannans from *C. ferrea* showed 96% inhibition of replication of DENV-2 in Vero cells while from *D. gardneriana* and *A. pavonina* exhibited 94% and 77% inhibition, respectively, at 25 μg/mL. The degree of sulphation in galactomannan was correlated to the anti-viral property and reported to be an inhibitor of DENV-2 entry. These sulphated galactomannans were found to be devoid of cytotoxicity at the concentrations of 25, 50 and 100 μg/mL up to 7 days in Vero cells [83].

7.5.4.7 Acetylenic Acids and Other Acids

Polyacetylenic acids anacolosine ((*E*)-tridec-2-en-4-ynedioic acid), octadeca-9,11,13-triynoic acid, (13*E*)-octadec-13-en-9,11-diynoic acid and (13*E*)-octadec-13-en-11-ynoic acid from *Anacolosa pervilleana* (Olacaceae) leaves exhibited IC_{50} values of 2.5 ± 0.1, 2.7 ± 0.4, 2.2 ± 0.5 and 2.7 ± 0.4 μM, respectively, in DENV NS5 RdRp assay. The CC_{50} values of all these acids except anacolosine were between 20 and 30 μM in Vero cells (octadeca-9,11,13-triynoic acid, 30 μM; (13*E*)-octadec-13-en-9,11-diynoic acid, 23 μM; (13*E*)-octadec-13-en-11-ynoic acid, 30 μM). The CC_{50} of anacolosine was more than 420 μM, and the presence of an additional acidic group in this compound would have probably prevented its entry through cell membrane showing a scanty cytotoxicity [84].

Acetone extract of *Pavetta tomentosa* (Rubiaceae) leaves showed anti-dengue activity in vitro. The extract inhibited viral infection by 92% in C6/C36 cells at the concentration of 125 μg/mL. Phytochemical analysis of *P. tomentosa* extract by gas chromatography-mass spectrometry revealed the presence of hexanedioic acid, bis(2-ethylhexyl) ester, 2,6,10,14,18,22-tetracosahexane, 2,6,10,15,19,23-hexamethyl-(ALL-E)-3,7,11,15-tetramethyl-2-hexadecen-1-oic acid, n-hexadecanoic acid, octadecanoic acid, vitamin E and 1-naphthalene propanol. The role of these constituents and their mechanism behind anti-viral activity of *P. tomentosa* should be explored further to develop them as a therapeutic option for dengue infection [85].

7.5.4.8 Coumarins

Two coumarin derivatives A and B (Fig. 7.8) isolated from the ethanol extract of *Mammea americana* (Clusiaceae) seeds were found to be active with EC_{50} values of 9.6 μg/mL and 2.6 μg/mL in DENV infected Vero cells [55]. The CC_{50} for coumarins A and B were reported as 3150 and 549.8 μg/mL, respectively. Based on the mechanism reported for other coumarins from *Myrtopsis corymbosa* [86], coumarins A and B were considered to exhibit anti-dengue activity by the inhibition of NS5 protein of DENV. In addition to anti-dengue activity, coumarins were reported to be effective in controlling endothelial dysfunction in diabetes. All these earlier reports are favourable towards the development of coumarins A and B of *M. americana* as anti-dengue compounds and also for treating endothelial dysfunction associated with severe dengue infections.

7.5.4.9 Tatanan A

Acorus calamus (Acoraceae) is another TCM, which had been investigated for anti-dengue activity. *A. calamus* is traditionally used for the treatment of neurodegenerative disorders. The root extract of *A. calamus* was identified to exhibit anti-dengue activity in DENV infected BHK-21 cells. Further fractionation and separation by column chromatography led to the isolation of 12 compounds from the root extract of *A. calamus*. Among these 12 compounds, tatanan A (Fig. 7.8) was found to be more effective in inhibiting viral infection in DENV-2 infected BHK-21 cells ($EC_{50} = 3.9\,\mu M$, $CC_{50} > 1000\,\mu M$), reducing the level of DENV E and NS1 proteins [87].

7.5.4.10 Resveratrol

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a phytoalexin (Fig. 7.8) produced in plants in response to any stress as a defence mechanism. Earlier reports have indicated anti-viral activity of resveratrol against influenza A virus, human immunodeficiency virus, Epstein-Barr virus, enterovirus 71 and respiratory syncytial virus [94–98]. Zainal et al. recently reported the anti-dengue activity of resveratrol and the molecular mechanism involved in it. In this study, treatment of DENV infected Huh 7 cells with resveratrol showed significant reduction in DENV viral particles and NS3 protein. This study explored the role of resveratrol on HMGB1, a non-histone DNA binding protein for its anti-dengue activity. Resveratrol could effectively inhibit the translocation of HMGB1 from the nucleus to cytoplasm and subsequent release of HMGB1 to extracellular fluid in host cells, thereby inducing the interferon-stimulated genes (ISG) of host cells. Induction of ISG is an important mechanism in host cells to prevent replication of virus and control infection.

Translocation of HMGB1 from the nucleus to cytoplasm in host cell leads to the upregulation of pro-inflammatory genes resulting in DHF and DSS [89, 90]. Extracellular HMGB1 has been found to cause vascular endothelial leakage in DENV infection. Thus, inhibition of translocation of HMGB1 and induction of ISG in host cells by resveratrol had been identified as the mechanism for its anti-dengue activity [88]. This study could pave way for the development of anti-viral compounds similar to resveratrol, which inhibits the target protein HMGB1 of host cells.

The review on anti-dengue activity of phytochemicals other than flavonoids, alkaloids and terpenoids reveals their rich chemical diversity. Honokiol, a lignan, gains importance because of its effect on various targets of DENV including E, NS1 and NS3. A single molecule could prevent virus entry and also inhibit its replication. The activity profile of gingerol is similar to the alkaloid carpaine that they both showed anti-thrombocytopenia effect. Like oxindole alkaloids, α-mangostin and resveratrol have the ability to act as direct-acting anti-dengue and immunomodulatory molecules as well. α-Mangostin, geraniin and tatanan A have similar mechanism, as they all act on DENV E protein. Anti-dengue molecules or phytoconstituents exhibiting similar mechanism of action with respect to DENV target proteins could be considered for quantitative structure-activity relationship (QSAR) studies to generate a common pharmacophore for the design of novel anti-dengue molecules.

7.6 Herbal Extracts with Anti-Dengue Activity

An increase in the number of research publications on herbal medicines as anti-dengue agents has been observed in this decade (2011–2020) as there is no specific approved anti-dengue compound except the anti-dengue vaccine, Dengvaxia. The aforementioned studies demonstrated the anti-dengue activity of extracts or bioactive fractions only (Table 7.6). The intention of this section is to provide more insights on those extracts or bioactive fractions for further research towards the identification of active phytoconstituents to treat dengue infection.

7.6.1 *Doratoxylum apetalum*

Doratoxylum apetalum is native to Mascarene Islands, reported to contain polyphenols. The hydro-ethanol extract prepared from aerial parts of *D. apetalum* exhibited anti-viral activity against all four types of DENV in Huh 7.5 cells. The IC_{50} values of the extract were reported as 96.35, 16.75, 25.90 and 23.30 μg/mL against four types of DENV, which indicated that DENV-2 was highly sensitive for the extract of *D. apetalum* [99]. This study did not indicate any possible mechanism for anti-dengue activity; however, it was reported for another flavivirus, Zika virus. The time of addition assay in Zika

Table 7.6 List of herbal extracts with anti-dengue activity

Plant species	Extract	In vitro study	In vivo study
Doratoxylum apetalum	Hydroalcoholic extract of aerial parts	Anti-viral activity against all four types of DENV in Huh 7.5 cells [99]	NR
Lonicera japonica	Flower aqueous extract	Increased expression of miRNA let-7a causing inhibition of replication of DENV NS1 protein in Huh 7 cells [100]	Upregulation of miRNA let-7a in DENV-2 infected suckling mice and healthy human volunteers [100]
Hippophae rhamnoides	Leaf ethanol extract	Decreased TNF-α and increased IFN-γ in DENV infected human blood-derived macrophages [101]	NR
Senna angustifolia	Leaf ethanol extract	Inhibition of NS2B-NS3pro in Vero cells infected with DENV-2 [102]	NR
Tridax procumbens	Stem ethanol extract		NR
Vernonia cinerea	Leaf methanol extract		NR

NR Not Reported

virus-infected A549 cells showed that the extract of *D. apetalum* inhibited the viral infection at the early stage by preventing the entry of virus into host cells. Based on the observations reported for Zika virus, it could be proposed that *D. apetalum* inhibits entry of DENV also into host cells.

7.6.2 *Lonicera japonica*

Lonicera japonica (Caprifoliaceae), also called as honeysuckle, is a commonly used TCM for the treatment of infectious diseases. The aqueous extract of honeysuckle flowers was explored for anti-dengue activity in vitro and in vivo in a study reported by Lee et al. [100]. Interestingly, this study focused on the molecular mechanism behind the anti-dengue activity of honeysuckle extract, especially on its role in the expression of microRNA (miRNA) let-7a. In vitro study in Huh 7 cells revealed honeysuckle extract upregulated miRNA let-7a, thereby affecting the replication of viral NS1 protein.

Further, in vivo studies in DENV-2 infected suckling mice and healthy human volunteers also provided evidence on the upregulation of miRNA let-7a by honeysuckle extract. This study unravels the different molecular mechanisms for inhibiting viral replication in DENV.

7.6.3 *Hippophae rhamnoides*

Sea buckthorn (*Hippophae rhamnoides*, Elaeagnaceae) is a thorny nitrogen-fixing, deciduous shrub [103]. Monika Jain et al. analysed the therapeutic effect of sea buckthorn leaf extract against DENV infected human blood-derived macrophages and compared with standard antiviral drug, ribavirin. The extract maintained the viability of dengue-infected cells at par with ribavirin along with the decrease and increase in TNF-α and IFN-γ, respectively, at the concentration of 50 μg/mL. These observations suggested that the sea buckthorn leaf extract has a significant anti-dengue activity [101].

7.6.4 *Senna angustifolia*

Inhibitory activity of ethanol extract of *Senna angustifolia* (Leguminosae) leaf against DENV NS2B-NS3pro was significant ($IC_{50} = 30.1 \pm 3.4$ μg/mL) with almost negligible effect on cell viability by MNTD assay. However, it was less effective in countering the DENV-2 influenced cytopathic effects in Vero cells. This was supported by the reduction in plaque formation (26.3 ± 3.8%) and a lower inhibition on viral load (67.2 ± 6.3%) at 50 μg/mL. The authors reported that DENV NS2B-NS3pro

inhibitory property, effect on cell viability and viral load were probably due to the phenolic glycosides of *S. angustifolia* [102].

7.6.5 *Tridax procumbens*

Tridax procumbens (Asteraceae) stem ethanol extract inhibited DENV NS2B-NS3pro ($IC_{50} = 25.6 \pm 3.8$ μg/mL) with nearly insignificant impact on cell viability by MNTD assay. Additionally, DENV-2 influenced cytopathic effects in Vero cells were efficiently reduced with a substantial reduction in plaque formation (80.6 ± 6.1%) besides inhibiting the viral load (86.3 ± 2.7%) at 50 μg/mL. The flavonoid contents of *T. procumbens* might be responsible for enzyme inhibitory property, effect on cell viability and viral load [102].

7.6.6 *Vernonia cinerea*

Methanol extract of *Vernonia cinerea* (Asteraceae) leaf exhibited inhibitory activity against DENV NS2B-NS3pro ($IC_{50} = 23.7 \pm 4.1$ μg/mL). The effect on cell viability by MNTD assay was almost insignificant at 50 μg/mL, while DENV-2 influenced cytopathic effects in Vero cells were efficiently reduced. These facts were supported by considerable reduction in plaque formation (64.0 ± 9.4%) and significant inhibition on viral load (79.5 ± 4.3%). The imperative bioactive principles sesquiterpene lactones and phenolic compounds in *V. cinerea* leaf would have mediated the inhibition of DENV NS2B-NS3 protease, effect on cell viability and reduction of viral load [102].

7.7 Strategies for Anti-Dengue Drug Discovery

Developments in various in vitro assays have accelerated the discovery of anti-dengue molecules from plant sources. Plaque reduction assay is the most commonly used to screen plant extracts and their active fractions for anti-viral activity. Furthermore, in vitro assays such as time of addition, attachment and penetration assays help in establishing the anti-dengue profiles of phytoconstituents. Though direct-acting anti-dengue molecules are much needed, phytoconstituents with immunomodulation property could provide benefit in alleviating the complications such as DHF and DSS. During screening of plant sources for anti-dengue activity, it is worthwhile to consider their immunomodulation effect also.

In silico approaches have also gained significant importance in unravelling the mechanism of phytoconstituents. Multiple approaches involving viral target based, host target based and ligand based could be followed to achieve the goal of dengue drug development from plant sources. Also, information garnered through in vitro investigations could be analysed for SAR or QSAR studies in designing derivatives of phytoconstituents. Considering the merits of anti-dengue compounds belonging to flavonoids and indole alkaloids, novel molecules bearing chromane or indole nucleus can be validated for their potency against all DENV serotypes. Simultaneously, it will be noteworthy to expedite the work on fractionation and isolation of phytobioactives from plant extracts with anti-dengue property, followed by in vitro and in vivo exploration.

7.8 Conclusion

Dengue, an acute infection caused by arboviruses, has appreciably raised over the past few decades across the world and become a major concern of public health. In recent years, the disease has drawn the global attention owing to the non-availability of direct-acting anti-viral compounds for human use. Across countries, research is prioritized towards the development of safe and efficient agent to treat or to prevent the viral infection.

Plants and their derived products are very crucial in drug discovery as they are endowed with a wide variety of chemical diversity and act as a

large supply of many small molecules. These molecules, sometimes, are the source of therapeutic agents by themselves or act as prototypes for further development process. Numerous existing reports unveil the potential of phytoconstituents, extracts and their bioactive fractions as effective agents against dengue infection (Fig. 7.9), but at the same time, the plants/phytoconstituents pool has not been explored to the extent available.

However, in vivo studies reporting the effectiveness of phytoconstituents or bioactive fractions for anti-dengue activities are limited, thus impeding the process of drug discovery. Moreover, from the limited in vivo studies available, it is observed that the physiochemical and pharmacokinetic properties do not show favourable profile. These findings undermine the evidence of their potency as therapeutic compounds.

Furthermore, it is possible that there may be numerous in vivo studies in progress, but it is impractical to recognize them in the absence of formal registry of such studies. And in some cases, it is also anticipated that in vivo studies with non-significant results might not have been reported. Hence, there seems to be a huge unfilled gap between the number of in vitro studies and their corresponding in vivo studies. In this regard, the challenge in translating findings from in vitro studies to in vivo studies assumes importance and is likely to provide clear direction for the future studies in the quest of finding a successful active molecule.

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Anti-Influenza Drug Discovery and Development: Targeting the Virus and Its Host by All Possible Means

8

Olivier Terrier and Anny Slama-Schwok

Abstract

Infections by influenza virus constitute a major and recurrent threat for human health. Together with vaccines, antiviral drugs play a key role in the prevention and treatment of influenza virus infection and disease. Today, the number of antiviral molecules approved for the treatment of influenza is relatively limited, and their use is threatened by the emergence of viral strains with resistance mutations. There is therefore a real need to expand the prophylactic and therapeutic arsenal. This chapter summarizes the state of the art in drug discovery and development for the treatment of influenza virus infections, with a focus on both virus-targeting and host cell-targeting strategies. Novel antiviral strategies targeting other viral proteins or targeting the host cell, some of which are based on drug repurposing, may be used in combination to strengthen our therapeutic arsenal against this major pathogen.

Keywords

Antiviral · Drug repurposing · Replication · Entry · Immune modulator

Abbreviations

CoV	Coronavirus
COX	Cyclooxygenase
HA	Hemagglutinin
IAV	Influenza A virus
IFN	Interferon
M2	Matrix 2
NA	Neuraminidase
NOX	NADPH oxidase
NP	Nucleoprotein
p09	H1N1 2009-pandemic strain
PA	Polymerase acidic subunit
PB1	Polymerase basic subunit 1
PB2	Polymerase basic subunit 2
PPI	Protein-protein interaction
RdRP	RNA-dependent ribonucleoprotein complex
RIG-I	Retinoic acid-inducible gene-I
TNF-α	Tumor necrosis factor-α
vRNP	Viral ribonucleoproteins

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8.1 Introduction

Infections by influenza virus constitute a major and recurrent threat for human health. Influenza viruses are the causative agents of seasonal flu epidemics, associated with up to 1 billion infections and 300,000–650,000 deaths worldwide and consequently with a large economic price including hospitalization costs and missing working days [1, 2]. In addition, influenza A viruses (IAV) have been the cause of several pandemics in recent human history, from the Spanish flu H1N1 in 1918 to the more recent H1N1 2009 pandemic [3].

Together with vaccines, antiviral drugs play a vital part in the prevention and treatment of influenza virus infection and disease. During a normal influenza season, antiviral drugs are mainly used to treat critically ill patients, such as those hospitalized in intensive care. In a pandemic context, pending the availability of a vaccine, antiviral drugs are essential both to treat patients who have been infected and to prevent infection in those exposed, including healthcare workers. Today, the number of antiviral molecules approved for the treatment of influenza, based on the targeting of viral proteins, is relatively reduced and threatened by the emergence of strains with resistance mutations. There is therefore a real need to expand the prophylactic and reinforce the current therapeutic arsenal. This chapter summarizes the state of the art in drug discovery and development for the treatment of influenza virus infections, with a focus on both virus-targeting and host cell-targeting strategies (Fig. 8.1). Novel antiviral strategies targeting other viral proteins or targeting the host cell, some of which are based on drug repurposing, may be used in combination to strengthen our therapeutic arsenal against this major pathogen.

8.2 From Existing Classic Antiviral Drugs to New Pre-Clinical Candidates

8.2.1 M2 Ion Channel Blockers (Amantadine/Rimantadine)

Influenza A M2 is a multifunctional viral homotetramer protein [4]. Its transmembrane (TM) domain forms a proton channel. This channel is required for the acidification of the viral endosome formed after fusion and endocytosis of the virus within the host cell. This process allows viral ribonucleoproteins (vRNPs) to dissociate from the matrix 1 (M1) protein. The proton conductance mechanism relies on the conserved H37XXXW41 sequence which is responsible for selectively gating H^+ ions [5–8]. Channel blockers interfere with the proton conductance mechanism by binding to the transmembrane pore [9] (Fig. 8.2). When proton conductance through M2 is blocked by the adamantane drug, this dissociation is prevented, and the virus is no longer able to replicate. In recent years, adamantane drug-resistant mutants have become prevalent in circulating viruses. The most prevalent drug-resistant mutations are S31N, L26F, and V27A, all of which are located in the transmembrane region of M2 [11]. Figure 8.2a shows the strong interaction of amantadine with V27 in the upper part of the pore. Upon drug resistance V27A mutation, this interaction is lost. Recently developed spiro-amantadyl amine effectively binds to A27 of the pore (Fig. 8.2b) [10]. Recently, new amantadine derivatives effective against double mutants M2-S31N/L26I and M2-S31N/V27A viral strains have been developed by Musharrafieh et al. [12]. The antiviral efficacy of such compounds is summarized in Table 8.1. As a consequence of resistance mutations that

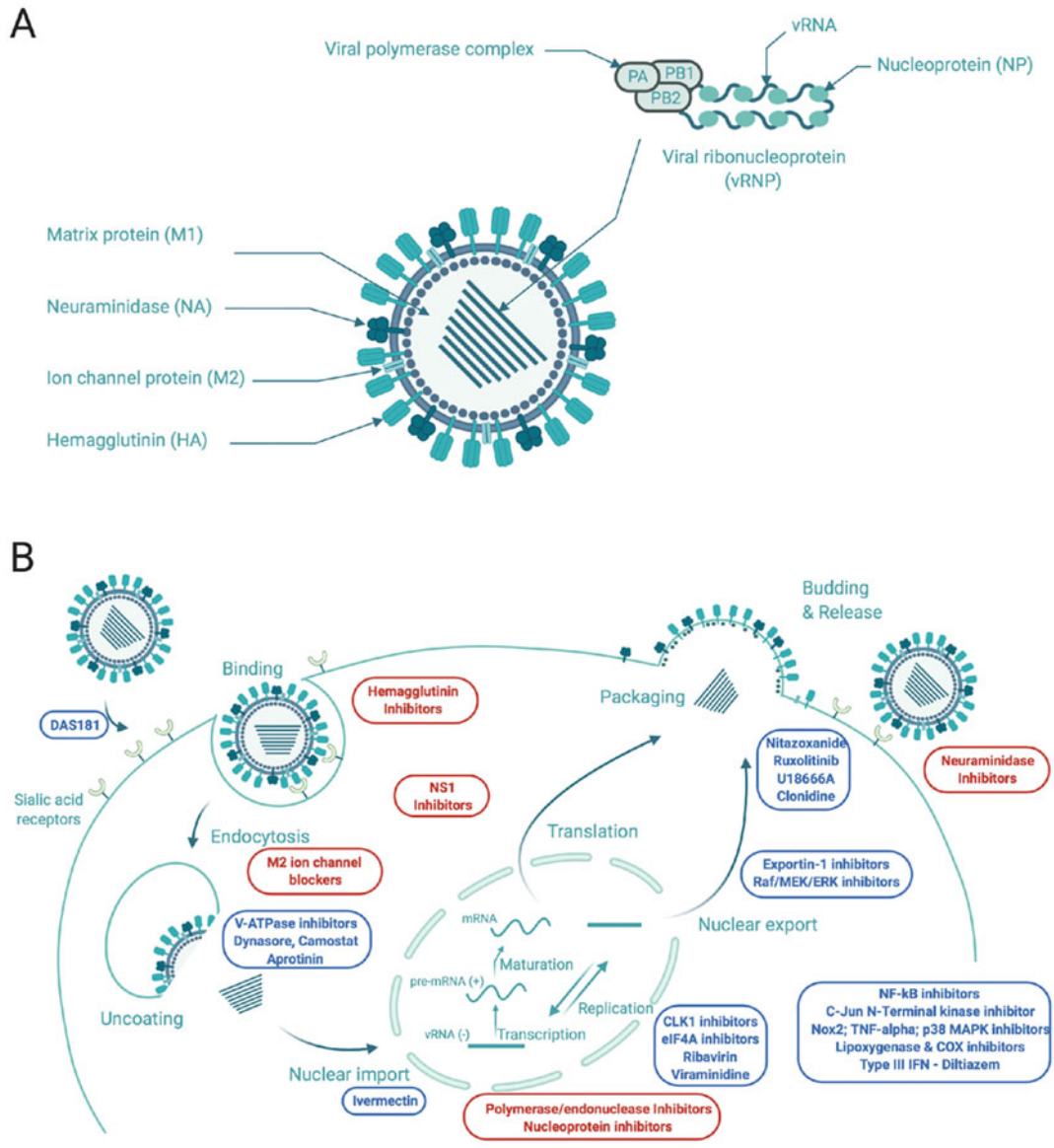

Fig. 8.1 Influenza viral particle and viral cycle; current state of anti-influenza drug discovery and development. (**a**) Influenza A virus (IAV) particle. The IAV genome is composed of eight ribonucleoprotein complexes (vRNPs), composed of single-stranded negative-sense viral RNA (vRNA) encapsidated by viral nucleoprotein (NP) and a viral polymerase complex (PA, PB1, and PB2) positioned at the extremity of the vRNA segment. Three viral proteins, hemagglutinin (HA), neuraminidase (NA), and ion channel protein (M2), are embedded within the viral membrane. Matrix protein 1 (M1) holds the vRNPs inside the virion. (**b**) The viral particle binds to sialic acid receptors and enters the cell via receptor-mediated endocytosis. Acidification of the endocytic vesicles leads to virus uncoating mediated by the M2 ion channel. vRNPs are then released into the cytoplasm and transported into the nucleus. There, the viral RNA-dependent RNA polymerase complex snatches the host mRNA caps to initiate the negative vRNA transcription. Transcribed vRNAs then undergo an mRNA maturation phase, before export to the cytoplasm to be translated. vRNAs are also replicated in the nucleus to generate new vRNPs in association with neosynthesized viral proteins. Progeny vRNPs are transported toward the cytoplasmic membrane with viral components to be packaged into new infectious particles which are formed by cellular envelope budding. Classic virus-targeting strategies are highlighted in red and virus-host-targeted strategies in blue. Figure created by BioRender.com

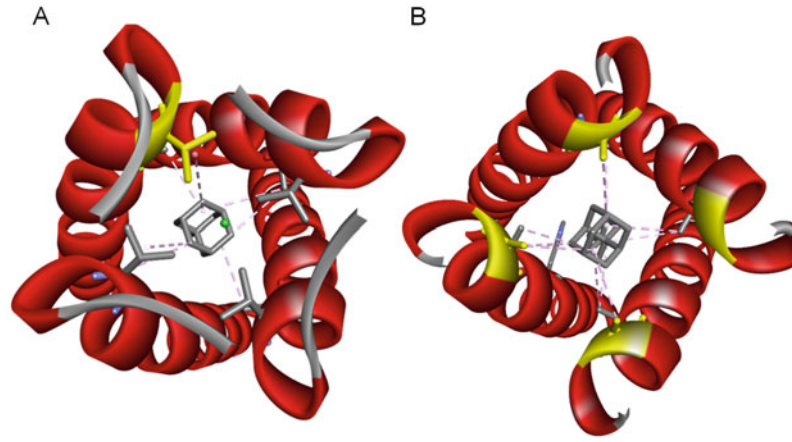

Fig. 8.2 Looking down the M2 channel in the presence of inhibitors: Structure of M2 WT and VA27 mutant in complex with amantadine and spiroamantadine. View down the pore channel in (**a**) WT-amantadine (V27 is colored in yellow, PDB ID 6BKK [9]) and (**b**) V27A-spiroamantadine complexes (A27 is colored in yellow, PDB ID 6NV1 [10])

Table 8.1 Summary of the activity and structures of the main antiviral compounds bound to their target, the proton channel M2 of influenza A or the neuraminidase NA of influenza A and B

Target	Compound	IC_{50}	PDB ID	Stage year approval)	References
M2	Amantadine	100μM (H1N1 WT) > 500μM (S31N) 15.7μM (WT channel [a,]) [13]	6BKK	Approved (1976)	Thomaston et al. [9], Cady et al. [14]
	Rimantadine	0.1μM (H1N1 WT) > 200μM (S31N)	2RLF	Approved (1994)	Schnell and Chou [15]
	Spiro-adamantyl amine	18.7μM (WT channel[a]) 0.2μM (V27A [a])	6BMZ 6NV1 6OUG	Pre-clinical	Thomaston et al. [9, 10]
NA	Oseltamivir (Tamiflu)	0.8 nM (N5 NA)	2HT7	Approved (1999)	Russell et al. [16]
	Peramivir	3.4 nM	2HTU	Approved (2014)	Russell et al. [16]
	Zanamivir	0.6 nM (N5 NA)	3CKZ	Approved (1999)	Collins et al. [17]
	Chebulinic acid Chebulagic acid	1.36 ± 0.36μM (H1N1 PR8) (Oseltamivir-resistant and H1N1 pdm09 viruses) CC_{50} > 100μM		Pre-clinical	Li et al. [18]
	Oseltamivir derivatives	0.66μM (H5N1)	Docking 150/430 cavity	Pre-clinical	Ai et al. [19], Jia et al. [20]; Zhang et al. [21]
	Triazole oseltamivir derivatives C1-modified oseltamivir derivatives	0.05–0.15μM (H5N1, H5N2, and H5N6) 0.1μM (H5N1, H5N6) 0.7μM (oseltamivir-resistant virus)	Docking 430 cavity	Pre-clinical	Ju et al. [22]

[a]Patch clamp assays [10]

appeared in M2 in H1N1/H3N2 circulating strains, both amantadine and rimantadine were removed from the WHO list of recommended anti-influenza agents for clinical use in 2009 [23].

8.2.2 Neuraminidase (NA) and Hemagglutinin (HA) Inhibitors

8.2.2.1 NA Inhibitors

NA inhibitors competitively inhibit terminal sialic acid residue removal from glycoproteins and carbohydrates found at the surface of host (mammalian) cells and influenza virus particles. Binding of virions to intact (uncleaved) sialic acid inhibits virion release. Among these NA inhibitors, peramivir, zanamivir, and oseltamivir carboxylate are the most frequently prescribed drugs and considered standard of care for influenza management (Table 8.1 and Fig. 8.3). Resistance to oseltamivir can be observed experimentally in a few cell passages and also found in the clinic. Typically, resistance originates from substitutions in the viral NA protein such as H274Y and I223R (predominant in H1N1 and H5N1 viruses) and E119V, R292K, or N294S (predominant in H3N2 viruses). Oseltamivir, peramivir and zanamivir are three NA inhibitors currently approved worldwide for the treatment of influenza A and B infections, oseltamivir being the most widely used. There is still a lot of debate about the effectiveness and real impact of inhibitors on the prevention and treatment of influenza. New oseltamivir derivatives, targeting either multiple sites or different NA cavities (as the "430" or the "150" cavity), have been recently developed. Some of these derivatives are very potent against multiple IAV and IBV strains, including oseltamivir-resistant ones (Table 8.1).

8.2.2.2 Hemagglutinin Inhibitors

The surface glycoprotein HA is associated with viral entry into host cells. HA binding to cell-surface, sialic-acid-containing glycans further enables fusion between the viral and host membranes in endosomal compartments. HA is composed of head (HA1) and stem (HA2/HA1) domains. As the regions on HA involved in binding and fusion are highly conserved, they are attractive sites for the design of new antivirals (Table 8.2). The broad-spectrum antiviral drug arbidol shows efficacy against influenza viruses by targeting the hemagglutinin (HA) stem region [24]. This molecule is currently licensed in Russia and China for the treatment of influenza and other infections [35]. A challenging strategy aiming at mimicking antibodies binding sites was successfully developed by Wilson et al., targeting the conserved stem region and more recently at the interface of the trimeric head region [13, 27, 36] (Fig. 8.4a). The binding sites of the binding sites for CBS1117 and JNJ4796 were both found in the stem region close to the fusion peptide, highlighting the possibility of further structure-based designed compounds [29]. De novo design of high-affinity trimeric proteins called "HA mini-binders" that bind influenza A hemagglutinin trimer at a conserved region binding site (Fig. 8.4b) [33]. These molecules were developed as alternative to antibodies. These and other compounds are summarized in Table 8.2.

8.2.3 Polymerase/Nucleoprotein/RNA inhibitors

8.2.3.1 Polymerase/Endonuclease Inhibitor (Favipiravir, Baloxavir Marboxil)

Influenza viruses transcribe and replicate their genome in the nucleus of infected cells by the means of a hetero-trimeric polymerase, PA, PB1, and PB2. The polymerase complex function requires the nucleoprotein NP, a protein associated with and protecting the segmented genomic RNA. Therefore, all four proteins are essential for replication. Whereas replication requires the generation of complementary positive polarity RNA intermediates (cRNA) that are then copied into progeny negative polarity segments (vRNPs), viral message is directly synthesized from vRNPs. Since the influenza virus polymerase is unable to form 5′ mRNA cap structures, its subunit PA is necessary for the generation of viral mRNAs via its endonuclease activity, transferring host mRNAs 5′-capped RNA primers in a cap-snatching mechanism. The endonuclease active site of PA-N terminal comprises a histidine and a cluster of three strictly

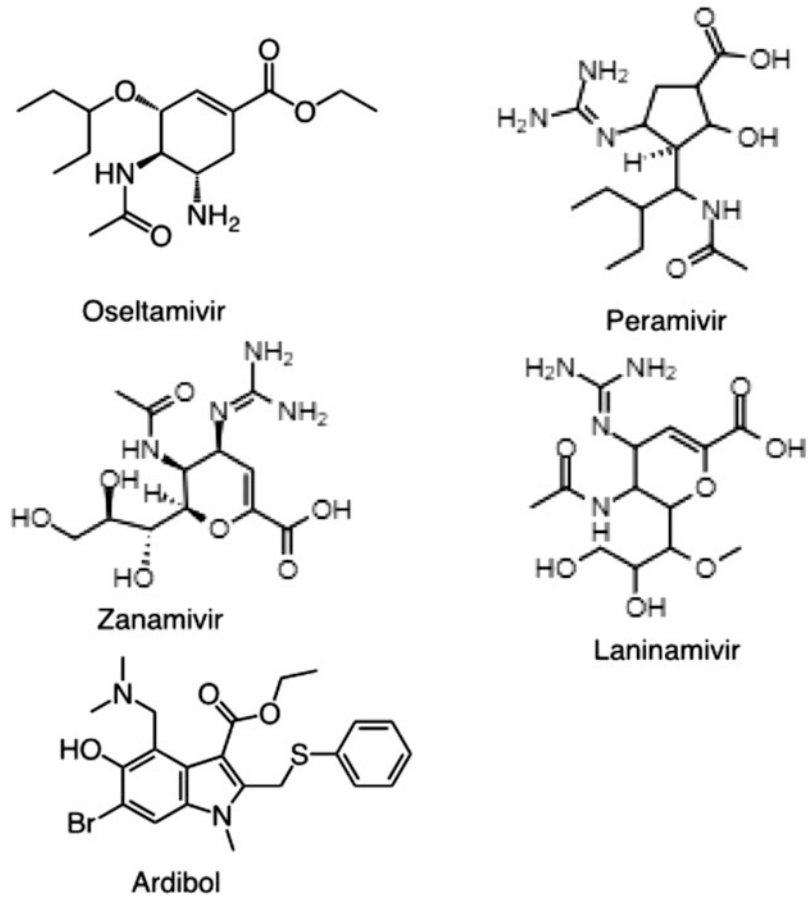

Fig. 8.3 Structures of the approved NA inhibitors

Table 8.2 Recent antiviral candidates targeting HA, their activity, and structures of their complexes with HA

Target	Compound/binding site	IC_{50}/CC_{50}	PDB ID	Stage	References
HA	Arbidol/stem region	4–12μM CC_{50} = 59μM	5T6S, 5T6N	Pre-clinical and clinical NCT03787459	Kadam and Wilson [24], Wang et al. [25], Wright et al. [26]
	F0045(S)/stem region	0.5–2μM (H1 HA)	6WCR	Pre-clinical	Yao et al. [13]
	JNJ4795/stem region	0.01–0.07μM (H1 HA)	6CF7	Pre-clinical	Van Dongen et al. [27]
	IY7640/stem region	0.5–7μM (H1 HA) CC_{50} > 800μM	Docking studies	Pre-clinical	Kim et al. [28]
	CBS1117/stem region	3μM For H5 HA	6VMZ	Pre-clinical	Antanasijevic et al. [29]; [30]); Hussein et al. [31]
	MB2746/stem region	0.3μM (H1 HA) CC_{50} > 100μM	Docking studies	Pre-clinical	Basu et al. [32]
	De novo design of "mini-binder" proteins	0.15–0.19 nM (H3 and H1 HA)	6KUY		Strauch et al. [33]
	penindolone		HA1 and HA2	Pre-clinical	Wu et al. [34]

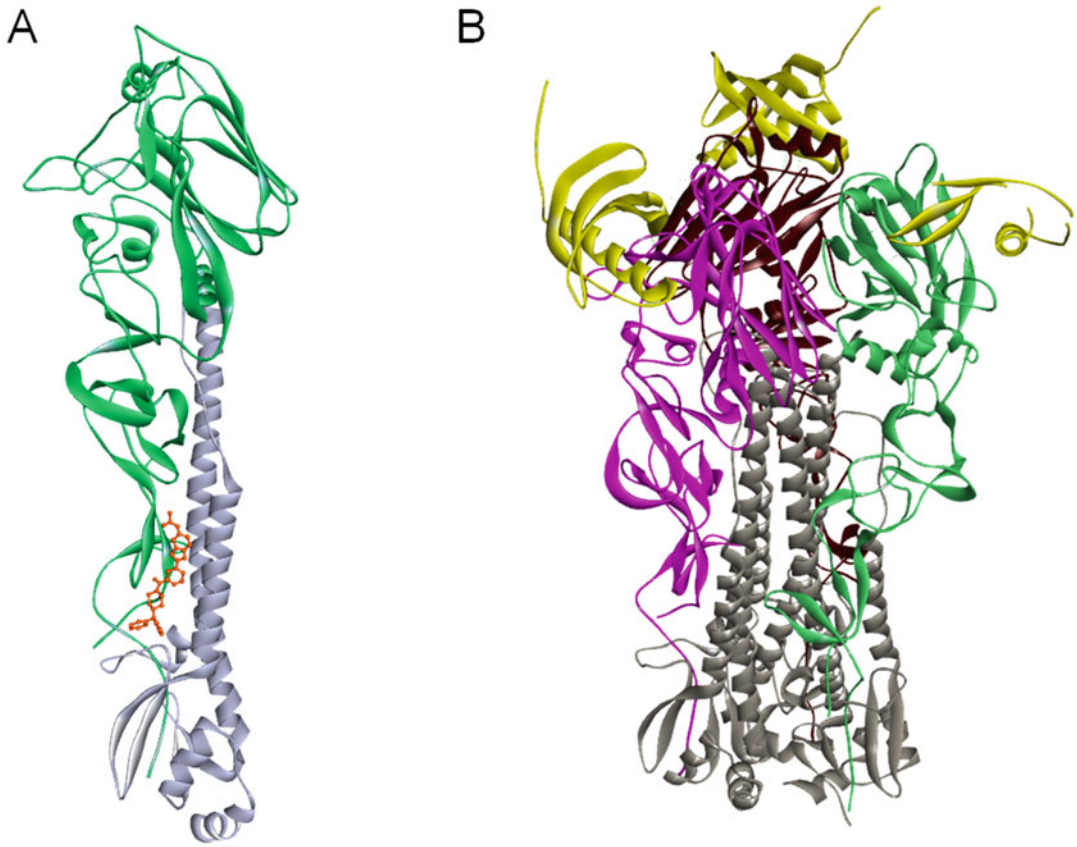

Fig. 8.4 Structure of some of the pre-clinical candidates targeting HA: (**a**) Structure of HA in complex with JNJ4796 shown in orange (PDB ID 6CF7) [27]. (**b**) Structure of trimeric HA in complex with mini-binder highlighted in yellow (PDB ID 6KUY) [33]

conserved acidic residues (Glu80, Asp108, Glu119), which coordinate (together with Ile120) one or two manganese or magnesium ions [37] (Fig. 8.5a). PB2 binds capped primers, the enzymatic activity for phosphodiester bond formation being associated with the PB1 subunit.

Several classes of inhibitors are in the clinics (Fig. 8.6): baloxavir (PA), favipiravir (PB1), and pimodivir (PB2, Fig. 8.5b).

8.2.3.2 Pre-clinical Compounds Targeteinf the Polymerase PA, PB1 and PA subunits, Escape Mutations and Resistance

Pre-clinical candidates, some of them being listed in Tables 8.3 and 8.4, are in development, benefiting from the recent insight provided by the structures of PA-PB1, PB1-PB2, and whole polymerase complex with or without RNA by X-ray crystallography [71–77] and cryo-electron microscopy [78–82]. The error-prone nature of influenza viral replication can rapidly generate point mutants for the selection of resistance that have seriously compromised the efficacy of influenza therapeutics. Escape mutations were identified under the pressure selection of PA inhibitors: the hotspot mutation for escape from baloxavir marboxil is located at PA residue 38, including several substitutions (PA I38T/M/F) [41]. Similarly, escape mutations from L-742.001 [42] and RO-7 [44] treatments were also characterized although in laboratory

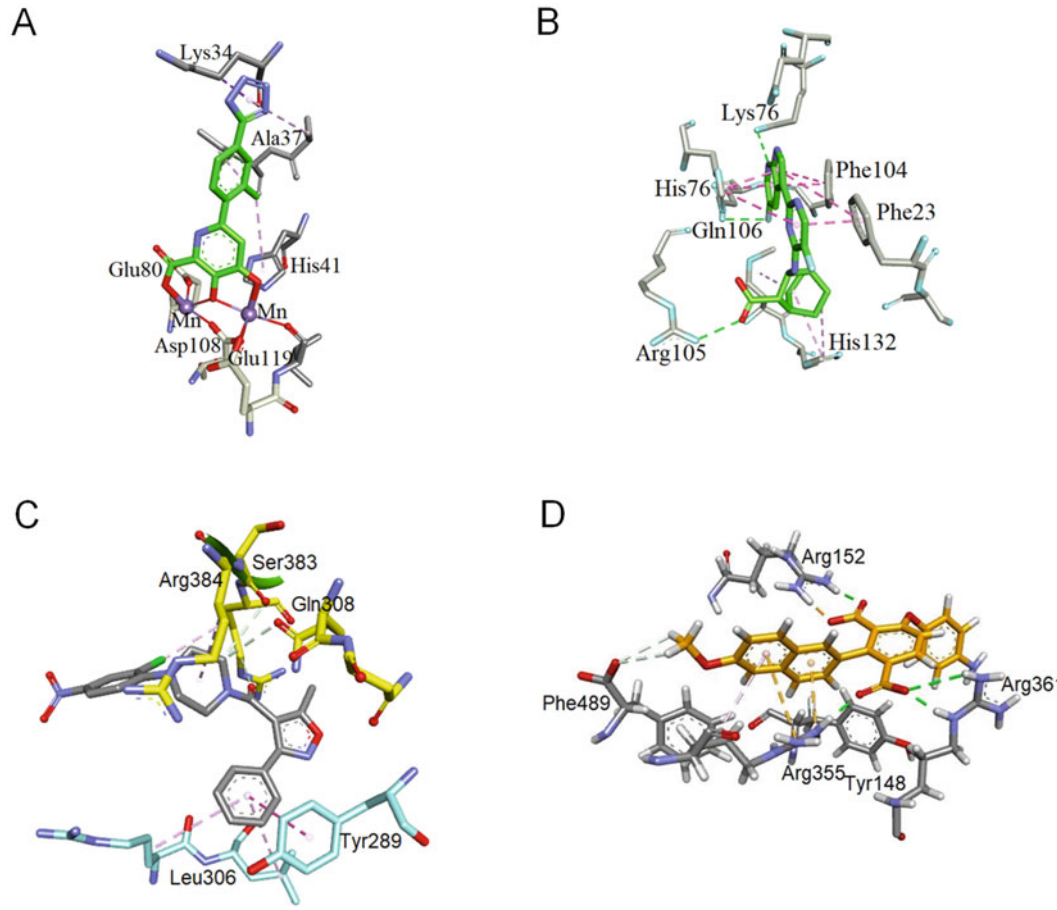

Fig. 8.5 Structure of some of the pre-clinical candidates targeting the polymerase. (**a**) Active-site PA N-terminal inhibitor compound 22 [38]; (**b**) PB2 inhibitor pimodivir [39] (the numbering is associated with this structure corresponding to the full-length PB2); (**c**) nucleozin-NP oligomeric complex PDB ID 5B7B; monomers A and B are in cyan and yellow, respectively; (**d**): naproxen F1-NP monomeric complex from docking studies [40]

resistance assays, escape mutants were not detected after multiple passages for L-742.001. While very tight affinities have been achieved by designing metal binding inhibitors to block the active site of the endonuclease activity in PA N-terminal (Table 8.2), the appearance of escape mutants often rapidly decreases their efficacy. Several recent reviews focus on the development of PA and polymerase inhibitors [83–86].

Different strategies have been undertaken to attempt overcoming induced resistance. Interfering with its proper assembly of the RdRP polymerase to inhibit function is pursued using protein-protein interaction (PPI) inhibitors. The advantage of such an approach is the relatively large interacting surface between the two proteins as compared to the binding site of an active-site ligand. Indeed, inducing simultaneous mutation of at least one residue on both proteins while maintaining their interaction is less likely to develop resistance and suggests that PPI inhibitors could be less prone to drug resistance than inhibitors of enzyme active sites. The recent identification of a single- domain antibody (nanobody) allowing to disrupt dimerization of FluA polymerase is among these lines [79]. PPI inhibitors have been developed based on the structural insight given by PA-PB1 crystal

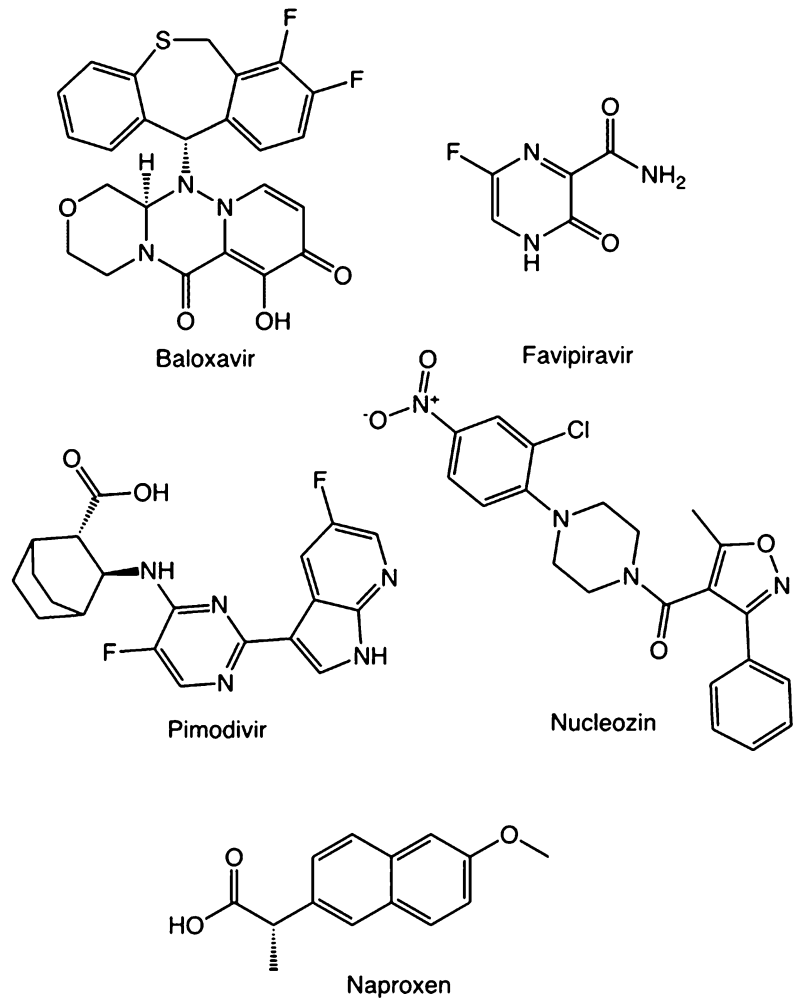

Fig. 8.6 Structures of the approved polymerase inhibitors and some pre-clinical candidates

structures in 2012 [87]. The inhibition of the polymerase PA-PB1 subunit interface has become an active field of research with the goal of remaining active against resistant strains to amantadine and to oseltamivir (Table 8.3). Recently, compound 12 was identified by structure-based screening of compounds targeting the PA-PB1 structure. No resistant virus was selected in vitro under drug selection pressure of compound 12a [48]. Moreover, derivatives of cyclothiophene and R151785 were found active against multiple strains of influenza A and B [50–52].

Based on the ability of PA-PB1 to bind viral RNA, it is likely that novel types of inhibitors could be developed by structure-based design [88]. Additionally, inhibitors targeting PA C-terminal [47] and its interactions with vRNA or with PolII could be effective targets, based on the accumulating wealth of structural data [74, 75, 79, 82] and deeper insight in the multi-protein assembly required during replication/transcription.

8.2.3.3 Broad-Spectrum Inhibitors

Favipiravir inhibits RNA viruses of the arenavirus, bunyavirus, flavivirus, alphavirus, norovirus, picornavirus, paramyxovirus, and rhabdovirus families, in addition to influenza viruses;

Table 8.3 Inhibitors of PA, PA-PB1 interactions, and PB1

Target	Compound	IC_{50}/CC_{50}	PDB ID	Stage	References
PA	Baloxavir marboxil	0.3–1μM (H1N1/H3N2)	6FS6 6FS9	Approved (2019) NCT02954354 NCT0294901	Omoto et al. [41]
	L-742,001	3μM (WT H1N1) 24μM (WT H1N1 pdm09) 236μM (H1N1 pdm09 PA F105S)	5CGV 5D9J	Clinical trial NCT01526785	Song et al. [42]
	RO7	16 nM (WT H1N1) 3 nM (H1N1 pdm09)	5VPX	Pre-clinical	Jones et al. [43]; Kowalinski et al. [44]
	Ana-0	0.8μM	Docking	Pre-clinical	Yuan et al. [45]
	Compound 22	110 pM	6E6W	Pre-clinical	Credille et al. [38]
	N-Acylhydrazone derivatives	11μM	5EGA	Pre-clinical	Carcelli et al. [46]
	"312"	37μM (H1N1, H2N2, and H3N2)	PA–C-terminal	Pre-clinical	Lo et al. [47]
PA-PB1	Compound 12a	0.9–2.7μM (FluA amantadine- and oseltamivir-resistant, FluB)	Docking	Pre-clinical	Zhang et al. [48]
	Amino-acids adducts of diphenyl-pyridine derivatives	39 ± 2μM (H1N1)	Docking	Pre-clinical	D'agostino et al. [49]
	Cycloheptathiophene-3-carboxamide	0.2μM–0.7μM H1N1 pdm09, H1N1 oseltamivir-resistant, H3N2, influenza B	Docking	Pre-clinical	Desantis et al. [50]; Nannetti et al. [51]
	R151785	2.5, 5.0μM p09, H1N1 oseltamivir- and amantadine-resistant influenza B	Docking	Pre-clinical	Zhang et al. [52]
PB1	Favipiravir	Broad-spectrum		Approved (2014)	Yoon et al. [53]
	β-d-N4-Hydroxycytidine/ EIDD-2801	Broad-spectrum influenza, SARS-CoV2		Clinical trial NCT04405739	Sheahan et al. [54]; Toots et al. [55]

therefore, it is considered as a broad-spectrum drug [53]. This drug is incorporated into newly synthesized RNA by the viral polymerase in place of purines but not pyrimidines, resulting in increased frequencies of C-to-U and G-to-A transition mutations. Although the barrier for resistance is relatively high, this drug seems to present toxicity issues. N4-Hydroxycytidine (NHC) inhibits RSV and both highly pathogenic avian and seasonal influenza viruses as well as SARS-CoV-2 virus, thus being also a broad-spectrum antiviral candidate with oral efficacy [55].

8.2.3.4 Pre-Clinical Compounds Targeting the Polymerase PB2 Subunit

Crystal structure of the PB2 cap-binding domain has been exploited to develop different 7-methylguanine derivatives [59]. Pimodivir (VX-787) is an inhibitor targeting the polymerase PB2 subunit at the m^7 GTP-binding site, forming extensive stacking interactions with several aromatic residues His (Figs. 8.5b and 8.6). It inhibits influenza virus replication and reduced viral load in animal infection models of H3N2 and H1N1

Table 8.4 Inhibitors of PB2 cap-binding, PB1-PB2, NP, and NS1

Target	Compound/binding site	IC_{50}/CC_{50}	PDB ID	Stage	References
PB2	Pimodivir (VX787)	2.6 nM	4P1U	Approved (2017)	Byrn et al. [56]; Clark et al. [39]
	5,7-Difluoroindole derivative of pimodivir	11 nM	6S5V	Pre-clinical	Mcgowan et al. [57]
	D 715–2441	3.6–4.4μM (H1N1, H3N2, H5N1, H7N9)	Docking	Pre-clinical	Liu et al. [58]
	Cap analogs	7.5μM H3N2	4CB5	Pre-clinical	Pautus et al. [59]
PB1-PB2	PP7	1.4–9.5μM (strain-specific)	Docking	Pre-clinical	Yuan et al. [60]
NP	Nucleozin	0.07μM (H1N1) 0.16μM (H3N2) 0.33μM (H5N1Y287H)	5B7B	Pre-clinical	Kao et al. [61]; Pang et al. [62]
	Compound 3	0.1 μM (H1N1 and H5N1)	3RO5	Pre-clinical	Gerritz et al. [63]
	2-(4-Chloro-3,5-difluorophenylamino)thiazole-4-carboxamide derivatives	0.11μM	Docking	Pre-clinical	Shen et al. [64]; Woodring et al. [65]
	Naproxen Naproxen C0 (naproxen derivative 2) Naproxen F1 (naproxen derivative 4)	Broad-spectrum FluA and Sars-CoV2 16 ± 5μM (H1N) 2.9 ± 0.3μM (H1N1) 1.8μM (H1N1 pdm09) 1.3 ± 0.2μM (H1N1) 0.7μM (H1N1 pdm09, H3N2, resistant to oseltamivir)	Docking	Pre-clinical	Dilly et al. [40]; Lejal et al. [66]; Tarus et al. [67]
	Hydroquinolinone derivatives (NUD)	1.8–7.0μM (H1N1)	Docking	Pre-clinical	Makau et al. [68]
NS1	A22	≅ 1μM (H1N1 PR8)	Docking	Pre-clinical	Kleinpeter et al. [69]
	ML303	0.7–17μM (H1N1 pdm09, H3N2)	HTS	Pre-clinical	Patnaik et al. [70]

viruses, although potency was highest against H1N1 strains [39, 56]. Phase II clinical studies indicated that this drug is well-tolerated, reduced viral load, and resulted in slightly faster resolve of clinical signs. Further derivatives of pimodivir have been designed [57]. Targeting the PB1-PB2 interface by PPI inhibitors has been challenging: although PP7 exhibited antiviral activities against influenza virus subtypes A pandemic H1N1, H7N9, and H9N2, resistances have been unexpectedly detected in laboratory assays [60].

8.2.3.5 Pre-Clinical Compounds Targeting the Nucleoprotein or the Nucleoprotein-RNA Interactions

The nucleoprotein associated with viral RNA and the polymerase complex is essential for transcription and replication [77, 89, 90]. The assembly of NP-RNA oligomers into RNP has been determined by cryo-electron microscopy studies [77, 78, 89, 91]. In the X-ray structures of the NP [92], the protein adopts a trimeric structure. NP self-association to achieve trimer formation is

mediated by a flexible tail loop that protrudes into a pocket of the adjacent subunit, via the formation of a critical interaction between R416 of one subunit and E339 of the adjacent subunit. The R416A mutant lacking this interaction adopts a monomeric structure [93]. The native protein can also be purified in a monomeric form at low salt and concentration conditions [93–95]. The ability to modify the oligomeric state of NP is the structural basis of most NP inhibitors presently developed. Nucleozin was the first NP inhibitor developed as a molecule impeding nuclear accumulation. Nucleozin enhanced higher-order structures [61, 63]. Figure 8.5c shows the interactions of one of the nucleozin ligands found in the X-ray structure (PDB ID 5B7B) stabilizing the interface between two NP subunits [62]. Escape mutants to nucleozin have been identified in laboratory assays. The opposite approach to impede nucleoprotein self-association has also been pursued by disrupting the important salt bridge R416-E339 mediating NP oligomerization [64]. Recently, new compounds with high affinity for NP were designed stabilizing monomeric NP [65]. Impeding NP binding to viral RNA has been achieved by naproxen drug repurposing, naproxen being a known inhibitor of cyclooxygenase (COX) [66]. As NP oligomerization is enhanced by the presence of RNA, naproxen binding to NP reduced NP oligomers and favored monomeric NP. Docking and single mutation studies identified Tyr148, the only aromatic residue within the RNA binding groove, and residues of the C-terminal part of NP R355, R361 and Phe489 being involved in the interaction of naproxen with NP. Laboratory assays showed no resistance after eight cell passages infected with influenza A. Naproxen exhibited antiviral effects in mice models of influenza A infection [40, 66] as well as influenza B virus [96]. Further structure-based design yielded new naproxen derivatives with improved antiviral effects and selectivity for NP without COX inhibition (Figs. 8.5d and 8.6) [40, 67] (Table 8.4). Some of these derivatives were found inhibiting NP-PA interactions [40, 97]. Naproxen derivatives also present antiviral properties against oseltamivir-resistant strains [40]. Additional compounds with some similarity of their hydroxyquinoline scaffold to the methoxynaphthalene scaffold of naproxen called NUD were designed and were also found to be resistant in escape mutation laboratory assays [68].

8.2.4 Drugs Targeting the Non-structural Protein-1 (NS1)

NS1 has a plethora of strategies to inhibit the host immune response due to its ability to establish multiple protein-protein and protein-RNA interactions. NS1 hampers different pathways both in the cytoplasm and in the nucleus of infected cells. NS1 antagonizes interferon-mediated antiviral host response by binding to double-stranded (ds) viral RNA, thus protecting it from cellular factors, by blocking retinoic acid-inducible gene-I (RIG-I) and NF-kB activation. One pathway by which NS1 increases virulence is through the activation of phosphoinositide 3-kinase (PI3K) by binding to its p85β subunit [98]. NS1 has two structural domains – RNA-binding domain (RBD) and the effector domain (ED) – connected by a short linker (LR) and a disordered C-terminal tail. New drugs binding to NS1 effector domain have been designed with low micromolar antiviral efficacy [69] (Table 8.4).

8.3 Host-Targeting and Drug Repurposing Approaches for the Treatment of Influenza

Considerable progress has been made in understanding the interactions between influenza viruses and the host cell in recent years. In this context, and in light of the emerging problem of resistance to available classical antivirals, many studies have focused on targeting host factors to limit virus replication, but also to modulate host immune response. The targeting of host factors and/or signaling pathways makes sense in the context of virally induced hypercytokinemia (also known as “cytokine storm”), which is directly correlated with tissue injury and an

unfavorable prognosis of severe influenza [99]. Indeed, approaches to control or attenuate this disproportionate immune response are of particular interest and are the subject of numerous pre-clinical and clinical studies. As with all viruses, influenza viruses depend on cellular machinery for their replication and propagation. Many cellular factors essential for the replication of influenza viruses have been uncovered through genome-wide RNA interference approaches [100–103] but also more broadly through different integrated cell biology approaches using interactome and transcriptome data, for example [104, 105]. In order to list the different host-targeting strategies developed, a distinction can be made between molecules with a mode of action associated with a relatively well-defined stage of the viral cycle and molecules associated with the modulation of signaling pathways. It is these two main classes that will be described in the following sections.

8.3.1 Drugs Targeting Host Cell Component at Different Stages of Influenza Replication Cycle

The replication cycle of influenza viruses consists in distinct successive phases, 1) entry, 2) nuclear import of viral genome (viral ribonucleoprotein; vRNPs), 3) genome replication and protein synthesis, 4) nuclear-cytoplasmic export of vRNPs, and 5) plasma membrane transport and budding of neo-virions (Fig. 8.1). A number of molecules targeting host factors in these different steps, at different pre-clinical/clinical development stages, are known today.

Viral entry is a target of great interest, as it is likely to allow prophylactic approaches, by blocking the infection in its early stages. One of the most advanced strategies consists to target the viral receptor. DAS181 (Table 8.5) (Fludase, Ansun BioPharma) is a sialidase fusion protein that cleaves both the Neu5Ac α(2,3)- and Neu5Ac α(2,6)-Gal linkages of sialic acid on host cells. DAS181 is administered as an inhalable dry powder to deliver sialidase to the pulmonary epithelium for cleavage of sialic acids, which renders the cells inaccessible to infection by virus [131]. DAS181 was demonstrated to have broad-spectrum activity, given the conserved nature of influenza and parainfluenza viruses binding to respiratory epithelium. Pre-clinical in vitro and in vivo studies demonstrated that DAS181 has activity against a number of seasonal influenza strains including those containing the H274Y mutation (conferring resistance to oseltamivir), highly pathogenic avian influenza strains (H5N1), and pandemic 2009 influenza A (H1N1). This compound was assessed in different Phase I and Phase II clinical trials (NCT00527865, NCT01651494, NCT01037205) with results indicating a significant reduction of viral load in treated influenza patients [106] but with identification of respiratory adverse events and rapid clearance of the drug being consistent with the induction of antibodies against DAS-181 – this could be a limitation in the duration and dosages of such treatment [107]. Other approaches targeting viral entry have also been described (Table 8.5), e.g., targeting the endosome acidification step by inhibition of V-ATPase (e.g., bafilomycin A1, concanamycin) or inhibition of the internalization (e.g., Dynasore) or cleavage steps of hemagglutinin (e.g., camostat). Most of these strategies were primarily evaluated at the pre-clinical stage and have not been further evaluated as their efficacy was either limited or accompanied by cytotoxicity. One exception is the protease inhibitor aprotinin, which was approved as anti-influenza drug in Russia [112].

The step of **nuclear import of vRNPs** is a crucial one, for which there are today very few molecules with antiviral potential described in literature. Interestingly, it has been shown in vitro that ivermectin (Table 8.5), a well-known anti-parasite drug, was able to inhibit viral replication via inhibition of importins (IMPα/β) and therefore the nuclear import of vRNPs [116].

Targeting the **replication** stage of the virus is one of the earliest host-targeting strategies, with pioneer works on the antiviral efficacy of ribavirin in the 1970s [119]. However, this nucleoside analogue and its prodrug, less toxic, do not appear

Table 8.5 Drugs targeting host cell component at different levels of viral cycle stages

Viral cycle stage	Drug name	Mode of action	Research phase	References
Viral entry	DAS181	Sialidase – Removes sialic acid receptors	Phase I/II	Moss et al. [106], Zenilman et al. [107]
	Bafilomycin A1	V-ATPase inhibitors – Inhibits endosomal acidification	Pre-clinical	Yeganeh et al. [108]
	Concanamycin			Müller et al. [109]
	Diphyllin			Chen et al. [110]
	Saliphenylhalamide			Bimbo et al. [111]
	Aprotinin	Protease inhibitors – Inhibit HA0 cleavage	Approved (2011)	Zhirnov et al. [112]
	Camostat		Pre-clinical	Yamaya et al. [113]
	Dynasore	Inhibition of internalization		de Vries et al. [114]
	EIPA			
	Fattiviracin			Harada et al. [115]
Nuclear import of vRNP	Ivermectin	Inhibits importin-α/β		Gotz et al. [116]
Genomic replication and protein synthesis	TG003	CLK1 inhibitors – Regulation of splicing – Decrease in M2 mRNA expression		Karlas et al. [100]
	Clypearin			Zu et al. [117]
	Corilagin			
	Silvestrol	eIF4A inhibitors – Inhibit viral protein synthesis		Slaine et al. [118]
	Pateamine			
	Ribavirin	Nucleoside analogue	Approved (1986)	Durr et al. [119]
	Viramidine (ribavirin prodrug)		Phase III (HCV)	Sidwell et al. [120]
	Cyclosporin A	Inhibits host RNA polymerase II	Pre-clinical	Liu et al. [58]
vRNP nuclear export		Inhibits nuclear export of vRNPs		
	Verdinexor	Exportin 1 inhibitors		Perwitasari et al. [121]
	DP2392-E10			Chutiwitoonchai et al. [122]
	CI-1040	MEK inhibitor – Nuclear retention of VRNP complex		Haasbach et al. [123]
	UO126			Pleschka et al. [124]
	PBP10/BOC2	Formyl peptide receptor 2 antagonists – Raf/MEK/ERK inhibition		Courtin et al. [125]
	Trametinib	MEK1/2 inhibitor – Inhibition of vRNP export	Approved (cancer)	Schräder et al. [126]
	Dapivirine	Reverse transcriptase inhibitor – Inhibition of vRNP export	Phase III (HIV)	Hu et al. [127]

(continued)

Table 8.5 (continued)

Viral cycle stage	Drug name	Mode of action	Research phase	References
Apical transport and budding	Nitazoxanide	Anti-parasitic – Inhibition of HA maturation and transport	Phase III	Rossignol et al. [128]
	Ruxolitinib	Virion formation and vRNA incorporation inhibition	Approved (myelofibrosis)	Watanabe et al. [105]
	U18666A	Hydrophobic polyamine – Reduces plasma membrane cholesterol level and decreases virion egress	Pre-clinical	Musiol et al. [129]
	Clonidine	Alpha2-adrenergic receptors inhibitor – Inhibits transport of HA transport to plasma membrane		Matsui et al. [130]

to be options being considered for the treatment of influenza virus infections of influenza viruses, despite interesting preliminary in vitro and in vivo results [120] (Table 8.5). Other, more recent strategies propose to target **mRNA splicing**. Influenza viruses are known to hijack cellular splicing machinery to their benefit, making them extremely dependent on it [132, 133]. Several studies show that the inhibition of Cdc2-like kinase 1 (CLK1), involved in the alternative splicing of M2 gene of influenza, appears to be an interesting antiviral option, with several molecules available (TG003, clypearin, corilagin, Table 8.5). Of all its molecules, clypearin has relatively low EC50s and very low toxicity, making it an attractive potential antiviral candidate [100, 117].

While strategies to prevent the nuclear import of vRNPs are relatively uncommon, paradoxically there are many more therapeutic approaches to block the **nuclear-cytoplasmic transport of vRNPs**. Indeed, in contrast to the inhibition of importins, the inhibition of exportin 1 (XPO1) by verdinexor (XPO1 antagonist KPT-335) allows to significantly reduce viral production in vitro and in vivo [121]. Another compound, DP2392-E10, inhibits nuclear export of both viral NP and nuclear export protein (NEP). More specifically, in vitro pull-down assays revealed that DP2392-E10 directly binds cellular CRM1, which mediates nuclear export of NP and NEP – highlighting CRM1 as a target of interest [122]. With the same objective, other strategies consist to target the Raf/MEK/ERK signaling pathway, known to be involved in the export of vRNPs [134]. Several MEK inhibitor molecules have been studied for their ability to inhibit the replication of influenza viruses, such as CI-1040 or U0126 [124, 125]. Interestingly, Schräder and colleagues have demonstrated that trametinib (GSK-1120212), a licensed MEK inhibitor used for the treatment of malignant melanoma, efficiently blocks influenza viral replication of different subtypes in vitro and in vivo [126] (Table 8.5).

Apical transport and budding, the last part of the last major step of the replication cycle, is also the object of several antiviral strategies, notably by blocking the transport of viral proteins to the plasma membrane (e.g., clonidine; [130]) or the cholesterol pathway, which would reduce virion egress (U18666A; [129]). One of the most advanced strategies is nitazoxanide, which was first approved for parasite infections' treatment. Its antiviral properties against influenza virus were first reported by Rossignol et al. [128]. Interestingly, the proposed mode of action of nitazoxanide against influenza clearly differs from its anti-parasitic effects, acting at the post-translational level by selectively blocking the maturation of the viral glycoprotein HA. Consecutively, it impacted on intracellular trafficking and insertion into the host plasma membrane [135]. This drug is a potent antiviral against a large panel of circulating strains [136]. A Phase IIb/III trial showed the efficacy of nitazoxanide in treating patients with non-complicated influenza [137], with a further, currently assessed, Phase III clinical trial (NCT01610245).

Table 8.6 Drugs targeting host cell signaling pathway and host responses that are crucial for influenza replication cycle

Host signaling pathway/response	Drug name	Mode of action	Research phase	References
NF-kB pathway	Acetylsalicylic acid	Immune dysregulation Inhibition of caspase/vRNP export inhibition	Approved	Mazur et al. [138]
	Pyrrolidine dithiocarbamate		Pre-clinical	Wiesener et al. [139]
	SC75741		Pre-clinical	Ehrhardt et al. [140] Haasbach et al. [123]
	LASAG		Phase II	Droebner et al. [141] Scheuch et al. [142]
C-Jun-N-terminal-kinase	SP600125	C-Jun N-terminal kinase inhibitor – Immune dysregulation	Pre-clinical	Nacken et al. [143]
p38 MAPK	NJK14047	Immune dysregulation	Pre-clinical	Choi et al. [144]
HMG-CoA	Statins	Immunomodulation	Phase II	Fedson [145], Mehrbod et al. [146]
TNF-alpha	Etanercept	Anti-inflammatory drug – Prevents TNF-mediated lung injury and edema	Pre-clinical	Shi et al. [147]
Nox2	Apocynin	ROS scavenger, inhibits Nox2 activity	Pre-clinical	Ye et al. [148] Oostwoud et al. [149]
	Ebselen	ROS scavenger and glutathione peroxidase mimetic, inhibits Nox2	Pre-clinical	
Lipoxygenase and COX pathways	Celecoxib	Immune dysregulation	Phase III	
	Mesalazine	Immune dysregulation	Pre-clinical	Davidson et al. [150] Carey et al. [151] Zheng et al. [152]
Type III IFN response	Type III IFN	Induction of type III IFN response	Pre-clinical	Davidson et al. [153] Kim et al. [154]
	Diltiazem		Phase II	Pizzorno et al. [155, 156]

8.3.2 Drugs Targeting Host Cell Signaling Pathways and Host Response that Are Crucial for Influenza Replication Cycle

Our increased knowledge of signaling pathways that are crucial in the response to infection and/or those hijacked by the virus has allowed many research teams to explore complementary antiviral strategies that can be described here (Table 8.6). The targeting of the ref./MEK/ERK channel, mentioned above, could of course also have been listed here. At the crossroads of the regulatory pathways of the immune response and the stress response, the **NF-kB pathway** was one of the first to be studied (Table 8.6). In the context of cell biology approaches, it was initially shown that the anti-inflammatory drug acetylsalicylic acid (ASA) had interesting antiviral effect against influenza viruses in vitro and in vivo, via inhibition of the NF-kB activating IkkB kinase [138, 157, 158]. Several drugs targeting the NF-kB pathway have been evaluated since then, such as pyrrolidine dithiocarbamate or SC7574, with encouraging in vivo results [123, 139, 140]. BAY81–8781/LASAG (D,L-lysine

acetylsalicylate-glycine) (Table 8.6), a modified version of ASA, demonstrates in vitro antiviral activity against several human and avian influenza viruses. In a mouse infection model, inhalation of LASAG reduced lung viral titers and protected mice from lethal infection [141]. More recently, a Phase II proof-of-concept trial compared LASAG versus placebo in patients with severe influenza. Aerosolized LASAG was demonstrated improving the time to symptom alleviation compared to placebo, although the reduction of viral load in LASAG-treated group was not statistically significant [142].

Based on clinical observations, 3-hydroxy-3-methylglutaryl coenzyme A (**HMG-CoA**) reductase inhibitors such as statins (Table 8.6), approved for indication of cholesterol metabolism regulators, have demonstrated pleiotropic anti-inflammatory and immunomodulatory properties, which could increase survival of patients with severe influenza [145, 146]. However, most in vivo studies reported so far failed to clearly demonstrate such a beneficial effect for influenza patients [159–161]. Nevertheless, an association between statin treatment with a reduction of mortality in patients hospitalized with laboratory-confirmed seasonal influenza was highlighted in observational studies [162, 163]. A randomized placebo-controlled Phase II clinical trial (NCT02056340) to evaluate the potential beneficial effect of atorvastatin in improving the status severely-ill influenza-infected patients is currently undergoing. The combination of naproxen with clarithromycin and oseltamivir twice daily reduced the both 30- and 90-day mortality and length of hospital stay of patients hospitalized for A(H3N2) influenza [164]. Other approaches, at the pre-clinical validation stages, propose to target the **TNF-alpha** (etanercept) or **NOX2** (apocynin/ebselen) or lipoxygenase/COX pathway (celecoxib/mesalazine) pathways [147–152, 165]. A Phase III clinical trial is currently investigating the benefit of celecoxib for the treatment of severe influenza (NCT02108366). These molecules could be of interest to better control the inflammatory response, which is a very important aspect of the pathology.

Modulation of immune and inflammatory responses is a therapeutic avenue that has been much explored, but which may present risks given the ambivalent aspect of these pathways in relation to viral replication and the evolution of the pathology. Indeed, such treatment should stimulate induction of antiviral genes to control IAV spread, without driving immunopathology. In this context, **IFN-lambda** (Table 8.6) appears as a potent anti-influenza therapeutic, without the inflammatory side effects of IFN-alpha treatment [153]. Intranasal administration of IFN-λ2/3 was shown to significantly suppress infection of various influenza strains, including WS/33 (H1N1), PR (H1N1), and H5N1 in the mouse lung, and was accompanied by greater upregulation of ISGs [154]. More recently, using a transcriptome-based screening approach, we identified and validated diltiazem, a calcium channel blocker used as an anti-hypertensive drug, as a very promising host-targeted inhibitor of influenza infection. Interestingly, the study of the mode of action revealed that diltiazem was a strong induced or type III IFN [156]. An ongoing French multicenter randomized clinical trial is investigating the effect of diltiazem oseltamivir bi-therapy compared with standard oseltamivir monotherapy for the treatment of severe influenza infections in intensive care units (FLUNEXT trial NCT03212716).

8.4 Perspectives and Concluding Remarks

Among all the molecules listed in this chapter, some are already available on the market for other therapeutic indications and fall within the scope of drug repurposing. This is the case for naproxen, diltiazem, LASAG, or nitazoxanide, for example. Drug repurposing bypasses the long, risky, and expensive pre-clinical studies, an early clinical evaluation stage conventionally used for de novo drug development. It takes advantage of available resources, as extensive human clinical, pharmacokinetics, and safety data, as the starting point for the development [155] All these aspects make the repositioning of drugs a very interesting approach, in particular

to enable a rapid response to the need for new antiviral strategies in the context of the emergence of a virus with pandemic potential.

Another very interesting perspective is the interest in combining different antiviral approaches with each other, including classical approaches targeting the virus with those targeting the host cell. The concept of combining therapies has already been used successfully, notably in the design of antiretroviral treatments [166]. Combination therapy can have several objectives, such as reducing the risk of the emergence of resistance by simultaneously targeting several viral proteins and/or key host factors, but also increasing the effectiveness of the treatments by obtaining additive or synergistic effects.

While there is relatively little convincing evidence to support the use of conventional virus-targeting antivirals in combination [167, 168], there are interestingly a growing number of examples of combinations of oseltamivir with host-targeted approaches. For example, we have shown that the combination of diltiazem and oseltamivir provides a much greater reduction in viral titers in a reconstructed human epithelium model compared to single treatments [156]. More recently, Schloer and colleagues have shown that a combination treatment of an antifungal molecule, itraconazole, with oseltamivir achieves much greater antiviral activity compared to monotherapy, making it possible to consider reducing the concentrations of drugs used and thus possibly reducing the problems of adverse effects and emergence of resistance mutations [169]. These results open up interesting prospects for the development of future therapeutic strategies, particularly for the treatment of severe forms of influenza. The potential arsenal for fighting influenza virus infections is potentially very extensive, in particular thanks to the combination of new molecules targeting the virus, resulting from docking and structure-based design strategies, with approaches targeting cellular factors and signaling pathways. In this context, the quality and relevance of the pre-clinical models, as well as the quality of the tools for evaluating combinations of molecules, are important critical elements.

Beyond influenza viruses, many of the antiviral molecules described in this chapter have the potential for broader-spectrum use. Indeed, some virus-targeted strategies can target viral determinants with very strong similarities between different viruses. This is particularly the case with naproxen for which we have previously demonstrated antiviral activity against both influenza viruses and SARS-CoV-2 [66, 170]. This property is explained by the fact that the nucleoproteins N of enveloped, positive-sense, single-stranded viruses coronavirus (CoV) share with negative-sense single-stranded viruses such as influenza A virus the ability to bind to and protect genomic viral RNA without sequence specificity and to form self-associated oligomers. Despite their differences, viruses induce and divert many common cellular pathways. As a result, host-targeted approaches can identify molecules with a broad spectrum of antiviral activity. An example is diltiazem, for which we have shown antiviral activity against influenza viruses [156], but which has been shown to be effective against other respiratory viruses, such as SARS-CoV-2 [171, 172], due to its mode of action involving the type III interferon response. Efforts to identify anti-influenza molecules therefore open up very interesting prospects for the broader development of antivirals. In many ways, antiviral research on influenza viruses is pioneering in this area and provides a starting point for the study of other emerging viruses.

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9 Search, Identification, and Design of Effective Antiviral Drugs Against Pandemic Human Coronaviruses

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Abstract

Recent coronavirus outbreaks of SARS-CoV-1 (2002–2003), MERS-CoV (since 2012), and SARS-CoV-2 (since the end of 2019) are examples of how viruses can damage health care and generate havoc all over the world. Coronavirus can spread quickly from person to person causing high morbidity and mortality. Unfortunately, the antiviral armamentarium is insufficient to fight these infections. In this chapter, we provide a detailed summary of the current situation in the development of drugs directed against pandemic human coronaviruses. Apart from the recently licensed remdesivir, other antiviral agents discussed in this review include molecules targeting viral components (e.g., RNA polymerase inhibitors, entry inhibitors, or protease inhibitors), compounds interfering with virus-host interactions, and drugs identified in large screening assays, effective against coronavirus replication, but with an uncertain mechanism of action.

Keywords

Coronavirus · SARS-CoV · MERS-CoV · Remdesivir · Viral replication · RNA polymerase

Abbreviations

ACE2	Angiotensin-converting enzyme 2
CC_{50}	50% cytotoxic concentration
CL^{pro}	Chymotrypsin-like protease
CTSL	Lysosomal cysteine protease cathepsin L
DHODH	Dihydroorotate dehydrogenase
DPP4	Dipeptidyl-peptidase 4
EC_{50}	50% effective concentration
eIF4A	Eukaryotic translation initiation factor 4A
EMA	European Medicines Agency
FDA	Food and Drug Administration
GBPA	4-(4-Guanidinobenzoyloxy) phenylacetic acid
GFP	Green fluorescent protein
HCoVs	Human coronaviruses

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HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IC_{50}	50% inhibitory concentration
IL	Interleukin
JAK	Janus kinase
mAb	Monoclonal antibody
MERS-CoV	Middle East respiratory syndrome coronavirus
M^{pro}	Main protease
NiRAN	Nidovirus RdRp-associated nucleotidyltransferase
nsp	Non-structural protein
ORF	Open reading frame
PL^{pro}	Papain-like protease
RDP	Remdesivir diphosphate
SARS-CoV	Severe acute respiratory syndrome coronavirus
SI	Selectivity index = CC_{50}/EC_{50}
RdRp	RNA-dependent RNA polymerase
TMPRSS2	Transmembrane protease, serine 2
UTR	Untranslated region

9.1 Introduction

Coronaviruses are enveloped viruses with a positive-sense single-stranded RNA genome and a nucleocapsid of helical symmetry that constitute the largest family of viruses (*Coronaviridae*) classified in the *Nidovirales* order [1]. The *Coronaviridae* include two subfamilies, *Letovirinae* and *Orthocoronavirinae*, with the last one being represented by four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [2]. Coronaviruses were first discovered in the 1930s in chicken with acute respiratory infections, often associated with high mortality rates [3, 4]. Human alphacoronaviruses were isolated 30 years later from individuals suffering common colds [5].

So far, there are seven major strains of human coronaviruses (HCoVs) with variable risks and effects on human health. Most human coronaviruses are harmless or produce symptoms that are generally mild, although fever and sore throat and eventually pneumonia and bronchitis may develop in the more severe cases [6, 7]. Human coronavirus strains in this group include alphacoronaviruses (e.g., strains 229E and NL63 (HCoV-229E and HCoV-NL63, respectively)) and betacoronaviruses (HCoV-OC43 and HCoV-HKU1) [8]. Collectively, these viruses are responsible for 15–30% of respiratory tract infections each year.

However, HCoVs can be highly pathogenic and cause pandemics. The first serious outbreak of highly pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV) occurred in 2002–2003 in the Guangdong Province of China. The virus (SARS-CoV-1) infected around 8000 people and cause 774 deaths with a mortality rate close to 10% [9]. A novel HCoV emerged in the Middle East in 2012. This virus, designated as Middle East respiratory syndrome-CoV (MERS-CoV), was found to be the causative agent of highly pathogenic respiratory tract infections in Saudi Arabia and neighboring countries [10]. Current scientific evidence suggests that dromedary camels are a major reservoir host for MERS-CoV and an animal source of MERS infection in humans. With more than 2500 infections and 866 deaths (as of January 2020), the fatality rate of this MERS-CoV is estimated at 34.3% [11].

Those important pandemics have been widely surpassed across the world in social impact and death toll by a novel coronavirus designated as SARS-CoV-2, responsible for severe pneumonia with associated side effects that in the most severe cases require hospitalization and eventually may cause death, particularly in older people [12, 13]. This disease is known as COVID-19 (standing for coronavirus disease 2019). SARS-CoV-2 is a betacoronavirus with 70% genetic similarity to SARS-CoV-1 [14]. As in the case of SARS-CoV-1, bats seem to be the origin of this virus, and in fact, a nucleotide sequence similarity of 96% has been found in virus isolated from human patients when compared with related bat

coronaviruses [15, 16]. Despite showing a relatively low fatality rate (current estimates less than 1%), COVID-19 has claimed more than 1.2 million lives around the world, while the number of infected people by the SARS-CoV-2 has been estimated at 49.7 million (as of November 8, 2020) [17].

Despite significant progress in vaccine development, when this manuscript went to press in November 2020, there were no vaccines available, and only a few drugs were available to fight COVID-19. Therefore, there is an unmet medical need to develop novel antiviral drugs and better treatment options to combat this deadly disease. From the medicinal chemistry point of view, there are several general approaches that might be helpful to identify new therapies to combat human coronavirus infections. The first one is to screen chemical libraries containing compounds or drugs that have been proven to be effective against related coronaviruses. Examples are target-based virtual screening, cell-level phenotypic screening, and high-throughput screening based on target structures [18]. One additional possibility involves the development of new specific drugs based on targets already known such as the genome or enzymes of individual coronavirus species [19]. However, this process is time-consuming and may not respond quickly to situations where the highly pathogenic coronaviruses spread very rapidly.

A potentially efficient approach for drug development in the current situation would be to test whether existing antiviral compounds targeting previously known coronaviruses would be effective to treat infections caused by the emerging new viral species [18]. These three approaches are often used in combination to facilitate drug development. Targets to be exploited in this process include both viral and host molecules, relevant in host-pathogen interactions. In this review, we focus on recent progress in the development of potentially effective coronavirus inhibitors. Representative examples of compounds targeting different steps in the viral life cycle are described, and their privileged structures are also discussed.

9.2 Genomic Organization, Structure, and Replicative Cycle of Pandemic Coronaviruses

The size of the coronavirus genome ranges from 26.4 to 31.7 kilobases (the largest of all RNA virus genomes). It is a non-segmented positive-sense RNA that contains a 5′ cap structure and 3′ poly-adenosine tail that functions as mRNA for the translation of replicase polyproteins [20]. Two-thirds of the genome are occupied by two large open reading frames (ORFs 1a and 1b) that together constitute the replicase gene. Both ORFs are translated directly from the genomic RNA. A short overlapping sequence at the 3′ end of ORF1a combined with a −1 ribosomal frameshift directs the synthesis of the ORF1b that leads to the formation of polyprotein pp1ab. Autoproteolytic cleavage of polyproteins pp1a and pp1ab carried out by viral papain-like (PL^{pro}) and serine-type (M^{pro}, CL^{pro}) proteases renders 16 non-structural proteins (nsp1 to nsp16), including the viral RNA-dependent RNA polymerase (RdRp or nsp12). In SARS-CoV and MERS-CoV, PL^{pro} and M^{pro} are encoded by nsp3 and nsp5, respectively [21, 22].

The organization of the coronavirus genome follows the order: 5′-leader-untranslated region (UTR)-replicase (ORF1a, 1b)-S (spike)-E (envelope)-M (membrane)-N (nucleocapsid)-3′ UTR-poly(A) tail, with accessory genes interspersed within structural genes at the 3′ end of the genome (Fig. 9.1) [20]. The accessory genes (ORFs 3a, 3b, 6, 7a, etc.) are not essential for replication in tissue culture but could have important roles in pathogenesis [24]. SARS-CoV and MERS-CoV show differences in composition and ORF arrangement in the 3′ regions of their genomes (Fig. 9.1). Structural and accessory genes contain transcriptional regulatory sequences required for their expression.

SARS-CoV and MERS-CoV are enveloped, spherical viruses of around 120 nm in diameter. Among major structural proteins in those virions, the protein S makes the characteristic spike

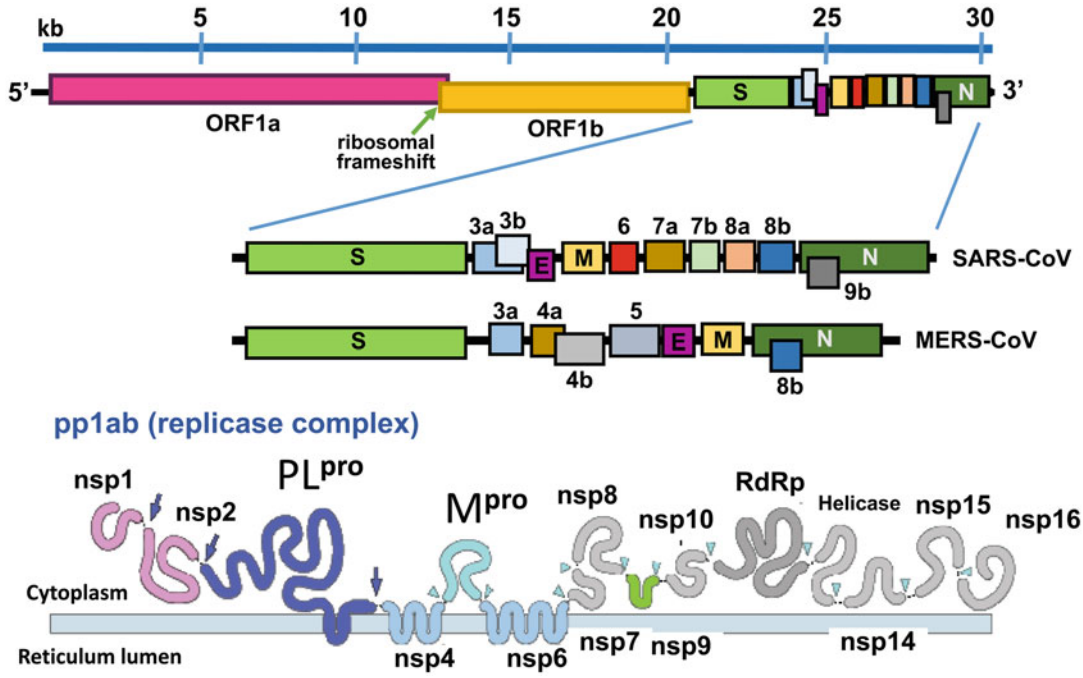


Fig. 9.1 Genomic organization and protein products of pandemic HCoVs. A scheme showing the genome and ORFs of SARS-CoV-1 is shown on the top. Differences in the arrangement and organization of S, E, M, and N coding sequences and ORFs 3a to 9b in SARS-CoV and MERS-CoV are shown in the middle. The structure of a *Betacoronavirus* replicase polyprotein (pp1ab) is shown below, together with proteolytic products nsp1 to nsp16. The polyprotein pp1a, derived from ORF1a, extends from nsp1 to nsp11. Expression of ORF1b is required for the synthesis of RdRp (nsp12), required for HCoV genome replication. Cleavage sites of PL^{pro} are indicated by blue arrows, while light blue triangles indicate the location of processing sites recognized by M^{pro}. Images have been adapted from Fehr and Perlman [20] and the ViralZone website [23]

structure on the viral surface. S (approx. 150 kDa) is a heavily glycosylated trimeric protein that attaches to the host receptor [25]. S trimers are composed of subunits S1 and S2, and the receptor binding domain locates at the C-terminus of S1. The M protein is the most abundant structural protein in the virion. It is small (25–30 kDa) and contains three transmembrane domains. The E protein facilitates assembly and release of the virus. It is small (approx. 8 to 12 kDa) and is found in small quantities in the virion. It has ion channel activity, important for the pathogenic effects of SARS-CoV-1 [26]. The N protein is composed of two separate domains, both capable of binding RNA. It has important roles in genome packaging as well as directing the viral genome toward the replicase-transcriptase complex. Some betacoronaviruses (e.g., murine hepatitis virus) also contain a hemagglutinin-esterase protein that binds to sialic acids and contains acetyl-esterase activity, but both strains of SARS-CoV and MERS-CoV lack this protein.

The replicative cycle of coronaviruses initiates with the attachment of the viral S protein to host receptors mediating virion endocytosis into the host cell (Fig. 9.2). Coronavirus host receptors are usually peptidases. In the case of MERS-CoV, this is dipeptidyl-peptidase 4 (DPP4), while SARS-CoV-1 and SARS-CoV-2 use angiotensin-converting enzyme 2 (ACE2). ACE2 is also used by the harmless HCoV-NL63 strain, while HCoV-229E gains entry into human cells by using aminopeptidase N. The structure of the SARS-CoV-2 S trimer has been determined in the prefusion conformation using cryo-electron microscopy [27]. In the predominant state of the

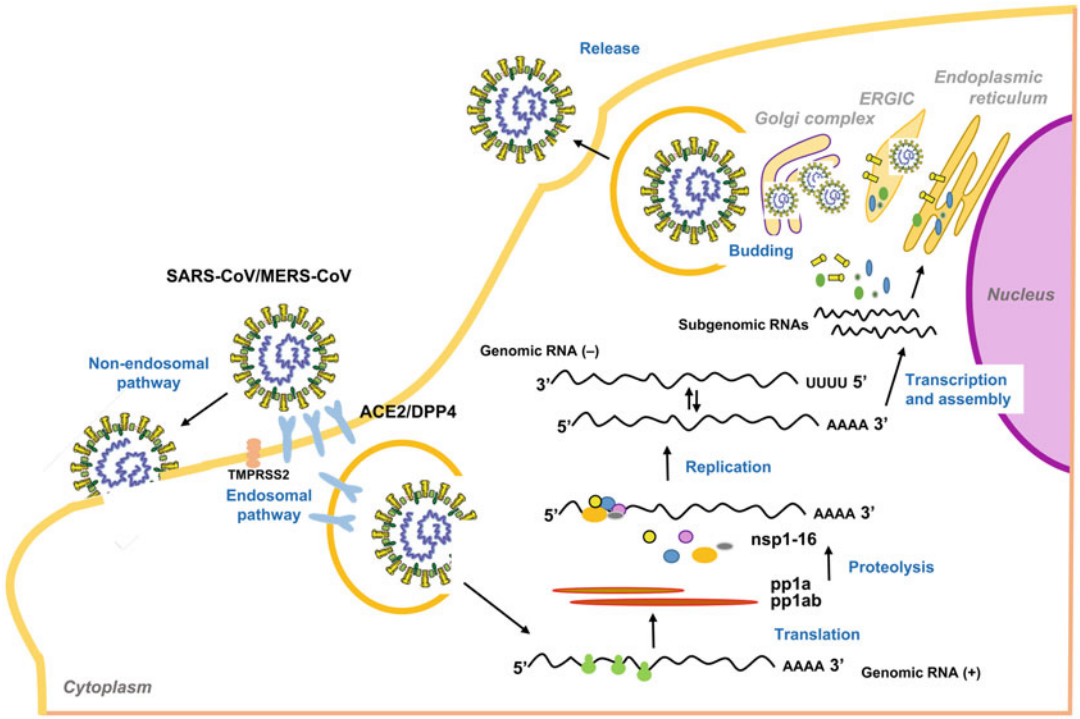


Fig. 9.2 Replicative cycle of a HCoV

trimer, one of the three receptor-binding domains appears rotated up in a receptor-accessible conformation.

The fusion of the viral envelope with the endosomal membrane occurs after acid-dependent proteolytic cleavage of the S protein by host cell cathepsin TMPRRS2 or another protease [28]. The process is facilitated by the formation of a six-helix bundle involving the S2 structure [29]. These events facilitate the release of the single-stranded genomic RNA into the cytoplasm, where synthesis and proteolytic cleavage of the replicase polyprotein take place. Replication occurs in viral factories. Double-stranded RNA is synthesized by the RdRp using the single-stranded RNA as template. Then, the double-stranded RNA is transcribed to produce genomic and subgenomic RNAs. The subgenomic RNAs are used for the synthesis of structural proteins (i.e., S, M, E, and N). These proteins are then inserted into the endoplasmic reticulum and move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) [30]. Viral genomes encapsidated by the nucleocapsid (N) protein bud into membranes of the ERGIC containing viral structural proteins, and form mature virions that are released by exocytosis.

9.3 COVID-19 Disease Progression and Therapeutic Intervention

As discussed above, SARS-CoV-2 infects the host using the ACE2 receptor, which is expressed in the lung, heart, kidney and intestine, mainly at endothelial cells [31]. The leading cause of mortality in patients with COVID-19 is hypoxic respiratory failure from acute respiratory distress syndrome. Successive cycles of viral replication lead to damage to the endothelium, particularly in the respiratory tract. Antiviral drugs should be effective in stopping disease at these initial stages. However, endothelial dysfunction may result in vascular inflammation (endotheliitis) and inflammatory cell infiltration, while shifting the vascular

equilibrium toward more vasoconstriction with subsequent organ ischemia, and inflammation with associated tissue edema. This may also result in the activation of coagulation pathways with potential development of disseminated intravascular coagulation and deregulated inflammatory cell infiltration, leading to a very serious and life-threatening disease [32, 33].

Many patients with severe COVID-19 show signs of a cytokine storm [34]. The high levels of cytokines (IL-2, IL-2R, IL-6, IL-7, IL-8, IL-10, IP10, G-CSF, MCP1, and MCP3) amplify the destructive process by leading to further endothelial cell dysfunction, disseminated intravascular coagulation, inflammation, and vasodilation of the pulmonary capillary bed. Therapeutic interventions in COVID-19 patients include bona fide antiviral drugs targeting different steps in the viral replicative cycle (e.g., remdesivir as an inhibitor of the viral RNA polymerase), but also drugs acting on the physiological consequences of the infection. In this group, steroid drugs and particularly dexamethasone can lower mortality rates in those patients who require invasive mechanical ventilation [35], while clinical trials involving the use of antibodies against specific cytokines (e.g., IL-1, IL-6, IL-17) have been considered to ameliorate the negative effects of cytokine storms [36–38].

In this review, we will focus on the description of compounds targeting viral components or directly affecting the interaction between host cell factors and coronaviruses.

9.4 RdRp Inhibitors

In May 2020, remdesivir, a nucleotide analogue inhibitor of the viral RdRp activity, became the first drug approved for emergency use for the treatment of COVID-19. In all viruses belonging to the order *Nidovirales*, the RdRp (nsp12) is a protein containing at least two domains, a nidovirus-specific N-terminal domain and a canonical RdRp domain occupying the C-terminal two-thirds of the protein (reviewed in [39]). RdRp domains contain six conserved motifs (A–F) involved in template and substrate recognition and catalysis of nucleotide incorporation. Amino acid sequence comparisons of nidovirus RNA polymerases showed the conservation of Asp residues in motifs A and C that coordinate two metal ions in the active site of the enzyme as in many other nucleic acid polymerases.

9.4.1 RdRp Structure and Mechanism of Action of Remdesivir

The structure of the SARS-CoV-2 RdRp has been recently solved by different groups using cryo-electron microscopy. Shortly after the first lockdowns in Europe due to COVID-19, Gao et al. reported the structure of full-length SARS-CoV-2 RdRp (nsp12) in complex with cofactors nsp7 and nsp8 at 2.9-Å resolution [40]. The complex was obtained after combining the purified proteins expressed in *E. coli*. In the structure, nsp12 contains a right-hand RdRp domain (residues Ser367 to Phe920) and the nidovirus-specific N-terminal extension domain (residues Asp60 to Arg249) containing a nidovirus RdRp-associated nucleotidyltransferase. Further analysis of the SARS-CoV-2 replicative complex led to the publication of the structure of the SARS-CoV-2 RdRp in an active form [41, 42], containing the three non-structural proteins (nsp7, nsp8, and nsp12), and more than two turns of RNA template-product duplex [41] (Fig. 9.3). Long helical extensions in nsp8 protrude along exiting RNA, forming positively charged "sliding poles" that explain the relatively high RdRp processivity required for replicating the long genome of coronaviruses. The nsp12 subunit binds to the first turn of RNA between its fingers and thumb subdomains, and the polymerase active site locates at the palm subdomain with conserved motifs A–E, where C has the conserved catalytic residues Asp760 and Asp761.

The RdRp of coronaviruses is an attractive target for the development of antiviral drugs. Classical broad-spectrum antiviral drugs targeting RNA polymerases of different viruses are ribavirin and favipiravir (Fig. 9.4). Coronaviruses have large genomes and relatively lower mutation rates

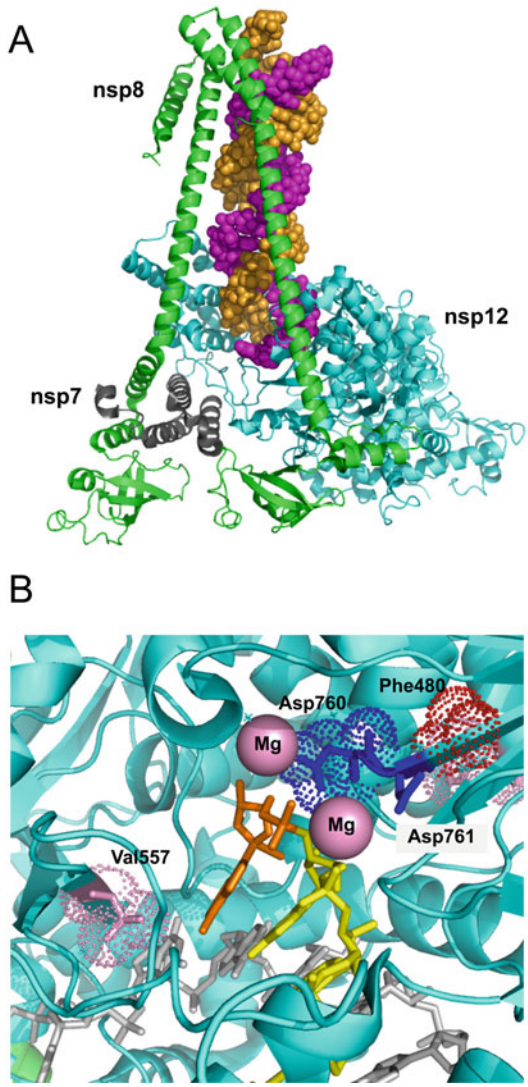


Fig. 9.3 Structure of SARS-CoV-2 RdRp and interactions of remdesivir at the nucleotide binding site. (A) Cryo-electron microscopy-based structure of the SARS-CoV-2 RdRp in an active form. The cartoon shows non-structural proteins nsp12 (RdRp) in cyan, nsp8 in green, and nsp7 (in dark gray), together with more than two turns of the RNA template-product duplex. Coordinates were taken from PDB file 6YYT. (B) View of the RdRp active site with the location of remdesivir (orange sticks) incorporated in the elongating RNA chain (yellow sticks). Active site residues Asp760 and Asp761 are shown in dark blue. Gray sticks are used to represent the RNA template. Coordinates were taken from PDB file 7BV2

compared to other RNA viruses, due in part to the presence of a proofreading 3′–5′ exonuclease encoded in the viral genome (nsp14) [43]. In addition to its exonuclease activity, nsp14 contains a guanine-N7-methyltransferase domain. Exonuclease-deficient mutants of mouse hepatitis virus and SARS-CoV-1 are viable but show an increased mutation frequency. However, knocking out the exonuclease activity in MERS-CoV and SARS-CoV-2 renders nonviable viruses [44]. The proofreading capability of nsp14 increases the fidelity of viral genome replication while making coronaviruses less susceptible to the mutagenic effects of nucleoside analogues such as 5-fluorouracil or ribavirin. Nevertheless, Shannon et al. [45] have recently shown that a recombinant SARS-CoV RdRp complex has an unusually high polymerization rate and a very low nucleotide insertion fidelity, facilitating the efficient incorporation of favipiravir into the viral genome and increasing C-to-U and G-to-A transition frequencies in the already low cytosine content SARS-CoV-2 genome. These findings suggest that lethal mutagenesis is responsible for the antiviral effect of favipiravir.

Remdesivir (Fig. 9.4) is a monophosphoramidate prodrug of an adenosine analogue with inhibitory activity against different types of viruses, most notably *Filoviridae* (e.g., Ebola and Marburg viruses), *Paramyxoviridae* (e.g., Nipah and Hendra viruses), *Pneumoviridae* (e.g., respiratory syncytial virus and human metapneumovirus), and *Coronaviridae* (e.g., SARS-CoV and MERS-CoV) [46–48]. In human airway epithelial cells, remdesivir showed EC_{50}s of 69 and 74 nM, against SARS-CoV-1 and MERS-CoV, respectively [49]. In addition, remdesivir was found to be effective against a broad range of coronaviruses, including circulating HCoVs, zoonotic bat coronaviruses, and pre-pandemic zoonotic coronaviruses [49]. Most importantly, remdesivir was also found to block SARS-CoV-2 replication in cell culture (EC_{50} = 0.77 μM; CC_{50} > 100 μM; SI > 129.87) [50].

After deblocking, remdesivir needs to be diphosphorylated in order to be incorporated by RdRps into the elongating RNA. Enzyme kinetics carried out with SARS-CoV-1, MERS-CoV, and

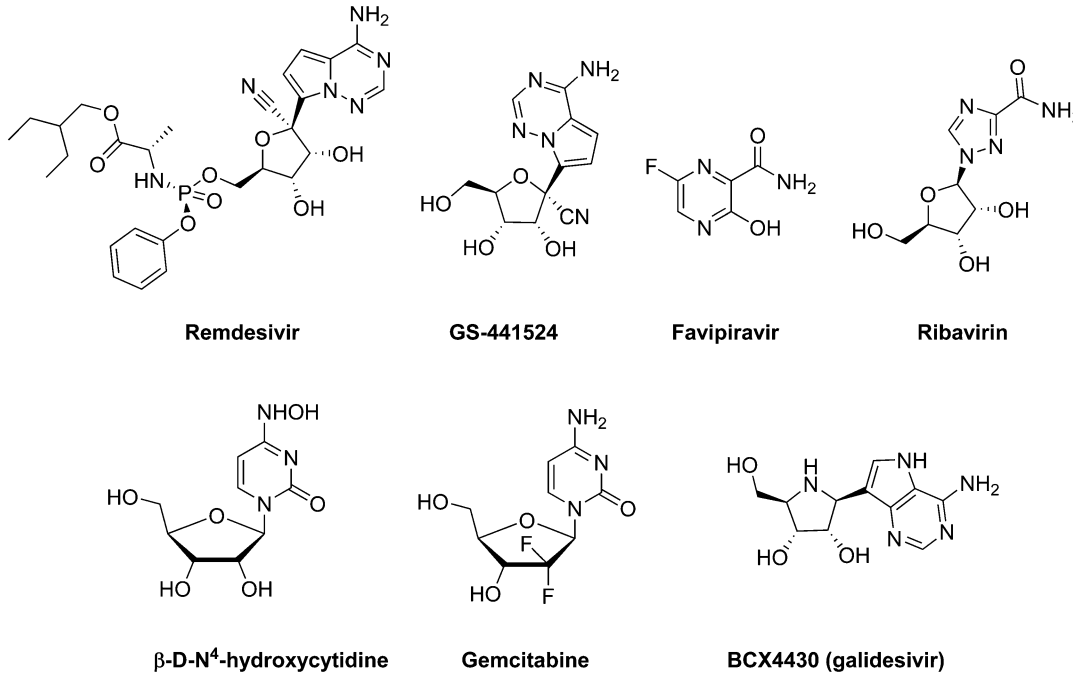


Fig. 9.4 Chemical structures of RdRp inhibitors

SARS-CoV-2 RdRps showed that delayed chain termination is the most plausible mechanism of action of remdesivir. The nucleotide analogue is incorporated into the RNA chain at position +1, but synthesis is halted after subsequent incorporation of three additional nucleotides [51]. Further studies of this group revealed a second mechanism of inhibition occurring at high ribonucleotide concentrations and involving the participation of the template sequence at the incorporation site [52]. In their model, the presence of remdesivir in the template sequence affects the incorporation of UTP, in a process modulated by the presence of Val557 and Ala558 at the RdRp nucleotide binding site [52].

The structure of SARS-CoV-2 RdRp in complex with a 50-base template-primer RNA and remdesivir at 2.5-Å resolution [53] shows the direct participation in RNA binding of 41 residues from nsp12 (26 in the template strand and 15 in the primer strand, all within 4 Å of the protein). Remdesivir is incorporated at the 3′ end of the primer (+1 position), but additional nucleotides interact with the template at positions +2 and +3. In agreement with the proposed mechanism of action (non-obligate chain termination), primer extension is immediately terminated when the concentration of the triphosphorylated form of remdesivir is high and the ATP/remdesivir diphosphate (RDP) ratio is low [53].

9.4.2 Remdesivir Resistance

Studies with remdesivir have not reported resistance associated with the use of the drug in pandemic coronavirus infections. Studies carried out with mouse hepatitis virus (a betacoronavirus) showed that the drug is more effective against viruses lacking nsp14 (exonuclease proofreading enzyme) than the wild-type mouse hepatitis virus [54]. In these viruses, successive passage in the presence of remdesivir selected for amino acid substitutions F476L and V553L in nsp12 that conferred up to 5.6-fold increased resistance to the drug. Their presence has a negative fitness cost in vitro and associates with viral attenuation in vivo [54]. The selected changes appear in motif

F (equivalent substitutions in SARS-CoV-1 are F480L and V557L) and affect residues of the fingers subdomain of RdRp, away from the polymerase active site. A substitution in the motif F of mouse hepatitis virus RdRp (i.e., V553I) has been associated with decreased accumulation of mutations over time (i.e., increased fidelity) [55], and recent SARS-CoV-2 RdRp inhibition studies with remdesivir derivatives showed that the balance between incorporation and excision properties of nucleotide analogues between the coronavirus RNA polymerase (nsp12) and the exonuclease (nsp14) is important for their specificity in nucleotide incorporation assays [56]. Evidence of remdesivir resistance in a COVID-19 patient has been recently reported and associated with the amino acid substitution D484Y in nsp12 [57].

9.4.3 Clinical Trials with Remdesivir

Remdesivir was originally used in clinical trials to fight Ebola disease together with specific neutralizing antibodies [58], although its efficacy was not demonstrated and the drug was not further developed. Later studies with rhesus macaques showed that prophylactic remdesivir treatment initiated 24 h before MERS-CoV inoculation strongly inhibited viral replication in respiratory tissues and prevented the formation of lung lesions [59]. Similar results were obtained in rhesus macaques infected with SARS-CoV-2 [60]. These and other studies suggested that remdesivir was a valuable drug to fight COVID-19, and several clinical trials were initiated as the pandemic advanced around the world. The first reported clinical trial evaluating the efficacy of remdesivir was carried out in China and showed that the drug reduced the hospitalization time, although differences with placebo controls were not significant [61]. Unfortunately, the trial was underpowered as researchers were not able to enroll enough patients to complete the statistically significant clinical study.

Approval of remdesivir for treating COVID-19 [62] came after a double-blind, randomized, placebo-controlled trial in hospitalized adults [63]. Patients received intravenously 200 mg of remdesivir the first day, and then 100 mg daily the next 9 days, or placebo for up to 10 days in the control group. Remdesivir was superior to placebo in shortening in 3–4 days the time to recovery in hospitalized patients with evidence of lower respiratory tract infection. Another trial showed that in patients with severe COVID-19, but not requiring mechanical ventilation, there was no significant difference in treatment efficacy and safety if remdesivir was given for either 5 days or 10 days [64].

Remdesivir is an expensive drug that needs to be administered intravenously. Some authors have argued that the prodrug was not designed for lung-specific delivery, and studies in non-human primates showed a relatively low half-life in serum (0.4 h), due to premature hydrolysis of remdesivir to GS-441524 (Fig. 9.4) [65]. However, this drug shows comparable efficacy in cell-based models of primary human lung and cat cells infected with coronavirus. Remdesivir is a C-nucleotide, and its original synthesis requires many steps and is not straightforward. Thus, the synthesis is not easy and not scalable. Further investigations should be focused on the potential use of GS-441524 over remdesivir for the treatment of COVID-19.

9.4.4 Favipiravir and Other Approved Nucleotide Prodrugs

Favipiravir (sold under the brand names Avigan, Avifavir, Areplivir, FabiFlu, and Favipira) was approved in Japan to treat influenza (Fig. 9.4). It is considered a broad-spectrum inhibitor of viral RNA polymerases [66], but several studies suggest that favipiravir induces lethal RNA transversion mutations, producing a nonviable viral phenotype. The favipiravir tissue culture EC_{50} for SARS-CoV-2 is in the high micromolar range ($EC_{50} = 61.9$ μM), well above the 0.77 μM value obtained for remdesivir under the same conditions [50]. The antiviral effect of favipiravir has also been shown in SARS-CoV-2-infected

hamsters when administered at high doses (1000 mg kg^{-1} day^{-1}) [67].

Favipiravir is a safe therapeutic option, although its pharmacokinetics are problematic, reaching low serum concentrations in critically ill patients. Nevertheless, some benefit has been detected in limited clinical trials and compassionate use [68, 69]. In a randomized clinical trial carried out in Japan, with asymptomatic and mildly ill COVID-19 patients, researchers showed that administration of favipiravir did not significantly improve viral clearance in the first 6 days, although they found a trend toward earlier viral clearance with the agent [70]. Avifavir (favipiravir) has been approved in Russia in June 2020, for emergency use to treat COVID-19 [71].

Sofosbuvir, tenofovir alafenamide, and abacavir are Food and Drug Administration (FDA)-approved oral drugs with well-established safety profiles. Sofosbuvir has been approved for the treatment of hepatitis C virus (HCV) infection, while tenofovir and abacavir are antiretroviral drugs. Enzymatic studies using SARS-CoV-2 RdRp have shown that their active metabolites 2′-F,Me-UTP, tenofovir diphosphate, and carbovir-triphosphate can be substrates of the viral polymerase and incorporated into the RNA chain, blocking its further elongation [72]. Further studies extended this notion to other nucleoside prodrugs (cidofovir, valganciclovir/ganciclovir, stavudine, and entecavir), although in the case of cidofovir-diphosphate (as well as 2′-O-methyluridine-5′-triphosphate), nucleotide incorporation caused delayed termination of RNA synthesis [73]. However, none of those drugs seemed to be effective in cell culture assays [74–76].

9.4.5 Other Nucleoside Analogues

Anti-coronavirus drug development has been challenged by their 3′–5′ proofreading exoribonuclease. This activity confers up to a 20-fold increase in replication fidelity compared with other RNA viruses. β-D-N^4-Hydroxycytidine is a cytidine analogue (Fig. 9.4) that has demonstrated potent, broad-spectrum antiviral activity against Venezuelan equine encephalitis virus, respiratory syncytial virus, influenza A and B viruses, chikungunya virus, and coronaviruses (reviewed by [77]). In cell culture, β-D-N^4-hydroxycytidine inhibits both murine hepatitis virus and MERS-CoV infections with EC_{50} values of 0.17 and 0.56 μM, respectively, and minimal cytotoxicity [78, 79]. The drug showed similar potency when tested in Vero cells against SARS-CoV-2 (EC_{50} = 0.3 μM) [80]. Unlike remdesivir, viral proofreading activity does not markedly impact sensitivity to β-D-N^4-hydroxycytidine inhibition, suggesting a novel interaction between the nucleoside analogue inhibitor and coronavirus RdRps. In addition, resistance to the inhibitor was modest and difficult to achieve in both murine hepatitis virus and MERS-CoV, hardly approaching twofold increased resistance after 30 passages [78]. In mice infected with SARS-CoV or MERS-CoV, both prophylactic and therapeutic administration of its oral prodrug molnupiravir (β-D-N^4-hydroxycytidine-5′-isopropyl ester, MK-4482, EIDD-2801), improved pulmonary function while reducing virus titer and body weight loss [80]. β-D-N^4-Hydroxycytidine is a promising drug candidate that is currently getting evaluated in phase 3 clinical trials. The drug is easy to synthesize and amenable to large-scale synthesis. This drug is also a promising alternative to remdesivir due to its high barrier to resistance. The different resistance profile of β-D-N^4-hydroxycytidine supports a potential therapeutic application of the drug in combination with remdesivir or an alternative to remdesivir treatment failures that require further investigation.

Other nucleoside analogues with demonstrated efficacy against HCoVs are BCX4430 (galidesivir), gemcitabine hydrochloride, 6-azauridine, mizoribine and acyclovir fleximer [77]. Gemcitabine hydrochloride (Fig. 9.4) appears to be the most potent in cell culture with EC_{50} values of 1.2 and 5 μM for MERS-CoV and SARS-CoV-1, respectively, although 6-azauridine was found to be highly potent against HCoV-NL63.

9.5 Helicase (nsp13)

As discussed in previous sections, in coronaviruses, the RdRp (nsp12) associates with nsp7 and nsp8 to form the RdRp holoenzyme that catalyzes the synthesis of viral RNA molecules. During the viral replicative cycle, this complex coordinates with several accessory factors. One of them is nsp13, a helicase that unwinds DNA or RNA in a ribonucleotide-dependent manner with 5′ -> 3′ polarity. In addition, nsp13 has RNA 5′-triphosphatase activity and could play a role in mRNA capping. The nsp13 helicase forms a stable complex with the elongation complex constituted by the RdRp holoenzyme [81]. Two molecules of nsp13 associate with the nsp12/$(nsp8)_2$/nsp7 complex, with N-terminal extensions of nsp8 interacting with the N-terminal domains of nsp13 (Fig. 9.5). The helicase nsp13 contains ribonucleotide binding sites and is also a potentially interesting target for antiviral drug development. Representative SARS-CoV helicase inhibitors include bananins (i.e., oligo-oxa-adamantanes conjugated with pyridoxal), 5-hydroxychromonone derivatives, the triazole SSYA10-001, and bismuth(III) ions [82–85]. In cell culture, SSYA10-001 inhibits the replication of MERS-CoV and SARS-CoV-1 with EC_{50} values of ~25 μM (selectivity index ≥20) and 7 μM (selectivity index ≥71), respectively, with no significant cytotoxicity at concentrations of 500 μM. Stronger inhibition against SARS-CoV-2 was observed with ranitidine bismuth citrate and related compounds [85]. In vitro, IC_{50} values of 0.69 and 0.70 μM were determined for the ATPase and DNA-unwinding activities of nsp13, with a selectivity index of 975. The inhibitory effects were attributed to an irreversible displacement of zinc (II) ions from the enzyme by bismuth(III) ions [85]. Ranitidine bismuth citrate also relieved virus-associated pneumonia in golden Syrian hamsters.

Interestingly, the structure of the RdRp holoenzyme bound to nsp13 unveiled a potential antiviral target through the discovery of ADP-Mg^{2+} bound to the RdRp-associated nucleotidyltransferase (NiRAN) domain. The enzymatic activity of the NiRAN domain is essential for virus propagation, but its target is unknown [86].

9.6 Coronavirus Entry: Antiviral Agents Targeting the Spike Glycoprotein

The spike glycoprotein S contains a receptor binding domain (RBD) in the S1 subunit. Its interaction with cellular receptors (e.g., DPP4 in MERS-CoV or ACE2 in SARS-CoV-1 and SARS-CoV-2) triggers a conformational change in S2 that facilitates fusion of the viral envelope with the host cell membrane [87]. Coronavirus inhibitors targeting the spike glycoprotein include the antiviral protein griffithsin (Fig. 9.6), the heptad repeat 2 (HR2) domain of the S2 subunit of HCoV-OC43 variants, and specific human monoclonal antibodies.

O'Keefe et al. [88] reported that griffithsin binds specifically to the SARS-CoV-1 spike glycoprotein and inhibits viral entry (EC_{50} = 0.61 mg/L and CC_{50} >100 mg/L). In addition, griffithsin was found to be effective against several HCoVs. A synthetic oligopeptide (OC43-HR2P), derived from the HR2 domain of the spike glycoprotein of HCoV-OC43, also showed fusion inhibitory activity against multiple HCoVs [60]. To obtain more potent inhibitors, researchers optimized peptide OC43-HR2P to obtain EK1 (Fig. 9.6) that showed higher antiviral activity and better pharmaceutical properties. The inhibitory activity of EK1 was demonstrated against SARS-CoV-1, MERS-CoV, HCoV-229E, HCoV-NL63, and HCoV-OC43 with EC_{50} values of 2.23 μM, 0.26 μM, 3.35 μM, 6.02 μM, and 1.81 μM, respectively [89]. More recently, Xia et al. [90] obtained a series of lipopeptide derivatives of EK1 and found that EK1C4 (Fig. 9.6) was the most potent inhibitor against SARS-CoV-2 S protein-mediated membrane fusion with an IC_{50} of 15.8 nM, >100-fold more potent than the original EK1 peptide, in pseudovirus infection assays.

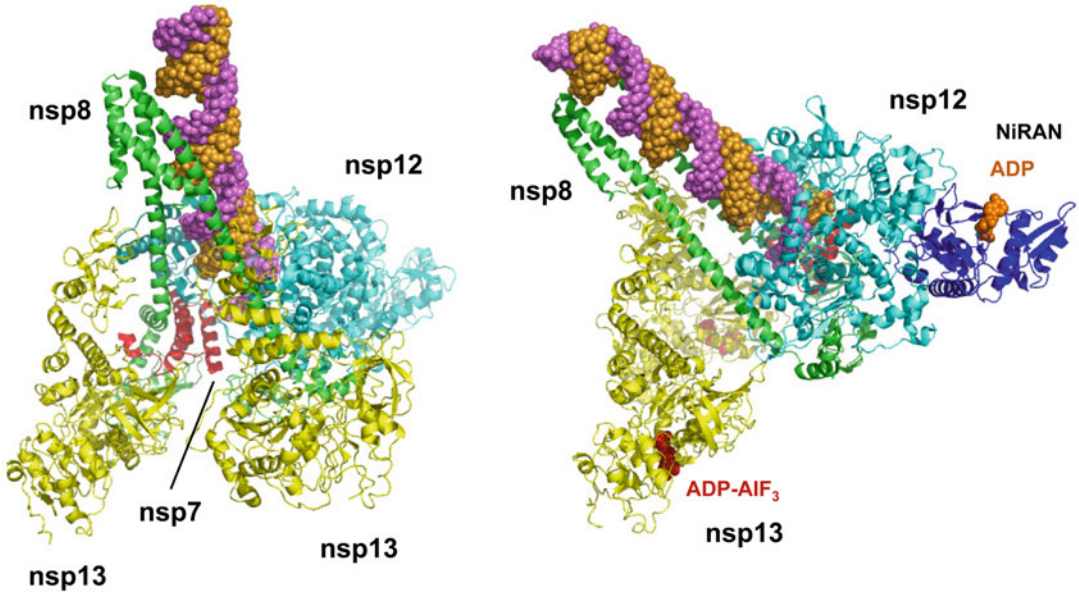


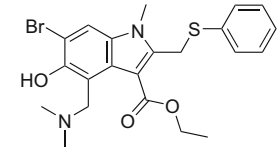
Fig. 9.5 Structure of the SARS-CoV-2 RdRp holoenzyme in complex with nsp13. Cartoons show non-structural proteins nsp12 (RdRp) in cyan, nsp8 in green, nsp7 in red, and nsp13 in yellow. The RNA template-primer is shown using a CPK model. ADP binding sites in nsp13 and the NiRAN domain of nsp12 are shown in red and orange, respectively. Coordinates were taken from PDB file 6XEZ

Using a similar approach, Zhu et al. [91] developed one HR2 sequence-based lipopeptide fusion inhibitor, termed IPB02 (Fig. 9.6), which showed highly potent activities in inhibiting SARS-CoV-2 S protein-mediated cell-cell fusion and pseudovirus transduction. The lipopeptide IPB02 inhibited SARS-CoV-1 and SARS-CoV-2 pseudoviruses with IC_{50}s of 0.251 and 0.08 μM, respectively.

In addition, umifenovir (Arbidol) has also been proposed as a potential SARS-CoV-2 replication inhibitor (EC_{50} = 4.11 μM and selectivity index 7.73) [92]. Umifenovir, an indole derivative (Fig. 9.6), has been licensed for decades in Russia and China against influenza and is considered a broad-spectrum antiviral drug. Based on structural data obtained with influenza virus hemagglutinin, it is believed that the drug interferes with membrane fusion. However, systematic reviews and meta-analyses failed to provide conclusive evidence to support the clinical use of Arbidol in SARS-CoV-2 infections [93, 94].

Structural or non-structural viral proteins contain epitopes that can be targeted by monoclonal

Griffithsin	SLTHRKFGGSGGSPFSGLSSIAVRSGSYLDAIIIDGVHHGGSGGNLSPTFTF GSGEYISNMTIRSGDYIDNISFETNMGRRFGPYGGSGGSANTLSNNVKVIQ INGSAGDYLDSLDIYYEQY
EK1	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL
EK1C4	^{N}EK1^{C}-GSGSG-PEG4-cholesterol
IPB02	ISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELK (cholesterol)



Umifenovir (Arbidol)

Fig. 9.6 Chemical structures of griffithsin, EK1, EK1C4, IPB02, and umifenovir (Arbidol). PEG4 represents a spacer composed of four units of polyethylene glycol

antibodies (mAbs) [95]. The effectivity of mAbs targeting the highly variable spike glycoprotein has been demonstrated for coronaviruses that infect animal models [96], as well as for SARS-CoV-2 and other HCoVs [27]. The degree of cross-reactivity between antibodies against the SARS-CoV and SARS-CoV-2 spike glycoprotein has been determined. Thus, CR3022 is a human SARS-CoV-specific mAb that binds the receptor binding domain of the S glycoprotein of SARS-CoV-2 with relatively high affinity ($K_{on} = 1.84 \times 10^5\ M\ s^{-1}$, $K_{off} = 1.16 \times 10^{-3}\ s^{-1}$, $K_d = 6.3$ nM). However, its epitope does not overlap with the receptor binding domain of SARS-CoV-2 [97]. Neutralizing nanobodies binding the SARS-CoV-2 spike have also been obtained, and their affinity for the receptor binding domain of the spike glycoprotein has been improved to reach K_d values of 12–40 nM. Their ability to block the viral interaction with the ACE2 receptor was shown in their subsequent characterization [98].

Antibodies such as CR3014 targeting the ACE2 binding site of SARS-CoV-1 did not bind SARS-CoV-2 S glycoprotein, implying that the differences in the receptor binding domains of SARS-CoV-1 and SARS-CoV-2 have a critical impact on the cross-reactivity of neutralizing antibodies [97]. JS016 is a human mAb based on neutralizing antibodies of a convalescent patient suffering COVID-19 that recognizes an epitope in S glycoprotein that overlaps the ACE2 binding site [99]. The protection conferred when SARS-CoV-2 was inoculated to rhesus macaques triggered the initiation of clinical trials sponsored by the pharmaceutical companies Junshi Biosciences (China) and Eli Lilly (USA).

Other neutralizing antibodies have been obtained in humanized mice as well as convalescent patients to prepare cocktails that could decrease the selection of viral mutants escaping antibody selective pressure [100]. These studies followed previous evidence showing that in fact, escape mutants were selected after treatment with a single potent neutralizing antibody [101]. More recently and using phage-display technologies, Li et al. [102] identified a bivalent human V_H domain (ab8) that showed high affinity for the receptor binding domain of the SARS-CoV-2 spike glycoprotein and the S1 subunit ($K_D = 19$ nM) as well as high avidity, outcompeting human ACE2 in binding the S glycoprotein. In animal studies with mouse-adapted SARS-CoV-2, neutralization of the virus was observed with wild-type mice at ab8 doses as low as 2 mg/kg. The authors also showed the prophylactic and therapeutic effect of ab8 in a hamster model of SARS-CoV-2 infection [102].

9.7 Coronavirus Protease Inhibitors

In coronaviruses, two-thirds of the viral genome encode non-structural proteins such as ORF1ab, ORF3a, ORF6, ORF7a, ORF8, and ORF10 (Fig. 9.1) which are responsible for transcription and viral replication [103]. ORF1a encodes a papain-like protease (PL^{pro}) and the main protease (M^{pro} also known as 3-chymotrypsin-like protease, $3CL^{pro}$) [19, 104, 105] During viral replication, the polyprotein (pp1a and pp1ab) undergoes extensive processing (i.e., maturation) by the two viral proteases, namely, M^{pro} and PL^{pro}, encoded by ORF1ab (Fig. 9.1). Non-structural proteins are highly conserved among coronaviruses [106]. Due to their essential roles in viral replication and because they differ significantly from human proteases, both are recognized as attractive targets for the development of therapeutics against SARS-CoV-1 and SARS-CoV-2 [19, 107]. In particular, the main protease M^{pro} is a promising target for inhibitor drug design in drug development [8, 108, 109]. Recently, breakthroughs related to the structural elucidation of key vital proteins of SARS-CoV-2, which include M^{pro}, PL^{pro}, spike protein S, and RNA-dependent RNA polymerase, have been reported. Moreover, the first disclosure of the X-ray structure of M^{pro} (6LU7, resolution 2.16 Å) [108] in complex with an irreversible peptide-like inhibitor (N3) represents an additional step toward a structure-based design of novel M^{pro} inhibitors.

From X-ray crystallographic studies, the structure of the M^{pro} has been well characterized as

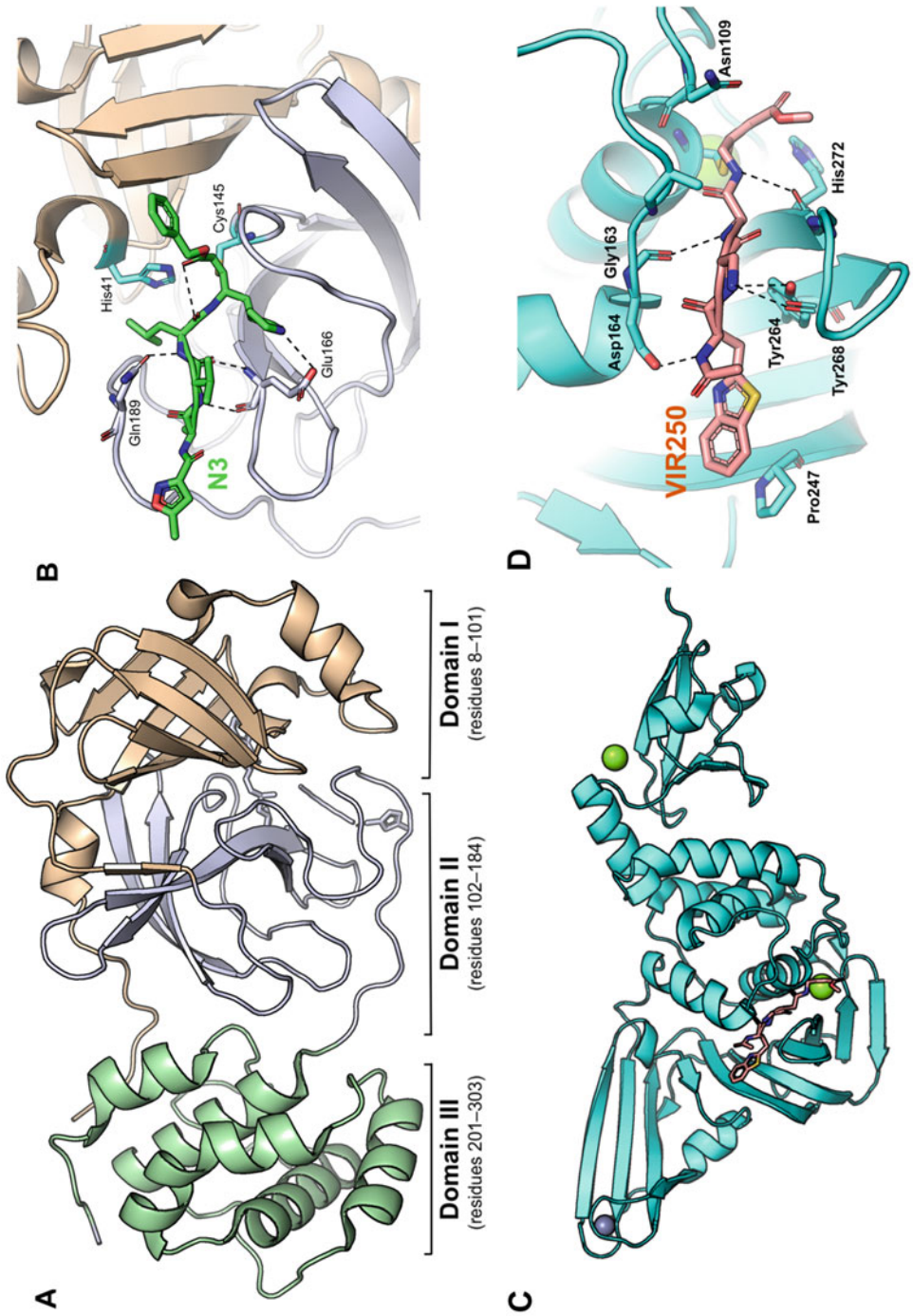


Fig. 9.7 X-ray crystal structure of SARS-CoV-2 M^{pro} co-crystallized with ligand N3 (A) and SARS-CoV-2 CL^{pro} co-crystalized with VIR250 (C). Ligand binding mode of N3 (C) and VIR250 (D) is shown. Key residues and important domains are highlighted

being a homodimer (Fig. 9.7) [8, 108]. Each monomer is composed of 3 domains (designated as I, II, and III), each of them containing approximately 100 residues (domain I, residues 8–101; domain II, residues 102–184; and domain III, residues 201–303). The active site including the Cys145-His41 catalytically dyad lies between the domains I and II. The substrate binding site comprises S1′, S1, S2, and S4 subsites which are highly conserved among coronaviruses. M^{pro} inhibitors showing activity against different coronaviruses are potential candidates for SARS-CoV-2, due to their high structural similarity.

The discovery and development of potential inhibitors for SARS-CoV-2 have been the top priority since the beginning of the SARS-CoV-2 outbreak [104]. Until now, several hundreds of compounds have been identified as potential candidates to treat SARS-CoV-2 infections. Many of them have been found in computational screening (or virtual screening) campaigns using natural product databases, through repurposing of approved drugs and clinical candidates, using virtual synthetically feasible-compound libraries, etc. [110, 111]. However, the efficacy of a large proportion of these reported compounds has not been experimentally verified; therefore, such compounds will not be discussed in this section. In addition to computational screening, the classical medicinal chemistry approach has also been used for hunting potential candidates for SARS-CoV-2, especially targeting SARS-CoV-2 proteases [112]. Thus, compounds from different chemical classes including small molecules, peptides, and peptidomimetics have been shown to inhibit the SARS-CoV-2 proteases. Moreover, the recent advancement in computation, high-throughput assays, biophysical methods, and synthetic organic chemistry disciplines offers an opportunity to design more potent inhibitors for M^{pro} through structure-guided approaches [113]. Representative examples of M^{pro} inhibitors are shown in Fig. 9.8.

Recently, Dai et al. [114] designed peptidomimetic derivatives (11a and 11b) using a structure-guided approach, by reaching S1′, S1, P1, and P2 sites of M^{pro} (Fig. 9.8). Both compounds showed excellent activity against SARS-CoV-2 M^{pro} with IC_{50} values of 0.053 and 0.040 μM, for 11a and 11b, respectively. Subsequently, co-crystallization of these compounds with M^{pro} also revealed a covalent bond with catalytic Cys145 residue, through aldehyde atoms in 11a and 11b.

In 2005, with the help of protein homology modeling, Yang et al. [115] designed compound N3 that irreversibly inhibits various coronaviruses, such as HCoV-229E (IC_{50} = 4.0 μM), feline infectious peritonitis virus (IC_{50} = 8.8 μM), and mouse hepatitis virus A59 (IC_{50} = 2.7 μM). It is interesting to note that the authors also wrote, "N3 could provide an effective first line of defense against future emerging coronavirus-related diseases. Moreover, it also suggests that incorporation of Michael acceptor with the peptidyl portion specific for proteases would be a good starting point for the development of inhibitors against viral Cys or Ser proteases." Exactly 15 years later, the same research group has experimentally verified that compound N3 is a potent inhibitor of the SARS-CoV-2 M^{pro} (Fig. 9.7b,c). Besides, Jin et al. [108] also used a drug repurposing approach to identify potential inhibitors for SARS-CoV-2 M^{pro}. Of >10000 compounds screened with a high-throughput assay, 6 compounds (ebselen, disulfiram, tideglusib, carmofur, shikonin, and PX-12) showed good inhibitory activity against SARS-CoV-2 M^{pro} (IC_{50} values within 0.67 and 21.4 μM), and some of these compounds also showed antiviral activity. More recently, Zhang et al. [116] reported high-resolution X-ray structures of unliganded SARS-CoV-2 M^{pro} (6YB7, 1.25 Å) and in complex with α-ketoamide inhibitors (compound 13b) (6Y2F, 1.95 Å).

In addition to covalent inhibitors, Su et al. [117] reported that baicalein could also inhibit SARS-CoV-2 M^{pro} (IC_{50} = 0.94 μM). Using fluorescence resonance energy transfer assays, Ma et al. [118] have screened a library containing various known protease inhibitors and identified four potential inhibitors of SARS-CoV-2 M^{pro}. These antiviral compounds were GC-376 (IC_{50} = 0.030 μM), boceprevir (IC_{50} = 4.13 μM),

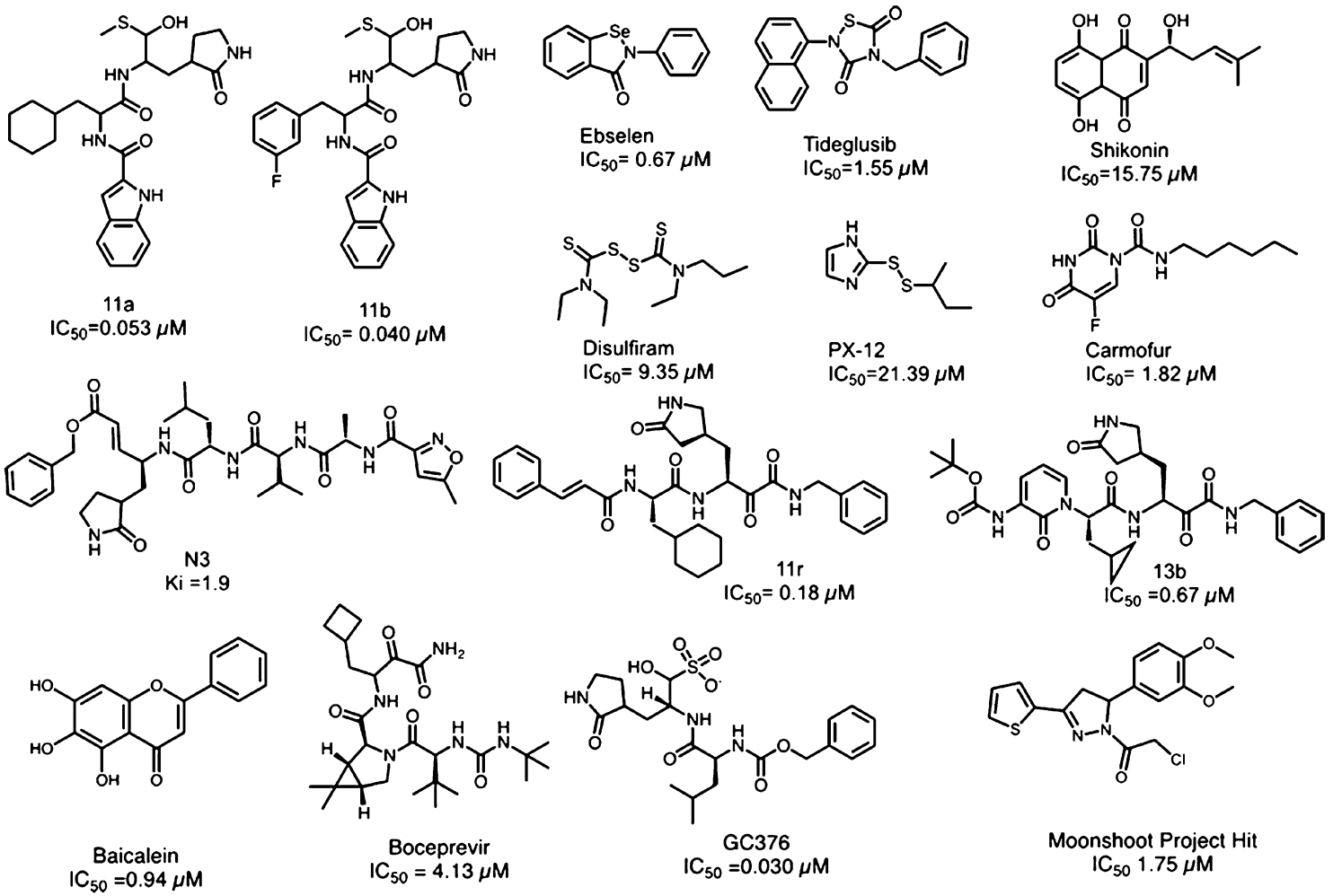


Fig. 9.8 Representative molecules that inhibit SARS-CoV-2 M^{pro} (compound ID is given as per their original publications)

and calpain inhibitors II (IC_{50} = 0.97 μM) and XII (IC_{50} = 0.45 μM) (Fig. 9.8).

In addition, papain-like cysteine protease (PL^{pro}) of SARS-CoV-2 is also a key enzyme for viral replication and a promising drug target for SARS-CoV-2 [119–121]. Although there are not many reported SARS-CoV-2 PL^{pro} inhibitors, a recent study by Rut et al. [121] provided a rational ligand design approach for the development of SARS-CoV-2 PL^{pro} inhibitors. Researchers used a combinatorial substrate library containing natural amino acids that are easily recognized by the protease. Interestingly, two structurally similar compounds VIR250 (Fig. 9.7d) and VIR251 showed promising activity against SARS-CoV-2 PL^{pro}.

While this chapter was under revision, three key papers were published on protease enzymes. The study by Rathnayake et al. [122] reports a series of dipeptidyl compounds containing a piperidine moiety. All the synthesized compounds were screened against M^{pro} enzymes of various coronaviruses, including SARS-CoV-2. Several of these compounds showed strong inhibition profiles in both fluorescence resonance energy transfer and cell-based assays, particularly compound 6e (Fig. 9.9) that showed the highest potency against SARS-CoV-2 (EC_{50} = 0.15 μM). Subsequently, Hattori et al. [123] reported antiviral activity of indole-based chloropyridinyl ester derivatives and showed excellent activity against SARS-CoV-2 for GRL-0820 (EC_{50} = 15 μM) and GRL-0920 (EC_{50} = 2.8 μM) (Fig. 9.9). The study also compared the efficacy of GRL-0820 and GRL-0920 with nine other known compounds, using the same assay at the same time. The result shows that both compounds (GRL-0820L, EC_{50} = 23 μM, and GRL-0920, EC_{50} = 8.6 μM) were active against SARS-CoV-2, and show good efficacy in comparison to other antiviral drug candidates such as remdesivir (EC_{50} = 1.3 μM), lopinavir (EC_{50} = 19 μM), nelfinavir (EC_{50} = 3.1 μM), favipiravir (EC_{50} > 100 μM), hydroxychloroquine (EC_{50} = 5.1 μM), nitazoxanide (EC_{50} = 31 μM), and nafamostat (EC_{50} > 100 μM). In another study by Klemm et al. [124], researchers have used a systematic repurposing approach to find PL^{pro} inhibitors by screening more than 3500 drugs and clinical candidates. From the screening, compound rac5c (Fig. 9.9) was identified as an effective inhibitor, with an IC_{50} = 0.81 μM for SARS-CoV-2 PL^{pro}. The mechanism of inhibition of that compound was also studied, suggesting that rac5c inhibits self-processing of nsp3-green fluorescent protein (GFP) of PL^{pro}. These three studies lay the foundation for further developments of improved drug candidates for SARS-CoV-2 especially targeting the protease enzymes.

9.8 Antiviral Agents Targeting Host Cell Proteins

Coronavirus replication requires the host cell to provide necessary elements, including organelles, proteins, and enzymes, to support viral structure and function. When viral infection occurs, host cell receptors and associated enzymes play a crucial role in the process of attachment and fusion. At the same time, host cells produce cytokines and other molecules in response to infection. These cellular components and their interactions with the virus might be exploited as targets for antiviral intervention.

9.8.1 ACE2 Inhibitors

ACE2 is the receptor for SARS-CoV-1 and SARS-CoV-2 [125]. Researchers have shown that SARS-CoV-1 and SARS-CoV-2 share 79.5% identity in their full-length genomic sequence and have demonstrated that SARS-CoV-2 uses the same receptor and entry pathway as SARS-CoV-1 [126, 127]. Furthermore, it has been shown that ACE2 has higher affinity for the spike glycoprotein of SARS-CoV-2 than for that of SARS-CoV-1 [128]. Taken together, these data support the notion that ACE2 is a druggable target for the development of antiviral agents against SARS-CoV-1 and SARS-CoV-2.

Dales et al. [129] designed a series of ACE2 inhibitors based on enzyme characterization and substrate studies. Representative compounds shown in Fig. 9.10 exhibited excellent ACE2

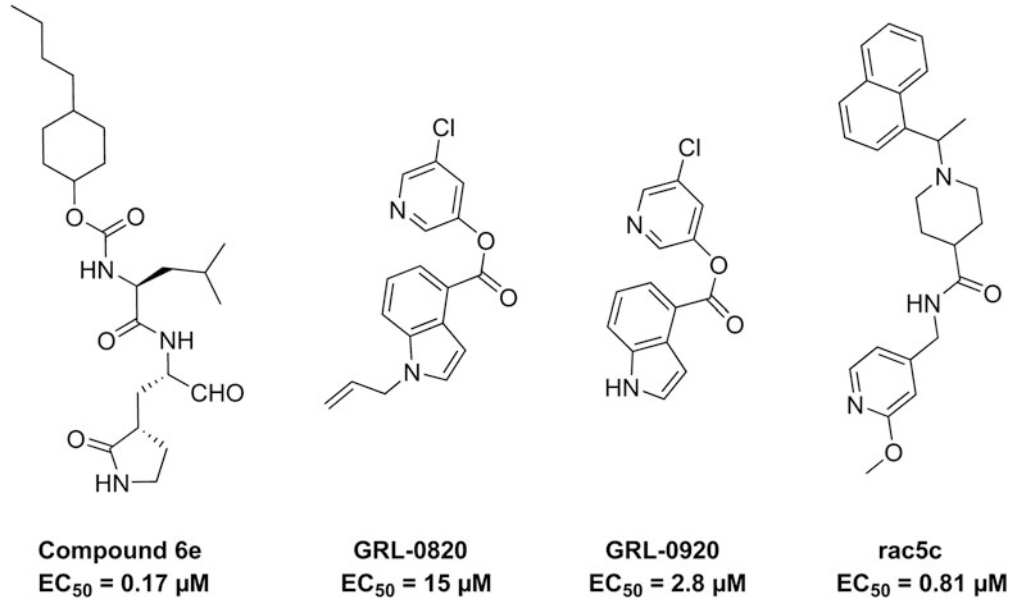


Fig. 9.9 Chemical structures of novel M^{pro} and PL^{pro} SARS-CoV-2 inhibitors

inhibitory activity and higher selectivity against ACE versus ACE2. The IC_{50}s of compounds 13, 15, and 16 against ACE2 were 4.2 nM, 1.4 nM, and 0.4 nM, respectively, but showed no activity against ACE (IC_{50} >10 μM) [129]. However, their potential antiviral effects have not been shown in cell culture and/or in vivo. Huentelman et al. [130] screened a chemical library containing 140000 compounds using in silico molecular docking and identified N-(2-aminoethyl)-1-aziridine-ethanamine (Fig. 9.10) as an effective ACE2 inhibitor with an IC_{50} of 57 μM. Interestingly, this compound blocks the membrane fusion process of SARS-CoV-1 while entering human embryonic kidney 293T cells with an EC_{50} in the micromolar concentration range [130].

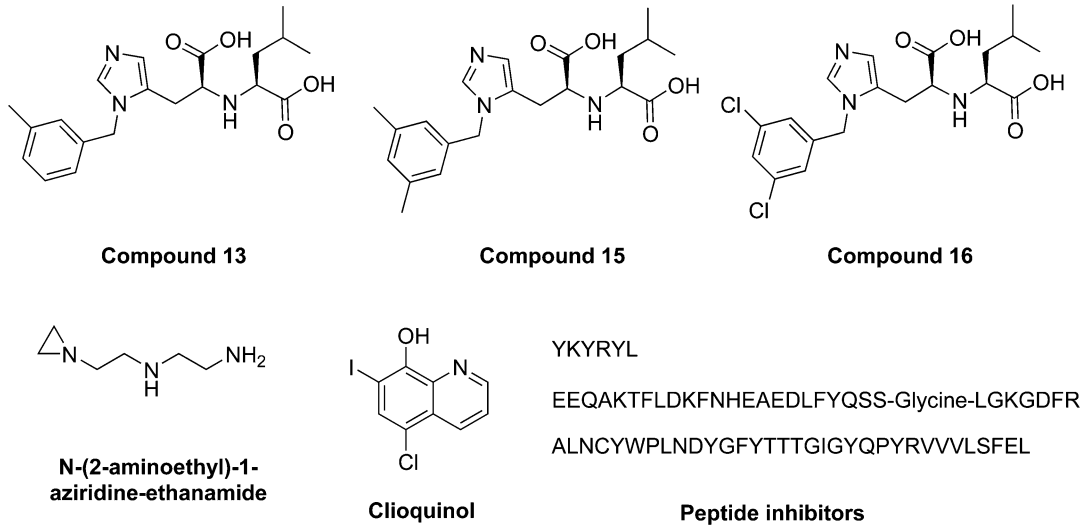


Fig. 9.10 Chemical structures of ACE2 inhibitors

Two derivatives of clioquinol (7-bromo-5-chloro-8-hydroxyquinoline and 5,7-dichloro-8-hydroxyquinoline) have been recently described as effective SARS-CoV-2 inhibitors [131]. Clioquinol (Fig. 9.10) and its derivatives show EC_{50} values in the low micromolar range (12.6–16.3 μM), interfering with ACE2 binding to the S glycoprotein. ACE2 is a zinc metalloprotease, and clioquinol is an established metal chelator and zinc ionophore.

Several peptides have been described as ACE2 inhibitors (see structures in Fig. 9.10). Those peptides mimic the structure of the receptor binding domain in the viral S protein and are involved in the interaction with ACE2. One of them is a 32-amino acid polypeptide containing the receptor binding domain sequence (residues 471–503 of the viral S protein) and shows an IC_{50} of 41.6 μM [132]. Based on similar principles, Han et al. [133] identified another peptide inhibitor that contained the two ACE2 motifs (residues 22–44 and 351–357) linked by glycine. This peptide showed potent anti-SARS-CoV-1 activity with an EC_{50} of 0.1 μM. A shorter peptide of six amino acids (YKYRYL) has also been shown to inhibit the proteolytic activity of ACE2, in Vero cells [134]. YKYRYL was found to reduce viral RNA levels by a factor of 10 and 600 at peptide concentrations of 10.5 mM and 14 mM, respectively, in comparison with control experiments carried out without the inhibitors.

9.8.2 Inhibitors Targeting the Surface Transmembrane Protease, Serine 2 (TMPRSS2)

The distribution of ACE2 receptors does not strictly correlate with the cell tropism of SARS-CoV-1 in the lungs, thereby indicating that other factor(s) could influence the infection efficiency and dynamics [135]. Cellular proteases are required for the cleavage of the SARS-CoV-1 spike glycoprotein and could modulate cell tropism [136]. Neutropilin-1 binds furin-cleaved substrates and is known to potentiate SARS-CoV-2 infectivity [137], although the mechanism involved has not been fully elucidated. In the absence of proteases in the cell surface, SARS-CoV enters cells by endocytosis. Viral S proteins are activated for fusion by the endosomal proteinase CTSL [138]. On the contrary, in the presence of serine proteases at the cell surface (e.g., trypsin and TMPRSS2), after receptor binding, spike glycoproteins are activated to trigger membrane fusion and direct the entry process [139, 140]. Treatment with furin inhibitors (e.g., decanoyl-RVKR-chloromethylketone and naphthofluorescein) blocks viral entry by suppressing cleavage of the spikes and the formation of syncytia [81].

TMPRSS2 contains domains corresponding to a type II transmembrane protein, a receptor class A domain, a scavenger receptor cysteine-rich domain, and a serine protease. The protease domain is thought to be cleaved and secreted into cell media after autoproteolytic cleavage.

Hoffmann et al. have recently demonstrated that SARS-CoV-2 uses the SARS-CoV receptor ACE2 for entry and TMPRSS2 for S protein priming, showing that camostat mesylate blocks viral entry [141] (Fig. 9.11). These findings were consistent with previous reports showing that a single treatment with camostat was sufficient to block MERS-CoV entry into Vero cells, a well-differentiated lung-derived cell line [142]. MERS-CoV S protein is able to induce cell-cell fusion in those cells, and syncytium formation was moderately inhibited by camostat at concentrations of 1–10 μM and completely inhibited at 100 μM. However, unlike in the case of furin inhibitors, camostat mesylate was unable to inhibit cleavage of SARS-CoV-2 spikes and block the formation of syncytia [143].

Camostat is an inhibitor of TMPRSS2 and related serine proteases and has been used clinically to treat chronic pancreatitis [144]. The efficacy of camostat mesylate can be limited by its conversion to the active metabolite 4-(4-guanidinobenzoyloxy)phenylacetic acid (GBPA) in serum. However, GBPA is also an inhibitor of TMPRSS2, and clinically approved doses of camostat mesylate may result in GBPA peak concentrations consistent with its antiviral activity [141]. Currently, there are several clinical

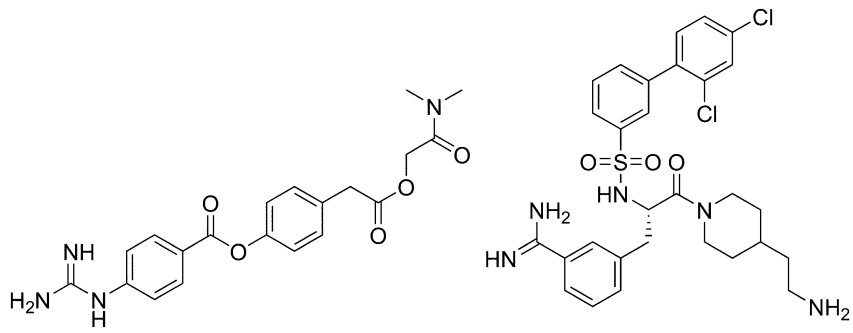


Fig. 9.11 Chemical structures of TMPRSS2 inhibitors

trials in progress to evaluate the efficacy of camostat mesylate as therapy against COVID-19. Despite being a repurposed drug, camostat mesylate was not detected as an effective antiviral drug candidate in screening campaigns using libraries of FDA-approved drugs [74, 76]. Interestingly, a related compound (nafamostat mesylate) was found to be a very effective inhibitor of SARS-CoV-2 replication in human lung cells, with an EC_{50} of 0.0022 μM [145, 146]. Apart from camostat and nafamostat, Pászti-Gere et al. [147] also showed the loss of expression of the serine protease domain of TMPRSS2 (missing 28 kDa band), when IPEC-J2 cells were exposed to I-432 (Fig. 9.11) at 50 μM for 48 h.

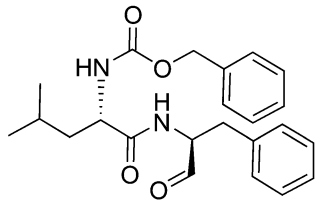
9.8.3 Endosomal Proteinase Cathepsin L (CTSL) Inhibitors

CTSL is a member of the human lysosomal cysteine protease family [148]. Using a cell-free membrane fusion system, it has been shown that CTSL is sufficient to activate membrane fusion by cleaving the SARS-CoV-1 spike glycoprotein and that SARS-CoV-1 infection can be blocked by specific inhibitors of CTSL [149]. Cleavage allows coronaviruses to enter cells through a cathepsin-independent mechanism, converting CTSL into an ideal target for the development of antiviral agents against coronaviruses [150].

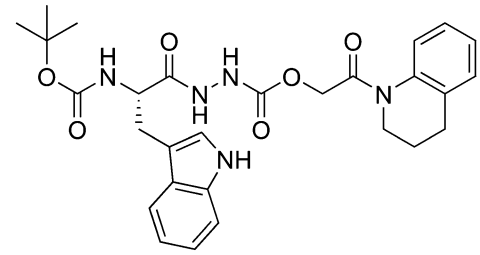
MDL28170 (Fig. 9.12) has been described as a potent CTSL inhibitor (IC_{50} = 2.5 nM) that blocks SARS-CoV-1 replication with an EC_{50} around 100 nM [149]. An oxocarbazate inhibitor (Fig. 9.12) has been shown to be effective against human CTSL in a time-dependent manner, with IC_{50}s of 6.9 nM (without preincubation) or 0.4 nM (measured after 4 h preincubation) [151]. This compound showed an EC_{50} of 273 nM in SARS-CoV-1 cell cultures. Teicoplanin (Fig. 9.12) is a glycopeptide antibiotic that inhibits CTSL in the late endosome. It blocks MERS-CoV and SARS-CoV-1 entry with EC_{50} values of 0.63 μM and 3.76 μM, respectively [127]. In addition, Zhou et al. found that another cysteine protease inhibitor (K11777, structure shown in Fig. 9.12) and its derivatives can also inhibit a variety of enveloped coronaviruses including SARS-CoV-1, MERS-CoV, and HCoV-229E by targeting cathepsin-mediated cell entry. The EC_{50}s of K11777 for SARS-CoV-1, MERS-CoV, and HCoV-229E are 0.68 nM, 46.12 nM, and 1.48 nM, respectively [144].

9.8.4 Compounds Interfering with Endosomal Acidification

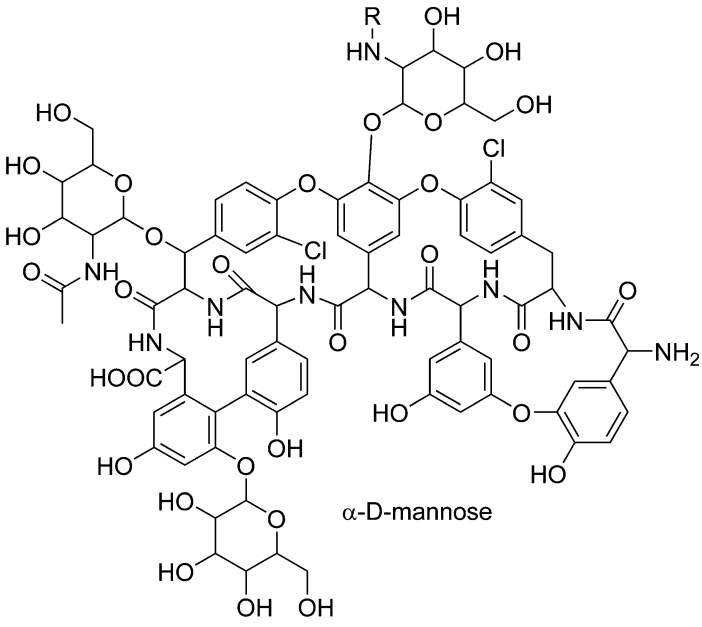
Viral entry involves receptor binding at the surface of the host cell, followed by endocytosis through the endosomal pathway [152]. In coronaviruses, the cleavage of the spike glycoprotein is dependent on endosomal acidification and requires the participation of associated acidic

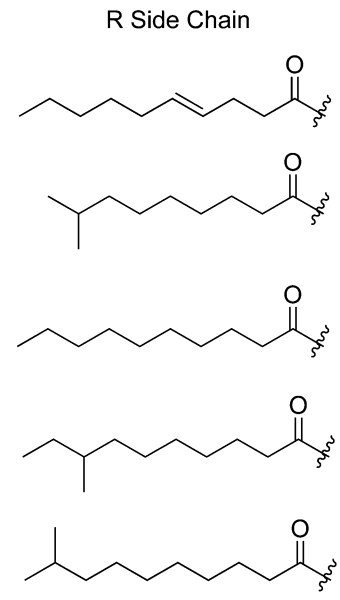


MDL28170

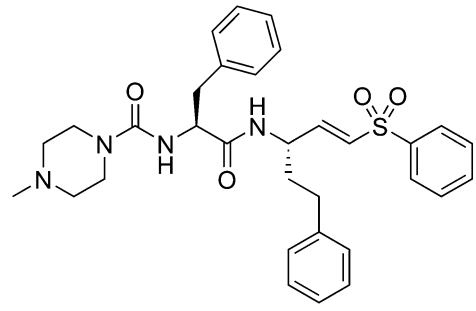


Oxocarbazate





Teicoplanin



K11777

Fig. 9.12 Chemical structures of representative CTSL inhibitors

pH-dependent endosomal proteases [153]. Without endosomal acidification, these proteases are unable to cleave the spike glycoprotein, and viral replication and infection is blocked.

Chloroquine (Fig. 9.13) is an antimalarial drug with a classical cationic amphiphilic structure. Under physiological conditions, its amino moiety exists mainly in an unprotonated form, which can spontaneously and quickly pass through cell or organelle membranes and accumulate in acidic intracellular compartments. When the concentration of chloroquine in acidic organelles increases, it gets protonated. Protonated chloroquine cannot easily pass through the organelle membrane, resulting in a pH increase. The organelle physiological and morphological properties will change, causing alterations and potential loss of function in enzymes required for proteolysis and post-translational modification [154]. Moreover, chloroquine exhibits antiviral effects by inhibiting the production of cytokines such as interferon γ and tumor necrosis factor (TNF) α and downregulating the expression of TNF α receptors [155].

In vitro cell culture studies have shown that chloroquine inhibits the replication of coronaviruses, such as SARS-CoV-1 (EC_{50} = 4.1 μM, CC_{50} > 128 μM, SI > 31), MERS-CoV (EC_{50} = 3 μM, CC_{50} 58.1 μM, SI 19.4), and SARS-CoV-2 (EC_{50} = 1.13 μM, CC_{50} > 100 μM, SI > 88.5) [50, 156]. Chloroquine has been approved by Chinese, South Korean, and Italian health authorities for the experimental treatment of COVID-19, but its medical benefit has not been proven. On 1 April 2020, the European Medicines Agency (EMA) issued guidance that chloroquine and hydroxychloroquine should be used only in clinical trials or emergency use programs [157]. Still, chloroquine has been under rigorous investigation as a potential COVID-19 therapeutic regimen [158], although as of August 2020, suspension of hydroxychloroquine administration remains halted, due to safety concerns. The United States FDA has also advised against the use of chloroquine and hydroxychloroquine in combination with remdesivir, due to the loss of effect of the RdRp inhibitor [159]. Further support of these measures has been provided by a randomized clinical trial showing that hydroxychloroquine had no clinical benefit when administered daily for 8 weeks as pre-exposure prophylaxis to hospital-based health-care workers exposed to patients with COVID-19 [160]. Hoffmann et al. [161] have shown that overexpression of TMPRSS2 (Sect. 9.2), a cellular protease that activates SARS-CoV-2 for entry into lung cells, renders SARS-CoV-2 infection of Vero cells insensitive to chloroquine.

9.8.5 Eukaryotic Translation Initiation and Elongation Factors and DEAD-Box RNA Helicases as Targets of Antiviral Drugs Against Coronaviruses

RNA helicases are involved in many cellular functions, including the unwinding of RNA duplexes and mRNA splicing and translation [162, 163]. DEAD-box proteins represent the largest group of proteins among the RNA helicase family. They are characterized by having several conserved motifs involved in intramolecular folding and RNA interactions [164]. The eukaryotic translation initiation factor 4A (eIF4A) was the first and most characteristic representative of the DEAD-box protein family and plays an important role in coronavirus replication [165].

Silvestrol (Fig. 9.13) is a specific eIF4A inhibitor isolated from the fruits and twigs of *Aglaia foveolata* [166]. It exhibits potent inhibitory activity on the expression of structural and non-structural proteins and the formation of viral replication/transcription complexes [167]. Silvestrol was found to be a potent inhibitor of cap-dependent viral mRNA translation in human embryonic lung fibroblast (MRC-5) cells infected with coronaviruses. Silvestrol blocked MERS-CoV and HCoV-229E replication in MRC-5

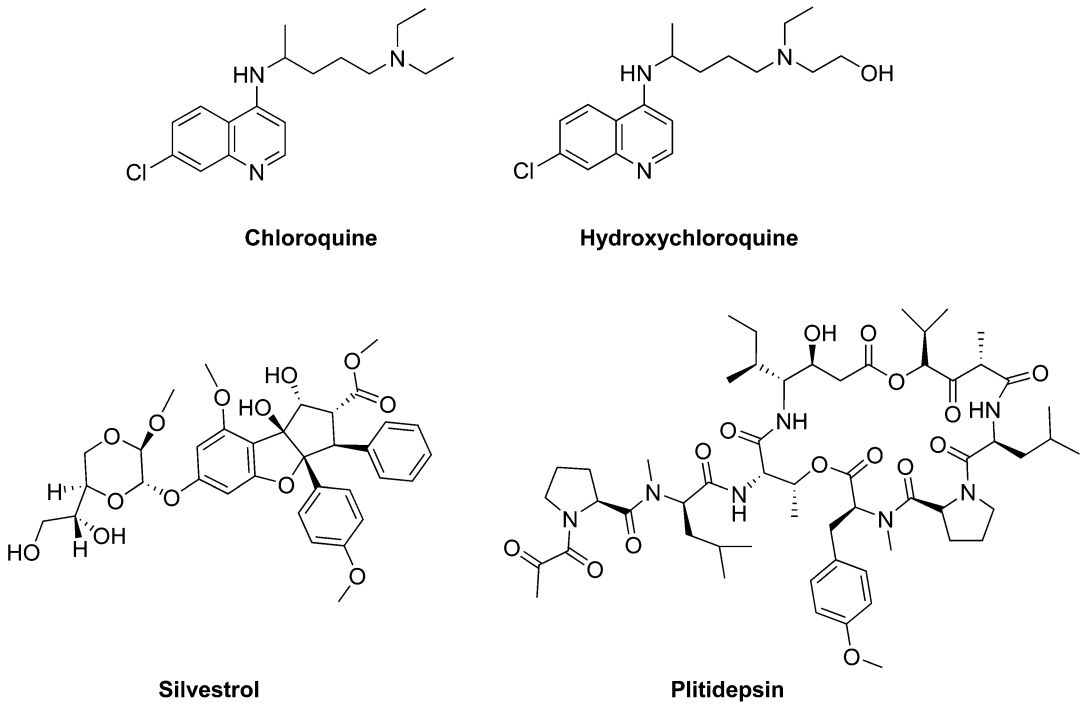


Fig. 9.13 Chemical structures of compounds interfering with endosomal acidification and translation initiation

cells with EC_{50} values of 1.3 and 3 nM, respectively, while keeping its CC_{50} values above 10 μM [167].

Early March 2020, the pharmaceutical company PharmaMar (Spain) announced that plitidepsin (also known as dehydrodidemnin B and marketed under the trade name Aplidin (Fig. 9.13)) was strongly active against SARS-CoV-2 in cell culture assays. Aplidin is a hematological cancer drug already approved in Australia and seeking approval in New Zealand and South Korea. Plitidepsin exerts its antitumoral effect by targeting the translation elongation factor eEF1A2 [168]. In the transmissible gastroenteritis coronavirus (TGEV), the nucleocapsid protein N interacts with eEF1A, and this interaction is important to support virus replication [169]. Knocking down eEF1A impairs TGEV replication in host cells. A proof-of-concept study to evaluate the safety profile of plitidepsin in patients with COVID-19 is currently recruiting patients (NCT04382066).

9.8.6 Comprehensive Interaction Maps Between SARS-CoV-2 and Host Proteins

Targeting host-virus interactions is expected to be an effective approach due to the high barrier to the development of resistance. Since the emergence of SARS-CoV-2 and the spread of COVID-19, there have been many large-scale attempts to identify effective inhibitors of viral functions. In one of those efforts, researchers cloned, tagged, and expressed 26 out of the 29 SARS-CoV-2 proteins in human cells and identified proteins that interacted with each of the viral proteins, using affinity-purification mass spectrometry [110]. They identified 332 high-confidence protein-protein interactions between SARS-CoV-2 and human proteins, including 66 druggable targets.

Pharmacological agents derived from this study and displaying antiviral activity included inhibitors of mRNA translation (zotatifin,

ternatin-4, and PS3061) and ligands of sigma-1 and sigma-2 receptors (haloperidol, PB-28, PD-144418, hydroxychloroquine, clemastine, cloperastine, progesterone, and siramesine) (Fig. 9.14). Sigma receptors have been considered as a type of opioid receptor, but pharmacological testing and functional analysis suggest that their function might be unrelated to them [170].

In cell culture assays, PB28 and zotatifin (Fig. 9.14) were the most potent inhibitors of virus replication with IC_{90} values of 0.278 and 0.037 μM, respectively [110]. PB28 was around 20 times more potent than hydroxychloroquine (IC_{90} = 5.78 μM) in those assays. Zotatifin is an eIF4A inhibitor, currently in phase 1 clinical trials for the treatment of cancer. During translation, eukaryotic initiation factor 4A (eIF4A) associates with eIF4H, a protein that interacts with SARS-CoV-2 nsp9. Interestingly, the eEF1A inhibitor ternatin-4 [171] also showed potent antiviral effects. Taken together, these studies as well as those discussed in the previous section suggest that the rate of translation elongation is critical to obtain appropriate levels of viral proteins necessary to sustain infection. In the case of sigma receptors, cytotoxicity is an important issue, and the lack of selectivity of chloroquine and hydroxychloroquine for hERG (human ether-à-go-go-related gene) ion channels and other off-targets may be related to their adverse reactions.

9.8.7 Inhibitors of the Host Dihydroorotate Dehydrogenase

Viral replication depends on the nucleosides provided by the host cells. Therefore, enzymes involved in nucleoside biosynthesis in host cells are another potential target for the development of antiviral drugs [172]. Dihydroorotate dehydrogenase (DHODH) is a rate-limiting enzyme in the de novo pyrimidine biosynthetic pathway [173]. In addition, DHODH inhibition may interfere with RNA synthesis and modulate the immune response [174]. Taken together this evidence supports the notion of DHODH as being a suitable target for the development of antiviral drugs. Previous work has shown that DHODH inhibitors inhibit the replication of a wide variety of viruses, including Ebola virus, dengue virus, and vesicular stomatitis virus [175–177]. Among them, leflunomide and its active metabolite teriflunomide (Fig. 9.15) have been used clinically to treat autoimmune diseases [178].

Recently, Xiong et al. [179] have shown that two potent inhibitors of human DHODH, S312 and S416 (Fig. 9.15), as well as brequinar, leflunomide, and teriflunomide inhibit SARS-CoV-2 replication in Vero E6 cells. The most potent inhibition was observed with S416 (EC_{50} = 0.017 μM and SI = 10505.88), followed by brequinar (EC_{50} = 0.123 μM and SI = 1880.49) and S312 (EC_{50} = 1.56 μM and SI = 101.41). Leflunomide and teriflunomide showed EC_{50} values of 26.1 and 41.5 μM, respectively. S416 showed >40-fold greater EC_{50} and SI compared to remdesivir and chloroquine in the same assays [50]. S312 and S416 showed good pharmacokinetic profiles and broad-spectrum antiviral effects against various RNA viruses, such as Zika virus, Ebola virus, and influenza A virus (including oseltamivir-resistant strains) [179]. Leflunomide is currently being evaluated against COVID-19 in clinical trials [180].

PTC299 (Fig. 9.15) is a potent, orally bioavailable vascular endothelial growth factor (VEGF) inhibitor, targeting DHODH and causing cell growth inhibition and differentiation in leukemias. Luban et al. have shown that PTC299 is a robust, dose-dependent, and DHODH-dependent inhibitor of SARS CoV-2 replication (EC_{50} range, 2.0 to 31.6 nM) with a selectivity index of >3800 in cell-based assays [181]. PTC299 clinical trials in hospitalized individuals with COVID-19 have been recently initiated by PTC Therapeutics, based on the encouraging data of anti-SARS-CoV-2 and cytokine inhibitory activities, as well as previously established favorable pharmacokinetic and human safety profiles.

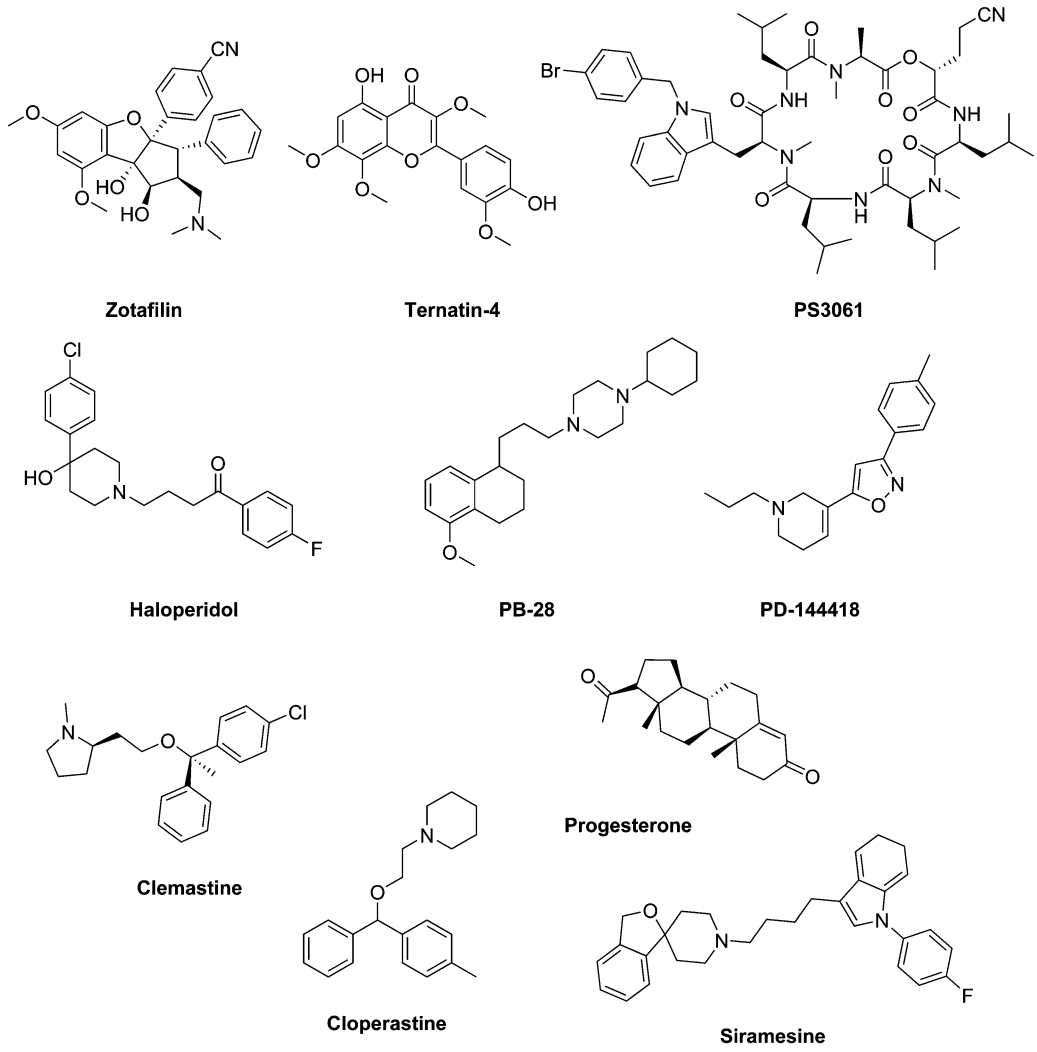


Fig. 9.14 Chemical structures of antiviral compounds interfering with host cell mechanisms, discovered through large screening analysis of pathogen-host cell interactions

9.8.8 Cyclophilin Inhibitors

Cyclophilins are cellular peptidyl-prolyl cis-trans isomerases that catalyze the isomerization of peptide bonds from *trans* to *cis* at proline residues, facilitating protein folding. Cyclophilins play a key role in the life cycle of many coronaviruses, including human coronaviruses 229E, NL-63, SARS-CoV-1, and MERS-CoV [182]. Cyclophilins bind cyclosporine A (Fig. 9.16), but unfortunately, this compound cannot be used against COVID-19 due to its strong immunosuppressive properties. Interestingly, alisporivir (Fig. 9.16) is a non-immunosuppressive cyclosporine A analogue with inhibitory activity against cyclophilins. Alisporivir was well tolerated by chronically infected HCV patients, when administered as a monotherapy (or combined with pegylated

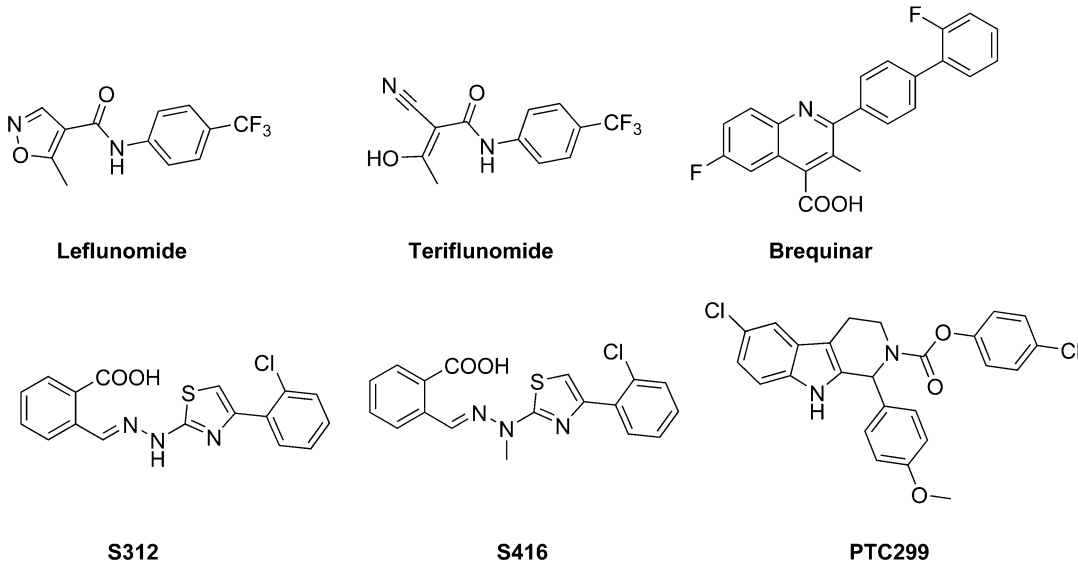


Fig. 9.15 DHODH inhibitors

interferon α and/or ribavirin) in phase 2 and 3 clinical trials [183]. In vitro, alisporivir inhibits the replication of HCoV-229E, HCoV-NL63, MHV, SARS-CoV, and MERS-CoV at low micromolar concentrations without cytotoxic effect. An EC_{50} of 0.46 ± 0.04 μM has been obtained for SARS-CoV-2 [184]. Phase 2 trials in patients with SARS-CoV-2 infection are currently underway.

9.8.9 Interferons as Therapeutic Options Against Coronavirus Infections

When host cells are infected with virus, they produce immunomodulatory molecules to combat the infection. Interferons are one of those types of molecules. There are three types of interferons: type I (α, β, ε, κ, and ω), type II (interferon γ), and type III (λ1, λ2, and λ3, also called interleukins

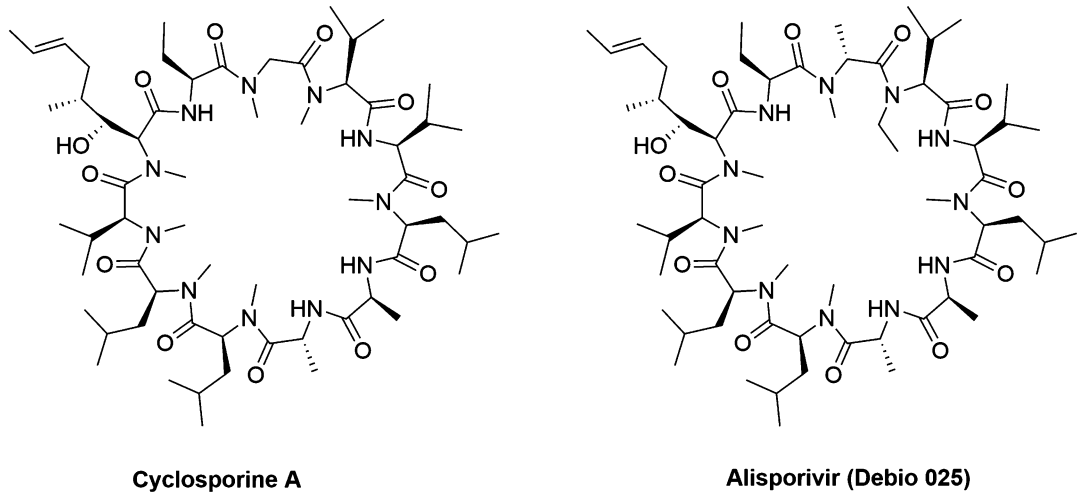


Fig. 9.16 Cyclophilin inhibitors

28α/β and 29) [185]. Interferons can activate interferon-stimulated genes which trigger the production of antiviral molecules [186]. Their mechanism of action involves interferon binding to cell surface receptors and activation of members of the Janus kinase (JAK) family, leading to changes in cell signaling pathways [187, 188]. A JAK1/JAK2 inhibitor known as baricitinib (Fig. 9.17) and approved for the treatment of moderate to severe rheumatoid arthritis has gained attention as a potential COVID-19 therapy [189]. In this context, the combination of baricitinib and remdesivir compared to remdesivir alone will be evaluated in phase 3 clinical trials (NCT04401579).

Type I and type II interferons were found to be effective against coronavirus infections, while some studies suggested that type I interferons could be more potent than interferon γ [190]. Falzarano et al. showed that the replication of HCoV-EMC/2012 could be blocked by a combination of interferon α 2b and ribavirin [191]. They found that by themselves interferon α 2b and ribavirin had EC_{50} values of 58.08 U/mL and 41.45 mg/L, respectively, when tested in HCoV-EMC/2012 replication assays using Vero RML6 cells. Additionally, virus-induced cytopathic effects in Vero cells disappeared after treatment with 62 U/mL interferon α 2b and 12 μg/mL of ribavirin. At the same time, a synergistic effect of interferon α and ribavirin has been reported in vitro for SARS-CoV-1 replicating in animal and human cell lines [192].

In any case, the role of interferons and the use of their recombinant forms in unrelated, highly pathogenic coronaviruses, SARS-CoV-1 and MERS-CoV, have been controversial in terms of their protective effects in the host [193]. Clinical studies have reported lack of interferon response in SARS-CoV-infected patients in spite of robust cytokine and chemokine productions. Nevertheless, interferon β 1a was found to inhibit SARS-CoV-2 replication in Vero E6 cells with EC_{50} values of 2–5 U/mL, a value that could be achievable in the clinical setting [194]. In addition, using primate epithelial cells (i.e., human Calu-3 and simian Vero E6), Felgenhauer et al. showed that type I interferon α and type III interferon γ were also capable of inhibiting the replication of SARS-CoV-2 [195]. In addition, the latter studies revealed that SARS-CoV-2 was more sensitive to interferon inhibition than SARS-CoV-1, opening the possibility of introducing them as preventive and therapeutic measures against COVID-19.

9.9 Antiviral Agents with Unknown Mechanisms of Action

Large-scale drug repurposing efforts allowed the discovery of novel antiviral compounds, as candidates for drug therapy against coronavirus diseases. Some of those molecules have a known mechanism of action and have been described in previous sections, but others remain without a clear target, and their role in viral infection is under investigation.

9.9.1 Nitazoxanide, Dipyridamole, Lycorine, Ivermectin, Suramin, Artemisinin, and Cenicriviroc

Several of these antiviral compounds were obtained using specific privileged structures. Nitazoxanide (Fig. 9.16) is a thiazolide derivative with broad-spectrum antiviral properties, licensed by the US FDA for the treatment of diarrhea and enteritis [196, 197]. Nitazoxanide inhibits the replication of different viruses including influenza virus, human immunodeficiency virus (HIV), and hepatitis B virus [198–200]. After oral administration, the active metabolite tizoxanide (Fig. 9.17) is generated in blood by deacetylation of nitazoxanide [201].

The replication of MERS-CoV and other coronaviruses can be blocked in vitro by nitazoxanide [202]. Wang et al. [50] have also reported that at low micromolar concentrations, this compound is also an inhibitor of SARS-CoV-2 replication (EC_{50} = 2.12 μM; CC_{50} > 35.53 μM; SI > 16.76). The proposed mechanism of action of nitazoxanide involves inhibition of the expression of the viral structural N protein.

Dipyridamole (Fig. 9.17) is an effective anticoagulant therapy with favorable and broad

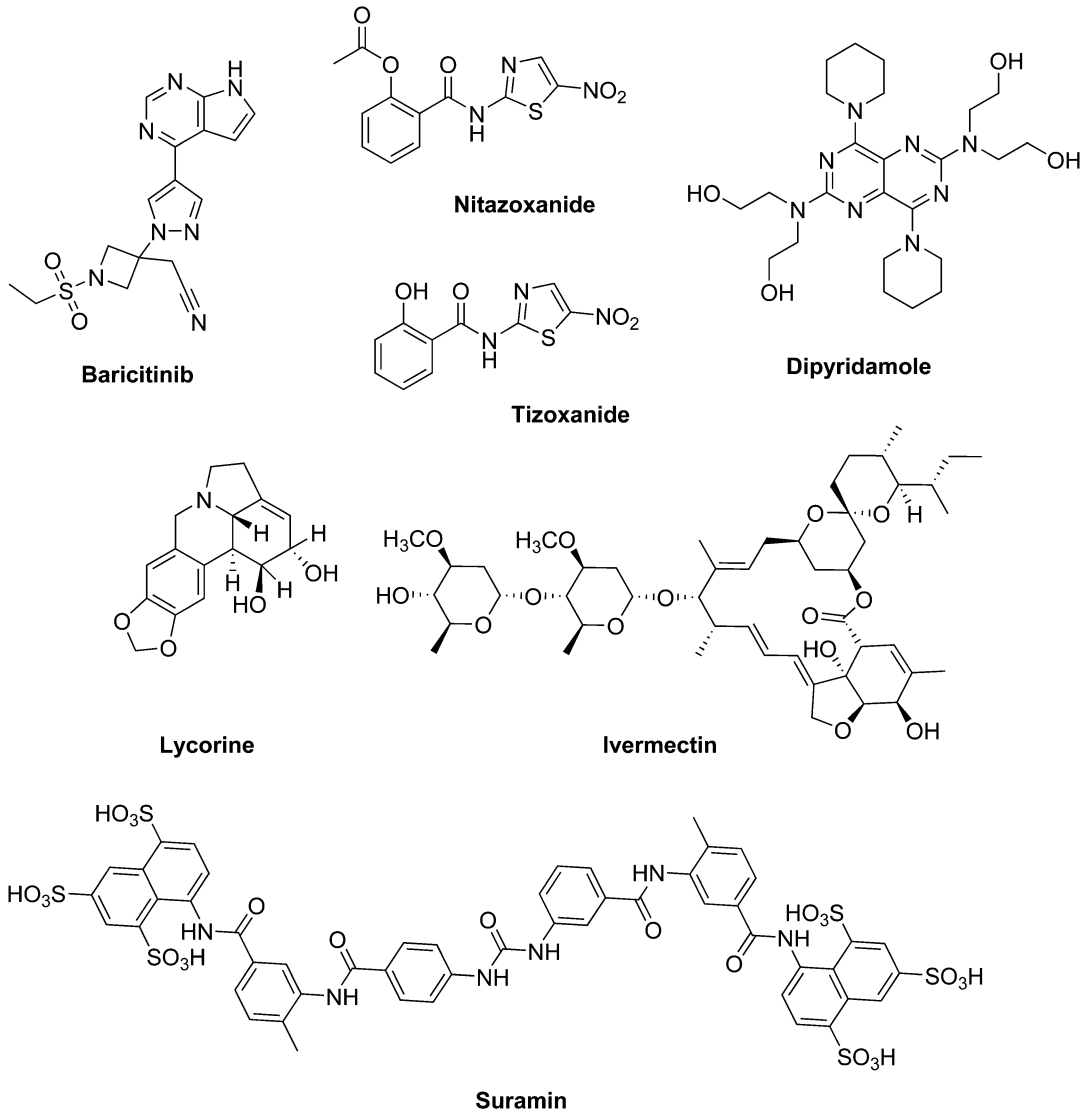


Fig. 9.17 Baricitinib, nitazoxanide, tizoxanide, dipyridamole, lycorine, ivermectin, and suramin

pharmacological properties [203]. Dipyridamole inhibits vasodilatation and platelet aggregation through different but complementary mechanisms, inhibiting phosphodiesterase and blocking nucleoside uptake [204]. Its mechanism of action may contribute to antiviral activity because nucleosides from host cells are essential for viral genome replication. It has been reported that dipyridamole inhibits the reactivation of herpesviruses [203].

Using virtual screening methods and a library of approved drugs, Liu et al. [205] identified dipyridamole as a lead compound, finding that this molecule inhibited the replication of SARS-CoV-2 with an EC_{50} of 100 nM in Vero E6 cells. In addition, they pointed out that patients who were infected with SARS-CoV-2 could benefit from dipyridamole through the mechanism of reducing viral replication, suppressing hypercoagulability, and enhancing immune recovery.

Natural products have played important roles in the process of drug development because of their unique structures. Lycorine (Fig. 9.17) is a natural product isolated from *Lycoris radiata* that shows many biological activities, including its ability to block viral infection. It has been reported that lycorine can inhibit the replication of dengue virus, HCV, coronaviruses, enterovirus 71, and avian influenza virus [206–209]. Lycorine was also found to be a potent inhibitor of the replication of SARS-CoV-1 in Vero E6 cells (EC_{50} = 15.7 nM, CC_{50} = 14.98 μM, SI = 954) [210], as well as other HCoVs, such as HCoV-OC43 (EC_{50} = 0.15 μM, CC_{50} = 4.37 μM), HCoV-NL63 (EC_{50} = 0.47 μM, CC_{50} = 3.81 μM), and MERS-CoV (EC_{50} = 1.63 μM, CC_{50} = 3.14 μM) [211]. As expected lycorine is also effective against SARS-CoV-2 (EC_{50} = 0.31 μM, CC_{50} > 40 μM, SI > 129) [212]. The mechanism of action of lycorine is not clear although it seems to inhibit protein synthesis while exhibiting cytostatic effects by targeting the actin cytoskeleton, affecting cell division.

Ivermectin (Fig. 9.17) is used for the prevention, treatment, and control of river blindness (onchocerciasis). The drug showed antiviral effects against positive-sense single-strand RNA viruses, including SARS-CoV-2, with an EC_{50} of around 2 μM in Vero/hSLAM cells [213]. However, despite being a repurposed drug, the inhibitory doses used in cell culture are relatively high. This would mean that the amount required in humans would be very large, and therefore, ivermectin does not look promising as an effective treatment for COVID-19 [214]. Ivermectin is a host-targeting agent that impairs nuclear import by interacting with the importin α/β1 heterodimer.

Suramin is a symmetric molecule containing six aromatic systems (four benzene rings and a pair of naphthalene moieties) (Fig. 9.17). Used as a medication to treat African sleeping sickness, recent work has shown that it inhibits SARS-CoV-2 replication in cell culture with an EC_{50} around 20 μM (SI > 55) [215]. This value is well below the maximum attainable level in human serum. The mechanism of action of suramin is not clear, but interferes with early steps of the viral replication cycle. The authors suggest that it may attached to the viral surface impeding its entry, but studies with parasites suggest that suramin may be internalized in cells and interact with host proteins. It is possible that glycosaminoglycans found in the spike glycoprotein of SARS-CoV-2 help in the attachment of the virus to the host cell surface as found in many other viruses and in this context heparin and suramin could be effective inhibitors of infection [216]. Still, this idea needs to be supported by solid evidence. Apart from suramin, artemisinins were also characterized as molecules acting after viral entry but in the early steps of the SARS-CoV-2 replication cycle [217]. Representative molecules of this class include arteannuin B, artesunate, and dihydroartemisinin, with EC_{50} values of 10.3 to 13.3 μM.

Interestingly, in addition to HIV protease inhibitors whose clinical efficacy was not demonstrated despite initial promise, a recent report has shown that cenicriviroc, a small-molecule chemokine receptor antagonist, active against HIV-1 in vitro, was found to have an inhibitory effect on SARS-CoV-2 replication, with EC_{50} values in the micromolar range [218]. Cenicriviroc has not been approved for clinical use against HIV. However, the approved chemokine receptor antagonist maraviroc seems to be inactive against SARS-CoV-2. The mechanism of action of cenicriviroc remains to be elucidated in the context of the coronavirus infection.

9.9.2 Drugs Derived from Large-Scale Compound Repurposing Screening

The emergence and rapid spread of SARS-CoV-2 around the world fueled a desperate search for medicines valuable to treat COVID-19. An obvious and straightforward approach has been focused on drug repurposing, mainly by screening compound libraries in viral replication assays. Among the most solicited libraries are those that contain drugs already approved for clinical use (e.g., FDA-approved drugs). Selected compounds

could enter advanced clinical trials, since safety issues were already considered at the time of their approval. Otherwise, new drug development could take more than 10 years [219].

Most of drug repurposing efforts used chloroquine, lopinavir (Fig. 9.18), and remdesivir as reference drugs with relatively low EC_{50} values (around 10 μM or less). Although effective inhibitors of SARS-CoV-2 replication in vitro, only remdesivir has gained approval for clinical use. Lopinavir and chloroquine failed to show any benefit in clinical trials against COVID-19 (for a review, see [180]). Lopinavir is an HIV protease inhibitor (reviewed in [220, 221]), and its target and mechanism of action in coronaviruses are unknown particularly considering that HCoV proteases belong to different families and show different active sites when compared to retroviral proteases. Despite these differences, it is still possible that HIV protease inhibitors retain some activity against SARS-CoV-2 M^{pro} and this could justify the effective inhibition of HCoV replication, also observed with nelfinavir [222] and atazanavir [223]. SARS-CoV-2 is susceptible to atazanavir in several cell lines, with EC_{50} values in the submicromolar range.

In a large screen of around 3000 FDA-approved and investigational new drugs carried out in South Korea, researchers found 24 drugs with EC_{50} values between 0.1 and 10 μM [74]. Two of them showed remarkable properties. Niclosamide (EC_{50} = 0.28 μM) was found to be a broad-spectrum antiviral, also effective against SARS-CoV-1 and MERS-CoV [224–226]. Niclosamide (Fig. 9.18) is an inhibitor of the S-phase kinase-associated protein 2 (SKP2), involved in autophagy by controlling the levels of beclin1 through ubiquitination [224]. The second drug (ciclesonide) which was less potent (EC_{50} = 4.33 μM) is an inhaled corticosteroid used to treat asthma and allergic rhinitis [227]. The effective concentration EC_{90} of ciclesonide (Fig. 9.18) for SARS-CoV-2 was 0.55 μM in differentiated bronchial tracheal epithelial cells [228]. Further studies showed that ciclesonide treatment of SARS-CoV-2 cultures selected drug-resistant variants with mutations in the nsp3 or nsp4 coding sequences [228]. Although the successful treatment of COVID-19 with ciclesonide has been reported [229], clinical trials are in progress to determine the potential value of the drug [180].

In another screening effort carried out in France with the Prestwick Chemical library, the authors found 15 hits with EC_{50} values of less than 50 μM and low cytotoxicity, in SARS-CoV-2 viral replication assays [76]. In this analysis, the most potent compounds were azithromycin (EC_{50} = 2.12 μM), spiperone (EC_{50} = 2.49 μM), and hydroxychloroquine (EC_{50} = 4.17 μM), all of them with SI values above 10. In the same assays, remdesivir showed an EC_{50} of 1.67 μM. Azithromycin, a macrolide antibiotic (Fig. 9.18), has been widely used in the clinical setting and evaluated in combination with hydroxychloroquine in clinical trials, so far with unconclusive evidence due to methodological shortcomings (for a review, see [180, 230, 231]). Spiperone is a D2 dopaminergic antagonist (Fig. 9.18), already identified as an antiviral molecule against polyomaviruses [232]. The mechanism of action in these viruses is not known, but inhibition apparently occurs early during infection but after viral entry.

Finally, the largest campaign reported so far involved the screening of approximately 12000 clinical-stage or FDA-approved small molecules [75]. The results of this work led to the identification of 100 molecules, including 21 known drugs that exhibit dose-response relationships. The EC_{50} values of 13 of them were compatible with achievable therapeutic doses. Apilimod (STA-5326) (Fig. 9.18), an inhibitor of the main endosomal phosphatidylinositol-3-phosphate/phosphatidylinositol 5-kinase enzyme PIKfyve and a drug initially identified as an inhibitor of the production of interleukins IL-12 and IL-23, was one of them (for a review, see [233]). Apilimod was developed for the treatment of cancer and autoimmune conditions. The inhibition of viral entry by apilimod and vacuolin-1 (another PIKfyve inhibitor) has been demonstrated for SARS-CoV-2 and Zaire ebolavirus [234]. Both inhibitory compounds cause distension of Rab5 and Rab7 subcompartments into small vacuoles. Other

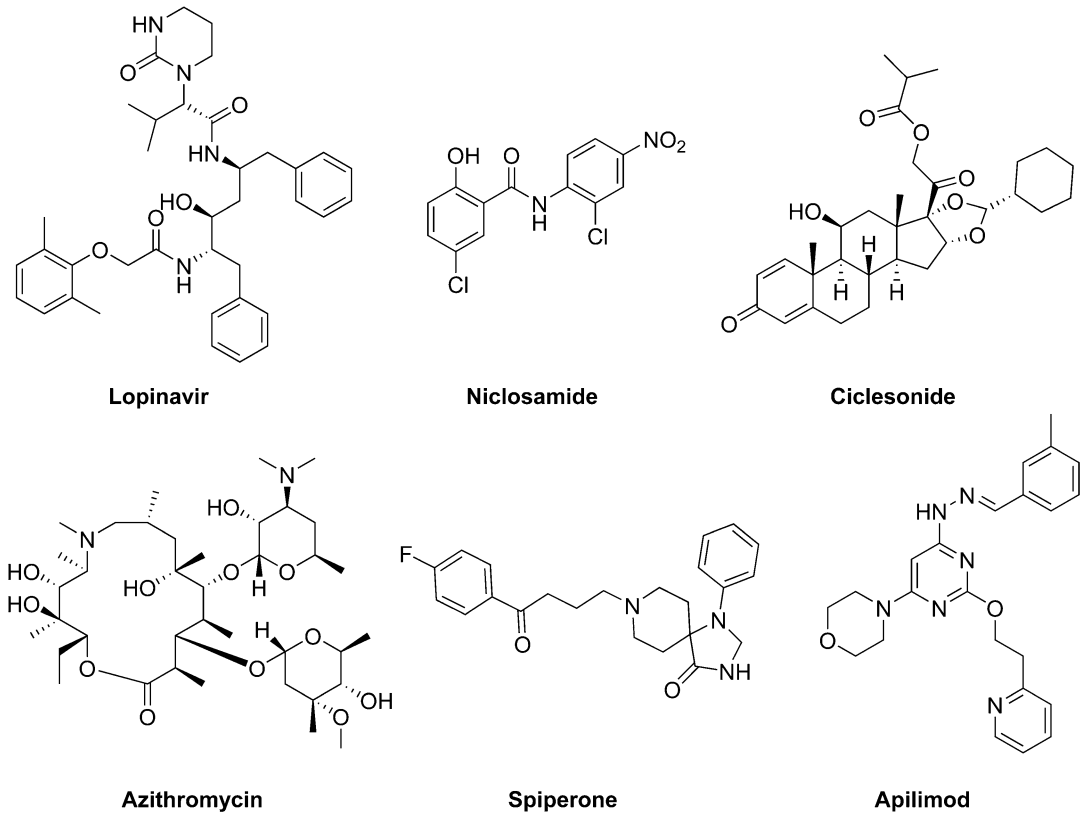


Fig. 9.18 Miscellaneous antiviral compounds derived from large-scale compound repurposing screening efforts

selected candidates in screening campaigns were cysteine protease inhibitors MDL-28170, Z LVG CHN2, VBY-825, and ONO 5334 [75]. Apilimod and other cellular kinase inhibitors have shown inhibitory potential in vitro against pandemic human coronaviruses (Table 9.1) and are considered attractive repurposed drugs for combating COVID-19 (for a review, see [235]).

9.10 Conclusions, Perspectives, and Future Developments in the Design and Development of Antiviral Drugs Against Coronaviruses

During the last two decades, outbreaks of SARS-CoV-1 in 2002–2003, MERS-CoV since 2012, and SARS-CoV-2 since 2019 constitute a major threat to public health. Unfortunately, despite improvements in the treatment of infected patients, there is lack of effective drugs, and mankind is anxiously waiting for vaccines to prevent coronavirus diseases, especially COVID-19. The approval of remdesivir has been a short step forward since the drug helps to shorten the hospitalization time when COVID-19 is not life-threatening. Other drugs (not considered antiviral compounds) such as dexamethasone decrease the mortality of the disease by combating the consequences of a disseminated viral infection that involves massive endothelial damage leading to cytokine storms and other systemic malfunctions.

In this situation, effective drugs preventing the hospitalization of infected individuals are urgently needed. In principle, these drugs should target viral components or interfere with host cell mechanisms involved in the viral replicative cycle. In only a few months, there has been an

Table 9.1 Repurposed cellular kinase inhibitors as potential therapies against COVID-19 [235]

Drug name	Type	EC_{50}[a]	Target for antiviral activity
Apilimod (STA-5326)	Investigational	<0.08 μM	PIKfyve
Baricitinib	Anticancer drug	NR	JAK1/JAK2 kinases
Dasatinib	Anticancer drug	2.1 μM	Src and Abl kinases
Dinaciclib (SCH-727965)	Investigational	0.127 μM	Cyclin-dependent kinase
Gilteritinib	Anticancer drug	0.807 μM	AXL kinase
Imatinib (STI-571)	Anticancer drug	9.8 μM	Abl kinase
Nilotinib (AMN107)	Anticancer drug	μM range	Abl kinase
Ralimetinib (LY2228820)	Investigational	0.873 μM	p38 MAPK
Saracatinib	Investigational	2.9 μM	Src and Abl kinases
Selumetinib (AZD6244)	Anticancer drug	NR	MEK1/ERK1/1/2 inhibition
Trametinib (GSK1120212)	Anticancer drug	NR	MEK1/2 inhibition

[a]Reported EC_{50} values were obtained in Vero E6 cells with SARS-CoV-2, except for saracatinib whose EC_{50} was determined for MERS-CoV. NR, not reported. Baricitinib is an oral JAK1/JAK2 inhibitor that was predicted, using artificial intelligence algorithms, to be useful for COVID-19 infection via proposed anti-cytokine effects (see Sect. 9.8.9) [189]

impressive progress toward the identification of potential targets of antiviral intervention, and some promising compounds have been identified. Nevertheless, candidates have to be evaluated in clinical trials before approval, and this is not an easy and quick task. It takes a lot of time and is expensive. Ideally, pharmaceuticals should be effective, affordable, and amenable to large-scale production, particularly considering the worldwide impact of COVID-19 and perhaps other coronavirus pandemics emerging in decades ahead.

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Peptide-Based Antiviral Drugs

10

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Abstract

Three types of chemical entities, namely, small organic molecules (organics), peptides, and biologics, are mainly used as drug candidates for the treatment of various diseases. Even though the peptide drugs are known since 1920 in association with the clinical use of insulin, only a limited number of peptides are currently used for therapeutics due to various disadvantages associated with them such as limited serum and blood stability, oral bioavailability, and permeability. Since, through chemical modifications and structure tuning, many of these limitations can be overcome, peptide-based drugs are gaining attention in pharmaceutical research. As of today, there are more than 60 peptide-based drugs approved by FDA, and over 150 peptides are in the advanced clinical studies. In this book chapter, the peptide-based lead compounds and drugs available for treating various viral diseases and their advantages and disadvantages when compared to small molecules drugs are discussed.

Keywords

Antiviral peptides · Oral bioavailability · Permeability · Solid-phase peptide synthesis · DNA recombinant technology · Half-life of peptides

Abbreviations

3LPro	3 L main protease
ACE-2	Angiotensin-converting enzyme-2
ARDS	Acute respiratory distress syndrome
BKV	BK virus
CLPro	Chymotrypsin-like protease
CMV	Cytomegalovirus
CoV	Coronavirus
DCC	N,N′-Dicyclohexylcarbodiimide
FDA	Food and Drug Administration
FMDV	Foot-and-mouth disease virus
Fmoc	9-Fluorenylmethoxycarbonyl
HAdV	Human adenovirus
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
HD	Human defensin

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HIV	Human immunodeficiency virus
HPIV	Human parainfluenza virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IAV	Influenza A virus
MERS	Middle East respiratory syndrome
PIV	Parainfluenza virus
PyBOP	Benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
SPPS	Solid-phase peptide synthesis
VSV	Vesicular stomatitis virus
WHO	World Health Organization

10.1 Introduction

Many of the most dangerous infectious diseases listed by the World Health Organization (WHO) are caused by viruses [1]. Some of the deadliest viruses are Marburg, Ebola, rabies, HIV, smallpox, hantavirus, influenza, rotavirus, SARS-CoV, MERS, and SARS-CoV-2 [1]. The infections caused by them are fatal to humans and also contribute to considerable burden to the economy in developed and developing countries. It is worth mentioning that the USA spends 15% of the healthcare-associated budget for treating infectious diseases. For some of the infections including the recently emerged COVID-19 caused by SARS-CoV-2, there is no exclusive medicine available for the treatment. In addition, certain viruses such as influenza virus have the ability to mutate at a faster rate, and the medicines discovered tend to become inactive [2]. In particular, the genes expressing neuraminidases and hemagglutinin are reported to undergo various mutations, and there were many variants of influenza viruses that exist, namely, H5N1, H5N2, H5N6, and H5N8. The hemagglutinin itself is known to exist in three subtypes, namely, H1, H2, and H3, while the neuraminidase is reported to exist in two phylogenic groups and nine subtypes. The first group includes N1, N4, N5, and N8 subtypes, and the second group includes N2, N3, N6, N7, and N9 subtypes [3–5]. Similarly, HIV gene expressing reverse transcriptase is also reported to exist in numerous variants such as K103N/Y181C, V106A/F227L, L100I, K103N, Y181C, Y188L, and E138K [6]. In these variants, either one or two amino acid residues in the catalytic site of the viral targets are mutated. Due to these mutations, the potency of the drugs is reduced drastically, and this contributes to the drug resistance of the viruses.

Currently, the multidrug-resistant microorganisms referred to as "superbugs" are the major threat to human healthcare. Their mutation rate is alarming, making the traditional drug discovery approaches incompetent. It is necessary to develop fast high-throughput screening techniques to handle such superbugs in the future. Otherwise, there is a higher chance of casualties due to viral infections as we have witnessed during World War II which can emerge again. When it comes to viruses, they have unique features that they cannot reproduce on their own and rely on the host cells for replication. The infection occurs due to the interaction between certain surface proteins in the virus and specific mammalian cell receptors [7–10]. The binding leads to the delivery of the viral genetic material within the mammalian cell which has the machinery to replicate and transcript the proteins necessary to build many copies of viruses. The genomics data of the virus provide sufficient information about the vital targets of the virus, and one can use this for developing potential antiviral drugs. The antiviral drugs can be either small molecule- or peptide-based. So far, pharmaceutical companies have mostly focused on the small molecule-based antiviral drugs due to the advantages listed in Table 10.1. However, now scientists have developed approaches to deal with many of the peptide-associated demerits, and so the development of peptide-based drugs is becoming the center of attraction in pharma companies [11, 12]. Easier and highly economical protocols to synthesize peptides in an industrial scale also add to this approach. In this book chapter, we discuss various peptide-based drugs for treating the viral infections. We provide an overview on the general aspects of peptide-based

Table 10.1 Advantages and disadvantages of small molecule drugs over peptide-based drugs

Properties	Small molecule drugs	Peptide-based drugs
Stability	Very stable	Can be digested by the stomach enzymes
Permeability	Permeable	Usually have limited permeability
Oral bioavailability	Good oral bioavailability	Poor oral bioavailability
Synthesizability	Easy to synthesize	Inefficient and expensive synthesis
Cost	Low cost to produce the drugs in bulk	Usually high cost for the bulk production
Efficacy and safety	Moderate	Very good
Prone to hydrolysis and aggregation	Mostly no	Yes and number of peptides are known to form aggregates

drugs and peptide synthesis and then discuss antiviral peptides.

10.2 Organics, Peptides, and Biologics as Drugs

Currently, available drugs can be categorized largely as small molecules (referred to as organics), complex and large-sized biomolecules (referred to as biologics), and the intermediate-sized peptides. Small molecules are easy to synthesize and have optimal pharmacokinetic and pharmacodynamic properties. However, they may bind to more than one specific target in human subjects and so have limited binding affinity and binding specificity. In contrast, the biological molecules have superior binding specificity but very poor pharmacodynamic and pharmacokinetic properties. They are also difficult to synthesize. Peptides, which are intermediate-sized biological molecules, are relatively easier to synthesize (when compared to biologics) and have favorable binding affinity and binding specificity. The disadvantage of peptide drugs is that they can be easily digested by the enzymes in saliva, in the stomach, and in serum (in particular peptidases in the gastrointestinal tract). The half-life time of most of the naturally occurring and synthetic peptides in serum is in the order of a few minutes [13, 14]. Also they suffer from poor oral bioavailability and permeability which have been overcome by the development of cyclic peptides or through the utilization of stable D-amino acid isoforms [14] or by suitably connecting to chemical tags [1] or chemical modification involving stapling [15–17], glycosylation [18], and PEGylation [19, 20]. Currently, only 2% of the drug market share deals with peptide-based drugs, while the share is almost 80% for the small molecule-based drugs. Given that the peptide-based drugs have superior binding affinity and binding specificity and reduced side effects, in the future, the pharmaceutical industries will focus more into developing peptide-based drugs.

10.3 Approaches for Improving Drug-Like Properties and Stability of Peptides

Modification of natural peptide backbone and side chains would presumably increase the stability of the peptides in the presence of peptidases and might significantly improve the drug-like properties. Over many years, several significant modifications are introduced in the peptide backbone [21–23] as illustrated in Fig. 10.1. Here we briefly discuss a few selected modifications.

10.3.1 Amino Acid Substitution Method

In this method, the substitution of the naturally occurring L-configured amino acid with the D-configuration, known as a D-amino acid, is carried out. Because peptides are recognized as being degraded by enzymes within a cell or the body, the incorporation of a D-amino acid might increase enzymatic resistance and increase

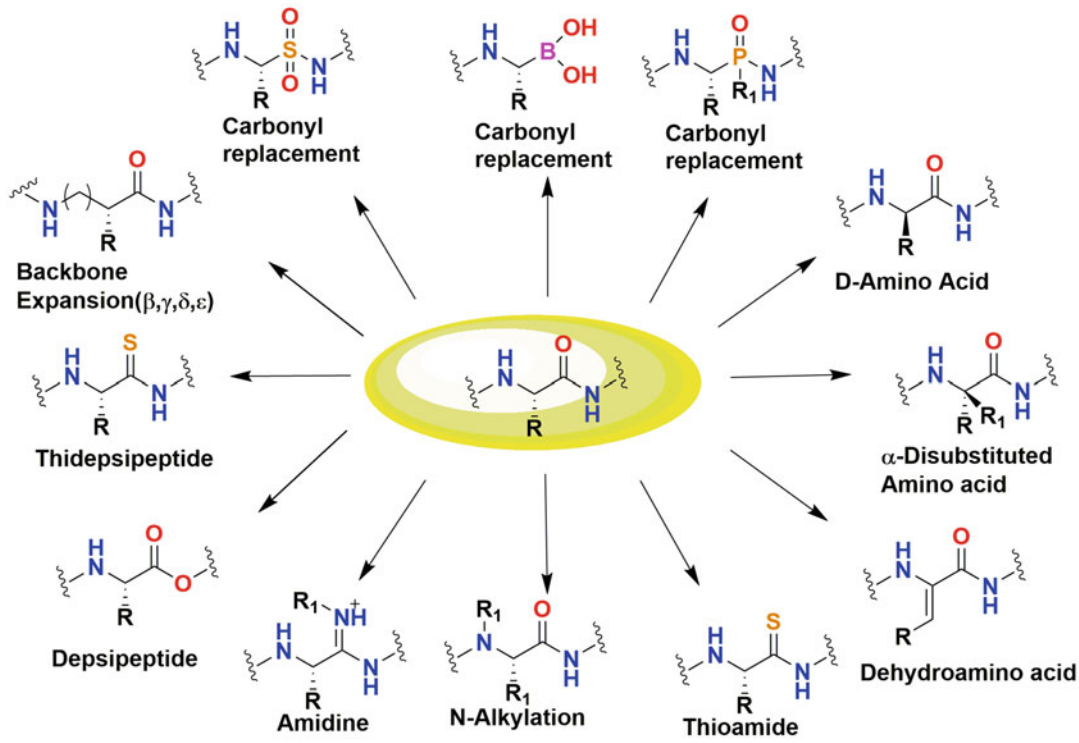

Fig. 10.1 Improving drug-like properties of peptides through various chemical modifications in peptide backbone and side chains

peptide stability [14, 24]. Additionally, it has been found in several peptide hormones that the incorporation of D-amino acids can increase peptide potency. In certain cases, the D-analogs had the same efficacy as the natural counterparts but had improved half-life periods in the gut and plasma and within cells [14]. Also this method deals with substitution of alanine at desired amino acid in the peptide. The change in structural conformation influences the peptide affinity toward the receptor.

10.3.2 Amino Acid Cyclization

In order to restrict the conformational freedom of peptide structures, a new approach called amino acid cyclization is followed. There are three different cyclization approaches, (1) side chain to side chain, (2) backbone to backbone, and (3) side chain to backbone approaches. Among the three cyclization processes, backbone cyclization approach has more potential in selectivity and affinity toward the receptor target. In backbone cyclization, the conformation freedom has been locked without changing the side chain pharmacophore. The well-known cyclization approaches are amide and disulfide bond formation. The former one is not limited to any residues, but the latter one requires at least two cysteine residues in the peptide. Further, as it is shown in the case of stapled E1P47 peptides which are HIV-1 entry inhibitors, the macrocyclization can be achieved through lactamization between the amine and carboxyl side chains of Lys and Asp or Glu amino acids, respectively [16]. In recent years, many sophisticated catalyzed macrocyclization methods have been developed which are referred to as:

1. Transition metal-catalyzed peptide macrocyclization.
2. Peptide macrocyclization by reversible iminoboronate linkages.
3. By imine formation.
4. Friedel-Crafts alkylation.
5. Ring-closing metathesis.
6. By oxadiazole formation [25].

10.3.3 Amide Bond Modification

The peptide bond normally possesses *trans* conformation, which are prone to biodegradation. Hence, the *trans* conformation of the peptide could reduce the ability to reach the site of action or affinity toward the receptor target. In this method, the modification of peptide bond through covalently and conformationally could lead to better stability and affinity toward the receptor target.

10.3.4 Peptidomimetic Method

In this method, the structural variations in natural amino acids are introduced through chemical reactions such as N-alkylation, C^{α}-substitution, cyclization, backbone extension, N-replacement, C^{α}-replacement, heterocyclic generation, and carbonyl replacement [26]. The peptidomimetics were shown to have improved stability against proteolysis and bioavailability and retained their bioactivities.

10.3.5 Stapling, PEGylation, and Glycosylation

In this approach, the natural peptide is modified with a stapling agent or covalently connected to functional units such as polyethylene glycol (PEG) [19, 20] or glycans with terminal sialic acid [27]. These modifications not only increased the stability but in certain occasions also increased the efficacy of the peptides. For example, the stapled analogs of L-E1P47 peptide, an HIV-1 fusion inhibitor peptide, showed greater inhibitor potency and strengthened proteolytic stability than the parent sequence [16]. Another m-xylene thioether-stapled peptide, hCS6ERE, showed inhibition to HIV-1 entry and replication comparable to that of T20, a 36mer peptide which is clinically approved for HIV treatment [16]. A hydrocarbon-stapled peptide referred to as P21S10 had improved potency for inhibiting the infection due to MERS-CoV and intervened with spike protein-mediated cell-cell fusion [17]. Further, the hydrocarbon stapling also improved the pharmacokinetic properties of P21S10 [17]. Moreover, a sialic acid-introduced HIV peptide drug, enfuvirtide (SL-ENF), showed 15 times increased half-life in rats when compared to natural counterpart [18]. It also retained the potency similar to natural counterpart, which suggests the chemical modification does not alter its inhibition capacity.

10.4 Chemical Space and Conformational Space of Peptides

Astronomical numbers of small molecules were proposed to exist based on the graph theory and by further applying the constraints related to valency of different types of atoms. It has been proposed that there are 10^{80} compounds having the atomic mass below 500 Da [28]. This gives greater opportunity to design active compounds that are specific to a biomolecular target. However, it is a challenge to screen the compounds from chemical space either computationally or using experimental high-throughput screening approaches. However, one can use various filters such as rule-of-five, lipophilicity, and molecular weight and then reduce the chemical space to an affordable size to facilitate the screening. One can also use the synthesizability as an additional parameter to identify drug-like compounds. If we carefully look, even the peptides offer enormous possibilities more than the small molecules as they have the possibility to exist in different conformational states. A tripeptide can exist in 2^4 conformational states as there are two peptide

bonds and two sets of *phi* and *psi* angles associated with each of these. So with increasing numbers of residues in peptides, the conformational states grow in size. In addition, there are 20 naturally occurring amino acids, and we can synthesize approximately 400 different dipeptides and 8000 different tripeptides. So, not only the number of possible peptides from a smaller number of residues is enormous, but also their conformational states are too many which provides the possibility to adopt a specific conformational state within the target-binding site. So, there is a higher possibility that the peptide-based drugs can control not only the wild-type virus but also the mutants as they have the conformational flexibility to maximize the interaction with the target independent of the small changes in the binding site residues.

10.5 Viral Infections in Plants, Animals, and Humans

Viral infections are known to occur in plants, fish, shrimp, animals, and humans, and we will mainly focus on those reported in humans. In plants, the virus infections lead to decrease in yield and in quality of the production contributing to economic loss. The viral infections in plants are associated with various symptoms such as stunting, mosaic patterns, yellowing, leaf rolling, ring spot, necrosis, and wilting [29]. There are many viruses reported in literature that affect plants. The list is not limited to those as follows: Tobacco mosaic virus, Tomato spotted wilt virus, Tomato yellow leaf curl virus, Cucumber mosaic virus, Potato virus Y, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus, Brome mosaic virus, Potato virus X, Citrus tristeza virus, Barley yellow dwarf virus, Potato leafroll virus, and Tomato bushy stunt virus [30]. Interestingly, plant viruses are not harmful to humans and animals as they can only reproduce in living plant cells.

Viruses also affect birds, animals, and livestocks. In particular, swine, dogs, cats, pigs, sheep, cattle, and horses are vulnerable to different kinds of viral infections. Border disease, hog cholera, bovine viral diarrhea, African swine fever, feline infectious peritonitis, Visna-maedi, caprine arthritis-encephalitis, equine infectious anemia, bovine leukemia, Aleutian disease, canine parvovirus, and swine influenza are the viruses known to affect birds and animals [31]. Foot-and-mouth disease virus (FMDV) is a member of the *Aphthovirus* genus in the *Picornaviridae* family, which is known to affect the ruminants and pigs.

Now we will list a number of viruses known to affect humans [32]. They are human bocavirus, parvovirus, KI polyomavirus, Melaka virus, WU polyomavirus, astrovirus MLB1, Bundibugyo ebolavirus, human bocavirus 2, human cosaviruses A–D, human cosavirus E1, astrovirus VA1, human papillomavirus 116, klassevirus, and Lujo virus. In addition, certain viruses have jumped from animals to humans, and these viruses are classified as zoonotic. And many of the viruses causing lethal infections belong to this category: HIV-1 (from chimpanzees), HIV-2 (sooty mangabeys); severe acute respiratory syndrome virus (SARS; horseshoe bats); hepatitis B, human T-lymphotropic virus (HTLV)-1 and HTLV-2, dengue, and yellow fever (all primates); human coronavirus OC43, measles, mumps, and smallpox (all livestock); influenza A (wildfowl); and hantavirus (rodents). There are also viruses known to attack bacteria, which are known as bacteriophages.

10.6 Peptide Drugs for Treating Viral Infection

The use of peptides for treating viral infection has been in practice since the 1980s. There are about more than 400 peptide-based drugs under clinical trials [33] with more than 60 approved for clinical use [34]. However, the number of peptide drugs approved for treating viral infections is only a few (as we will see in the case of HIV virus infection). In particular, cationic antiviral peptides extracted from different sources have been used to treat the infections due to herpes simplex virus types 1 and 2 (HSV) and cytomegalovirus (CMV) as well as inhibit the vesicular stomatitis virus.

The following general mechanisms were proposed behind the antiviral activity of the peptides:

1. They bind to the targets on the mammalian host cell surface which are involved in the interaction with surface proteins of viruses.
2. They bind to the viral targets, which are responsible for host cell infection [35, 36].
3. The peptides suppress the viral gene expression or inhibit the viral enzymes involved in the replication and transcription [37].

10.6.1 HIV

The first antiretroviral drug for HIV introduced 35 years ago was a small molecule inhibitor targeting HIV-1 reverse transcriptase [38]. Later small molecule drugs targeting reverse transcriptase and proteases have become popular due to their potency against multidrug-resistant variants of HIV and were used in combination with NtRTI. The fusion inhibitors were introduced as the third class of HIV inhibitors and target an earlier life-cycle stage of the virus and can be effective in preventing and inhibiting viral infection. The identification of peptide inhibitors having sequences similar to N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) units of gp41 opened up the possibility of using peptides as antiviral drugs for treating HIV infection [39]. The peptide sequences referred to as DP107 and SJ-2176 were able to inhibit the fusion peptides at μM and nM levels, respectively. Based on this more potent peptide, DP178, which is having more than 60% sequence identity to SJ-2176, has been identified for treating viral infection. This peptide drug is commercially referred to as T20 (enfuvirtide or Fuzeon) that has been approved by FDA in 2003 as the first fusion inhibitor. The sequence for T20 is reported in the DrugBank database as YTSLIHSLIEESQNQQEKNEQELLELDK-WASLWNWF. The lower half-life period, potency, and low genetic barrier for drug resistance of T20 are major problems limiting its use for HIV treatment [40]. Many strategies were explored to improve the potency and pharmacokinetic properties of this drug. Peptide-fatty acids and cholesterol-conjugated peptides were explored as alternative options to increase the bioavailability [9]. For example, the cholesterol-conjugated peptide C34 had showed increased serum lifetime [9, 41]. Similarly human serum albumin-conjugated C34 and T20 showed increased half-life but retained their potency as the parent peptides [41]. The compounds such as *sifuvirtide* and *albuvirtide* were developed as alternative to T20 with improved drug-like properties. The former compound is made of 36 amino acids having a sequence similar to the deep pocket of gp41 protein. It had improved potency and half period (20–26 h) than enfuvirtide [42, 43]. It also showed potency against T20-resistant strains of HIV [42]. The latter compound, albuvirtide, had a sequence similar to C34 except that the 13th serine is replaced by a lysine residue [41]. Such modification allows it to be conjugated with 3-maleimimidopropionic acid and made this compound to irreversibly bind to HSA, which essentially increased the half-life to 26 h in rats and to 102 h in monkeys [41, 44].

In general, peptides derived from CHR and NHR domains of gp41 can interact with their counterparts and can interfere with 6HB formation, which is essential for fusion of HIV virus and host cell. Other sets of peptides were targeting CD4 and co-receptor binding sites of gp120, a surface glycoprotein, which is covalently bound to gp41 transmembrane glycoproteins [45, 46]. The parent peptide RINNIPWSEAMM had binding affinity for both CD4 and co-receptor binding sites of gp120, while the triazole-conjugated peptides had many orders of improved binding affinity for the same target [46].

10.6.2 Influenza Virus

The influenza virus infection can be neutralized by peptide drugs in three ways [47, 48]: (1) the

drugs can inhibit the virus attachment to host cell membranes, (2) the drugs can disrupt viral envelope, and (3) the drugs can interfere with replication process of virus by inhibiting the polymerase. The viral attachment can be prevented by the peptides through the following mechanisms. In one mechanism, the peptides bind to sialic acid binding by blocking the receptor site of HA. In particular, HA binds to sialylglycoconjugates on the host cell surface, which is an essential step for the membrane fusion [3]. The peptide-based "sialic acid mimics" inhibitors have been developed from phage library containing random pentapeptides [3]. In particular, N-stearoyl pentapeptides ($C_{17}H_{35}$ CO-ARLPR-NH_2) have shown inhibitory activity for both subtypes H1N1 and H3N2. The IC_{50} values were, respectively, 1.9 and 1.6 μM for both subtypes. The residues in the sialic acid binding site of HA are shown by the arrow and labelled as B site in Fig. 10.2.. The residues Ser136, Asn137, Glu190, Leu194, Trp222, and Leu226 are responsible for binding to the pentapeptide as shown from molecular docking study (refer to Fig. 10.3) [3]. In particular, Ser136, Asn137, and Glu190 are involved in hydrogen bonding with peptide.

Based on the peptide sequence information of HA1 and HA2 units of hemagglutinin (as in gene accession number AY818135), 59 peptides each having 18 amino acids were developed to inhibit viral entry. In particular, HA-pep25 having sequence N-SKVNGQSGRMEFFWTILK-C showed antiviral activity (with $IC_{50} = 12.0 - 51.0$ μM) against multiple variants of influenza virus [49]. It targets host cell receptor and intervenes with the process of viral fusion protein entry. In addition to the HA fusion inhibition peptides, there are other therapeutic peptides against influenza virus, and they work by disrupting viral envelope and by inhibiting viral replication. The peptides referred to as LF C-lobe peptide 1-3, mucroporin-M1, and LL-37 belong to the former category, while peptides referred to as killer peptide, HNP-1, and Peptid-6 belong to the latter category. Interestingly, all these peptides are active against different subtypes of influenza virus [50].

10.6.3 West Nile Virus

West Nile virus is a single-stranded RNA virus responsible for West Nile fever and belongs to the family *Flaviviridae*. They infect humans by using vectors such as mosquitoes or ticks. The viral RNA is translated to single polyprotein, which is cleaved by viral and host cell proteases into structural (capsid, envelope, and pre-membrane) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [51]. Among these, the envelope protein is responsible for host cell receptor interaction, membrane fusion, and virion assembly and so is a potential target for antiviral therapy. Many antiviral peptide drugs against this virus have been identified using murine brain cDNA phage display library, which target this envelope protein, responsible for host cell infection [52]. Synthetic peptides having sequences in WNV E protein had inhibitory effect, and the IC_{50} values were reported to be in the range of 10 μM. The peptides with sequences TFLVHREWFMDLNLPWSSAGSTVWR (WN53) and TFLVHREWFMDLNLPWSSA (WN83) having sequences from domain I/II junction of E protein showed a significant inhibitory effect for WNV [52].

10.6.4 SARS-CoV

There are many potential targets identified in SARS-CoV for therapeutic development: the 3L main protease, papain-like protease, RNA-dependent RNA polymerase, helicase, and spike protein. Also the angiotensin-converting enzyme-2 (ACE2) of the human cell is also a potential target as it is involved in the interaction with spike protein during the infection process. A number of peptide drugs have been developed for these targets. Based on the residues in the HR1 and HR2 of spike protein, peptide inhibitors having binding affinity in the μM and submicromolar range were developed [53]. The peptide inhibitors developed and the sequences of the peptides are provided in Table 10.2. Through flexible molecular docking, three octapeptides, ATLQANEV,

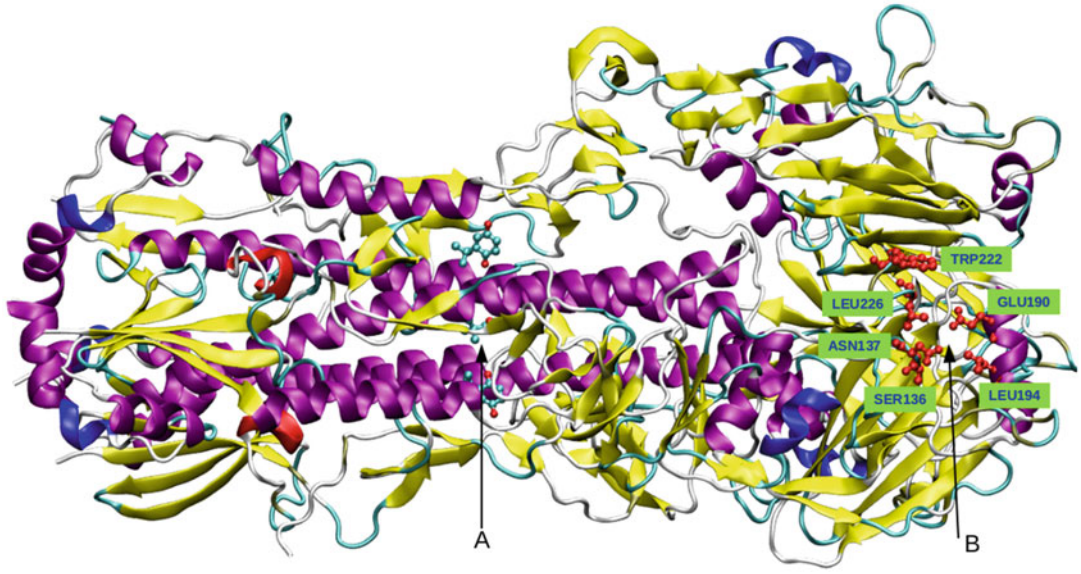

Fig. 10.2 Two characteristic binding sites (referred to as A and B) for hemagglutinin inhibitors. The molecules binding to A site intervene with the conformational change required by HA for membrane binding. The molecules binding to B site (also referred to as sialic acid binding site) intervene with the sialyl binding on the host cell surface

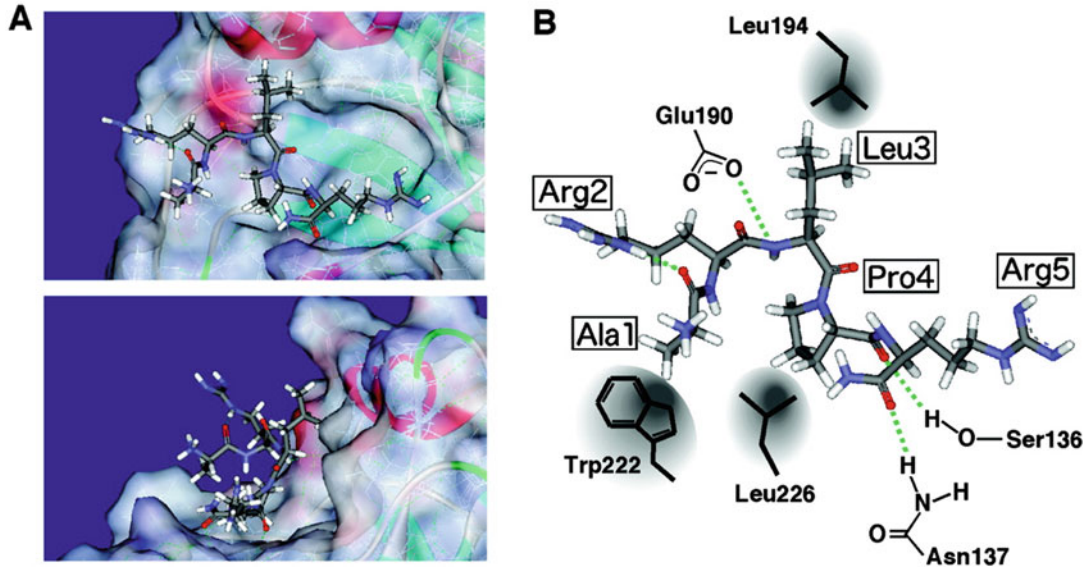

Fig. 10.3 The residues of HA interacting with pentapeptide are shown along with the binding mode within HA in the sialic acid binding site. (Taken from reference [3]. Permission to use has been generously granted by ACS)

Table 10.2 List of therapeutic peptides developed for treating COVID-19 infection. (Adapted from the reference [24]) The peptides inhibit the targets of spike protein in certain cases and ACE-2 receptor of human cells

Peptide	Target	Sequence
Ek1	HR2 domain of SARS-CoV	SLDQINVTFLDLEYEMKLEEAIKLEESYIDLKEL
HR1	HR2 domain of SARS-CoV	NGIGVTQNVLYENQKQIANQFNKAISQIQESLTTTSTA
HR18	HR2 domain of SARS-CoV	IQKEIDRLNEVAKNLNESLIDLQELGK
P6	RBD of S-protein of SARS-CoV	EEQAKTFLDKFNHEAEDLFYQSSGLGKGDFR
SARS WW-III	ACE-2 human	GYHLMSFPQAAP-HGVVFLHVTW
SARS WW-IV	ACE-2 human	GVFVFNGTSW-FITQRNFFS
RBD-11b	ACE-2 human	YKYRYL
S471–503	ACE-2 human	ALNCYWPLNDYGFYTTTGIGYQPYRWVLSFEL
SP10	ACE-2 human	STSQKSIVAYTM

AVLQSGFR, and ATLQAIAS, having inhibitory activity for 3 L main protease of SARS-CoV were identified. It is also shown that the residues His-41 and Cys-145 (referred to as catalytic dyad) are involved in the binding with the peptides [54].

10.6.5 MERS-CoV

MERS-CoV is another member of the coronavirus family, which was associated with MERS pandemic in 2014. In the case of MERS-CoV, after the ejection of RNA genome into host cell, it is translated into two polyproteins pp1a and pp1b in the cytoplasm [55]. These polyproteins are cleaved into 16 non-structural proteins with the help of 2 proteases, namely, PLpro acids (papain-like protease) and 3CL, in (3-chymotrypsin-like protease). So targeting the two main proteases is a potential therapeutic route for controlling the infection [56]. A number of peptidomimetic inhibitors having μM binding affinity for 3 L main protease have been developed [17]. Refer to the structure reported as 4RSP in pdb which shows key interactions between the 3 L main Pro and peptidomimetic compound. Further, a number of peptides which can disrupt the membrane fusion have been developed. The most effective fusion inhibitor is referred to as P21S10, which is having an EC_{50} value of 1 μM [17]. Also the synthesized stapled peptides such as P21S2, P21S4, P21S5, P21S8, P21S9, P21S8F, and P21S8ZF were also able to inhibit the MERS-CoV by serving as membrane fusion inhibitors. Based on the structure reported for fusion protein of MERS-CoV [57, 58], a peptide HR2P has been developed, which can potentially inhibit the replication and spike protein-mediated cell-cell fusion. In particular, this peptide had a sequence made of residues 1251–1286 of the HR2 domain of fusion protein. Further modification of the peptide with hydrophilic residues improved its stability, solubility, and antiviral activity [58].

10.6.6 SARS-CoV-2

SARS-CoV-2 associated with the recent pandemic infection, COVID-19, has genomic overlap with SARS-CoV to a larger extent, and so the therapeutic targets are identical for both viruses. A number of peptide-based lead compounds [59, 60] have been identified, which target 3 L main protease and spike protein. Once the viral genome is delivered into host cells, the replication and transcription are the processes responsible for virus multiplication, and proteases are the main functional enzymes involved in this. So, blocking proteases through suitable inhibitors will have therapeutic effects [11, 60, 61]. A number of peptide and peptidomimetic inhibitors have been developed for this target. In particular, many of the inhibitors are involved in the covalent bonding with CYS145 of 3 l main protease (3lpro). Many crystal structures for 3lpro and peptide/peptidomimetic inhibitors are reported in the protein database. Refer to the structures reported with PDB id 6 LU7 and 6YZ6 (oligopeptide), and Fig. 10.4 shows the 3lpro in complex with N3 inhibitor, which is covalently bound to the CYS145 residue [63]. The residues K31 and

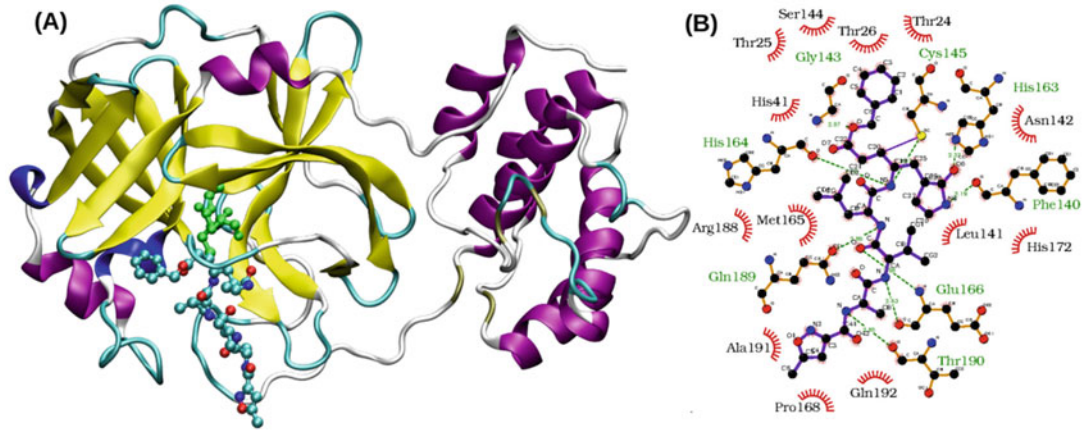

Fig. 10.4 (**a**) Binding mode of inhibitor N3 with 3 L main protease of SARS-CoV-2 as reported in the PDB (reference [62] LU7). The catalytic site residue, CY145, is shown in green color, and the inhibitor is shown. (**b**) Protein-ligand interaction diagram computed for 3 L main protease and N3 complex

K353 in the receptor binding domain of spike protein are involved in the binding to hACE-2-receptor [64]. In particular, these two residues have been referred to as hot spots in spike protein-ACE-2 receptor interaction and have been attributed to the increased contagious nature of SARS-CoV-2 when compared to SARS-CoV. Further, the affinity toward hACE-2 receptor has contributions from a number of hydrogen bonds, electrostatic interactions, and hydrophobic interactions. The residues involved in such interactions from spike protein of virus and host cell ACE-2 receptor are listed in Table 10.3. In principle, any peptides, which will weaken the interactions between the spike protein and host cell receptor, can serve as a therapeutic candidate. A number of peptides have been designed that can bind to the interfacial region of spike protein: ACE-2 complex [65]. Further, the strategy is to design peptides that are homologous to virus binding domains of ACE-2 receptor and facilitate the disruption of interaction due to hot spots in the protein-protein interface region [65]. Along this line, the peptides based on ACE-2 regions (such as 21 − 43, 27 − 38, 22 − 44, 22 − 57, and 22 − 44 − linker−351 − 357 amino acid residue ranges of ACE2) are synthesized and studied for their binding with spike protein. In particular, 23mer peptide binder referred to as SBP1 (having sequence similarity with ACE-2 alpha-helix domain) with μM binding affinity has been synthesized from fast-flow peptide synthesis [65].

10.7 Strategies for Developing Peptide Therapeutics for Viruses with Envelope Proteins

Viral infection occurs through a very general mechanism for the viruses having envelope proteins [8, 10, 66]. Certain proteins in the surface of viral envelope are involved in the interaction with receptors in mammalian cells. Depending upon the virus nature, the receptors can vary. In the case of SARS-CoV and SARS-CoV-2, the hACE-2 is the mammalian cell receptor mediating the membrane fusion of the spike protein. In the case of MERS-associated coronavirus cellular, receptor dipeptidyl peptidase-4 (DPP4) on the surface of the host cell is responsible for infection [67, 68]. So, a general strategy is to develop peptides having sequence similarity to cell receptor binding domain, which eventually can intervene with protein-protein interaction between spike protein and human cell receptor. A number of such peptides have been identified, and their activities with spike protein were

Table 10.3 Important residue-wise interactions between the spike protein of SARS-CoV-2 and human ACE-2 receptor and their nature of interactions

	SARS-CoV2 S-protein	ACE-2 receptor
Hydrogen bonding		
1	K417	D30
2	G446	D38
3	Y449	Q42
Electrostatic		
1	G502	K353
2	K417	D30
3	E484	K31
Hydrophobic		
	F486	M82
	Y489	K31
	Y505	K353

evaluated. Table 10.2 shows a list of such peptides for SARS-CoV from receptor binding domain of spike protein and ACE-2 viral binding domain. These peptides are shown to interrupt protein-protein interactions that are essential for the viral infection. Another novel approach is to exploit the viral membrane disruption properties of defensins like peptides. Since these peptides are positively charged and amphipathic (having both regions of hydrophobic and hydrophilic), they can bind to membranes and exert antiviral activities through the solubilization of lipids or by forming pores to disrupt membrane [62, 69]. This antiviral mechanism is generally known as lipid envelope antiviral disruption and has been demonstrated in the case of HCV. In particular, the peptides referred to as AH and C5A are amphipathic sequences, which were derived from N-terminal region of NS5A involved in membrane association [69, 70].

10.8 Sources of Antiviral Peptides

Antiviral peptides can be obtained from various sources available in nature or from various animals. They can also be synthesized in the laboratories and can be expressed using microorganisms. Figure 10.5 illustrates different routes to obtain antiviral peptides.

10.8.1 Peptide Synthesis

Peptides having up to 30 amino acids can be produced in bulk using chemical synthesis methods referred to as solution-phase synthesis and solid-phase synthesis [71–75]. Peptides having more number of residues are synthesized using convergent peptide production approach, where many peptide fragments are ligated to get the large-sized peptides. The chemical ligation approach [73, 76] was proposed by Kemp and colleagues in the 1970s. In this, N-terminal of one fragment is chemically ligated to the C-terminal of another fragment, and during this process, the remaining functional groups are protected from reaction suitably.

Peptides could be chemically synthesized in solution phase by attaching different amino acids through activating the carboxylic group into reactive ester group, which is an easily leaving group, while the other ends of the amino acids are protected using a protecting group such as butyloxycarbonyl (Boc) and esters. The free amino group of the amino acid acts as a nucleophile (where the C-terminal is converted to an ester) that attacks the activated C-terminal carbon (where the N-terminal is protected) and forms an amide bond also known as peptide bond (Fig. 10.6) [71]. The popular coupling reagents are DCC, HBTU, HATU, and PyBOP. Though solution-phase peptide synthesis is economic to make short hydrophobic peptides in large

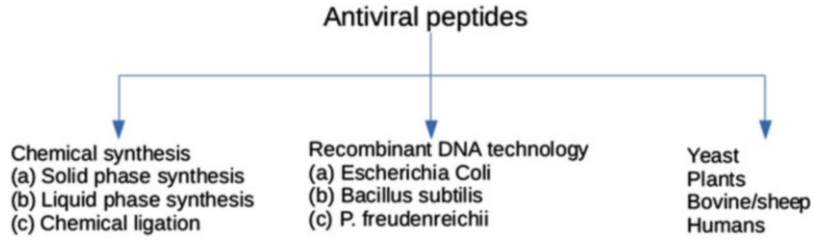

Fig. 10.5 Scheme showing various sources of antiviral peptides

quantities, long, hydrophilic, or charged peptides are not only expensive and time-consuming but also difficult to synthesize with high yields and purity [72].

To overcome the disadvantages of the solution-phase peptide synthesis, in 1969, Bruce Merrifield developed a method that revolutionized the synthesis of peptides, because it provided a much faster way to produce peptides in much higher yields and purity. Subsequent refinements in the method now allow a reasonable yield of a peptide containing 100 amino acids to be synthesized in 4 days [2]. In Merrifield's method, the N-terminal-protected amino acid was covalently attached to the solid support, then the protecting group was removed using deprotection solution, and then another N-terminal-protected amino acid was added along with other activating groups that resulted in the attachment of the amino acid to the resin [75]. So,

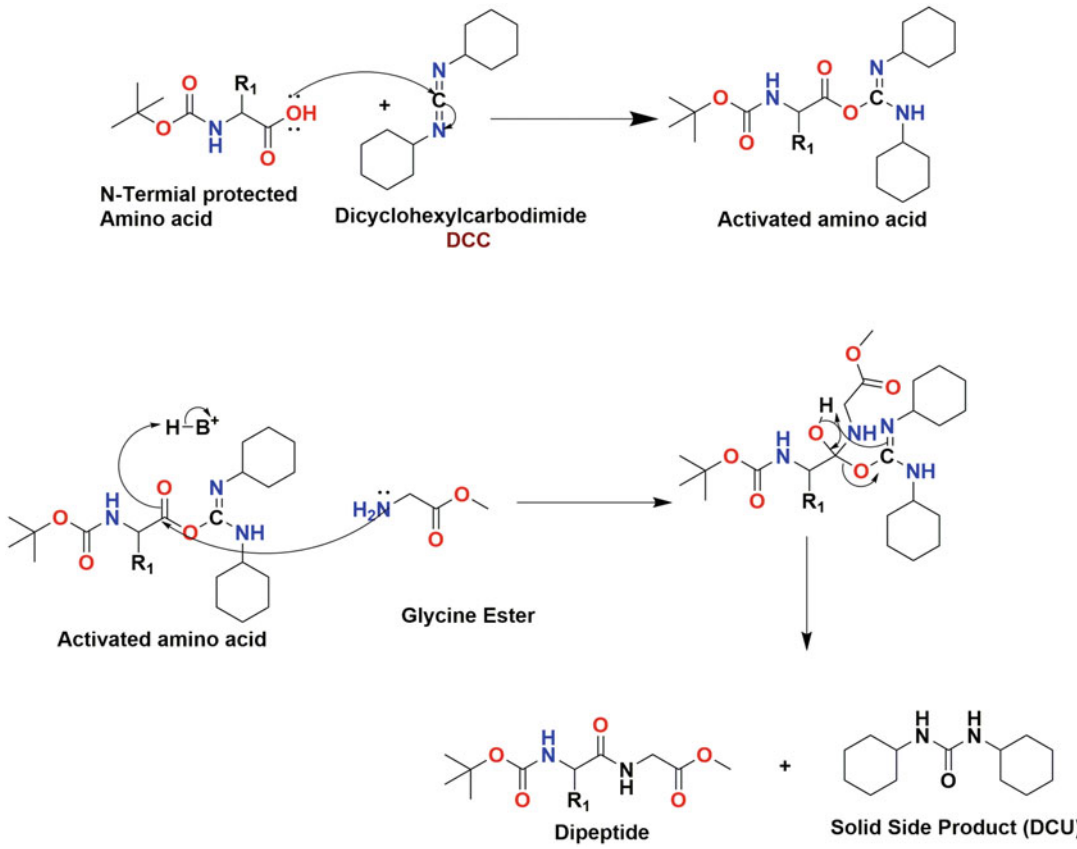

Fig. 10.6 General scheme for solution-phase peptide synthesis using "Boc" chemistry

this process of deprotection and coupling will continue till the desired peptide length is reached. Once the peptide is synthesized, it will be cleaved from the resin using cleavage cocktails. Merrifield used TFA/DCM for deprotection and DCC for coupling, and for peptide cleavage, strong acid like hydrofluoric acid was used. Benzyl derivative is used for the side chain protection. The backbone and side chain protecting Boc and Benzyl groups are acid labile. So, while deprotecting the N-terminal Boc, some of the side chain protecting group lost on every step of deprotection. Further, during deprotection, some of the peptide-resin linkages are also lost. To overcome this, the mild reaction condition for the peptide synthesis has been developed. This has been achieved by reworking the overall protecting group strategy, so that the exposure to acidic reagents is reduced. A range of basic labile protecting groups was therefore investigated for the solid-phase peptide synthesis application. In 1972, Louis A. Carpino introduced the Fmoc group which was proved as an efficient group for solid-phase peptide synthesis [77]. Fluorenylmethoxycarbonyl derivatives are cleaved by organic bases with the initial formation of non-volatile and reactive dibenzofulvene with the potential for addition or polymerization reactions. This problem is largely avoided in solid-phase synthesis, where simple washing steps eliminate soluble co-products. Solid-phase peptide synthesis (SPPS) is now widely used as a standardized method (Fig. 10.7) and automated to make it more efficient to make peptides of choice with high purity and good yields. This method also facilitates synthesis of peptidomimetics, peptides with longer lengths, and difficult sequences in a short period of time.

10.8.2 Recombinant DNA Technology

There are a number of disadvantages in using the chemical synthesis for peptide production: (1) the extremely high cost associated with synthesis and purification, (2) the solubility of many peptides, and (3) toxic side products are generated due to the use of solvents and reagents in the chemical synthesis. Recombinant DNA technology serves as an alternative approach for synthesizing antiviral peptides in a large scale. In peptides of length 20–50 residues and in peptides having complex secondary structures with multiple disulfide bonds, this approach is most suitable. In this approach, peptides are produced using an expression system like *Escherichia coli*. In general, multiple expression systems including mammalian cell lines, insects, and plants can be used. The first peptide drug produced using this approach is *insulin* (having 51 amino acids), and that was approved in 1991. Followed by that, other peptide drugs such as *calcitonin*, *ecallantide*, and *teduglutide* were also approved. A number of antiviral peptide drugs were also synthesized using recombinant DNA technology. For example, cyanovirin-N (CV-N) having an anti-HIV property was produced recombinantly using *E. coli.*

10.8.3 Other Sources

Peptides are also extracted from sources such as fungus, plants, insects, animals, and humans [78]. The antimicrobial/antiviral peptides such as LL37 (cathelicidin) [79] and HNP1 (alpha-defensin) are extracted from humans, while TAP (beta-defensin) is extracted from bovine. The AMPs such as plectasin and alamethicin are extracted from fungi while kalata B1 (cyclotide) and RTD-1 (theta-defensin) are from plants and monkeys, respectively. In particular, the defensins are cationic, amphipathic peptides having 29–42 residues folded into alpha-helix- and beta-sheet-like secondary structures [80]. The defensins exhibit their antiviral activities to multiple viruses such as respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), HSV, HIV, IAV, BKV, HPV, and HAdV [7, 35, 47, 81–88]. Interestingly, there are different antiviral mechanisms proposed for the defensins depending upon the target virus. The proposed mechanisms are (1) envelope disruption (as in RSV and HPIV), (2) blocking of receptor binding (HSV and HIV), (3) fusion inhibition (IAV and HIV), (4) blocking reverse transcription (HIV),

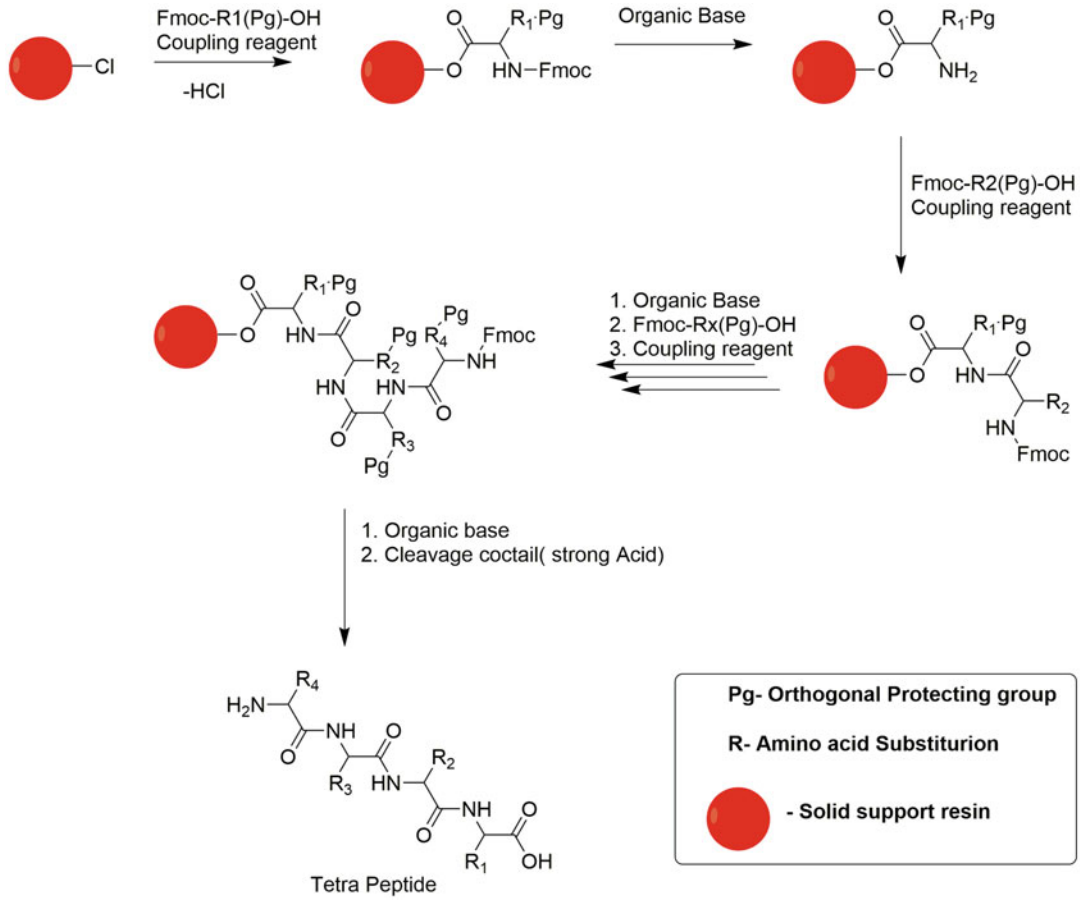

Fig. 10.7 General scheme for solid-phase peptide synthesis using "Fmoc" chemistry

(5) blocking uncoating (HAdV), (6) blocking gene expression (HSV and HIV), and by (7) preventing nuclear transport (HPV).

10.9 Challenges and Opportunities

There are many challenges to employ peptides as drugs, and we list them here.

1. Intravenous administration of drugs as the oral administration would lead to their degradation in the digestive tract.
2. Peptides have a shorter half-life in the stomach as they are quickly broken down by proteolytic enzymes.
3. Quick hepatic (liver) and renal (kidney) clearance of peptides from the blood circulation.
4. Peptides face significant physiological obstacles.
5. Peptides sometimes lead to poor selectivity, the activation of different cell structures, and adverse effects due to their unanticipated conformational flexibility [12].
6. High production cost and market price.
7. Peptide antigenicity.
8. Commercial production scale.
9. Challenges in searching and identifying novel peptides and the associated technologies [89].

The *SWOT* analysis of naturally occurring peptides, according to *Fosgerau* and *Hoffmann*, is as follows [90]:

Strengths:

(a) Good efficacy, safety, and tolerability.
(b) High selectivity and potency.

(c) Predictable metabolism.
(d) Shorter time to market.
(e) Lower attrition rates.
(f) Standard synthetic protocols.

Weaknesses:

(a) Chemically and physically unstable.
(b) Prone to hydrolysis and oxidation.
(c) Tendency for aggregation.
(d) Short half-life and fast elimination.
(e) Usually not orally available.
(f) Low membrane permeability.

Opportunities:

(a) Discovery of new peptides, including protein fragmentation.
(b) Focused libraries and optimized designed sequences.
(c) Formulation development.
(d) Alternative delivery routes besides parental.
(e) Multifunctional peptides and conjugates.

Threats:

(a) Immunogenicity.
(b) New advancements in genomics, proteomics, and personalized medicine.
(c) Significant number of patent expiries.
(d) Price and reimbursement environment.
(e) Increasing safety and efficacy requirements for novel drugs [90].

Despite their demerits, peptides have several advantages over small molecule drugs [91]. Since peptides are naturally occurring biologics, they are safe and have a greater efficacy, selectivity, and specificity. Unlike synthetic substances, peptides are degraded into their component proteinogenic amino acids without leading to metabolites. Figure 10.8 brings out potential properties of peptides and small molecules, which direct the drug efficacy [92].

10.10 Design of Antiviral Peptide Drugs

Below we will focus on experimental and computational approaches to develop various peptide drugs against different viral infections. Computational screening methods require very thorough understanding of viral infection mechanisms and the mammalian receptors involved in the infection and information about the vital targets responsible for replication and transcription. The viral genomes carry all required details for developing antiviral therapeutics. Through gene mining, one can identify vital targets of the viruses. By using the primary structure (sequence of the enzyme) and the three-dimensional structures of the homologous proteins available in protein databank, one can propose 3D structure for various viral targets. Once the structure is available, one can use structure-based approaches for developing peptides against the virus [58, 60, 67, 93–95]. The method used to propose the 3D structure for a given sequence by using a template structure is referred to as homology modeling [96], and it requires high sequence similarity between the target sequence and template sequence for proposing reliable models. A number of active tripeptides such as FRG, FRV, FHV, YRV, FRT, FRS, and FRI are designed against the neuraminidase target of influenza virus using a combined molecular docking and molecular dynamics approach [97]. Using AutoDock-based free energy calculation approach a tetrapeptide, Boc-Ile-ΔPhe-Trp-OCH3 has been identified as a potential inhibitor against HIV-1 integrase [67]. Recently, for SARS-CoV2, the peptides to block fusion core are also designed using molecular docking and molecular dynamics simulations [48]. Experimentally, the peptides from library can be screened against specific targets for their activity, and there are developments to screen numerous peptides automatically.

10.11 FDA-Approved Antiviral Therapeutic Peptides

Further we briefly discuss some of the peptide-based drugs that are successfully developed and approved by FDA for the treatment of various viral infectious diseases. *Alisporivir* (Fig. 10.9a) is used to treat hepatitis C virus. This cyclic peptide is a cyclophilin inhibitor [98]. The

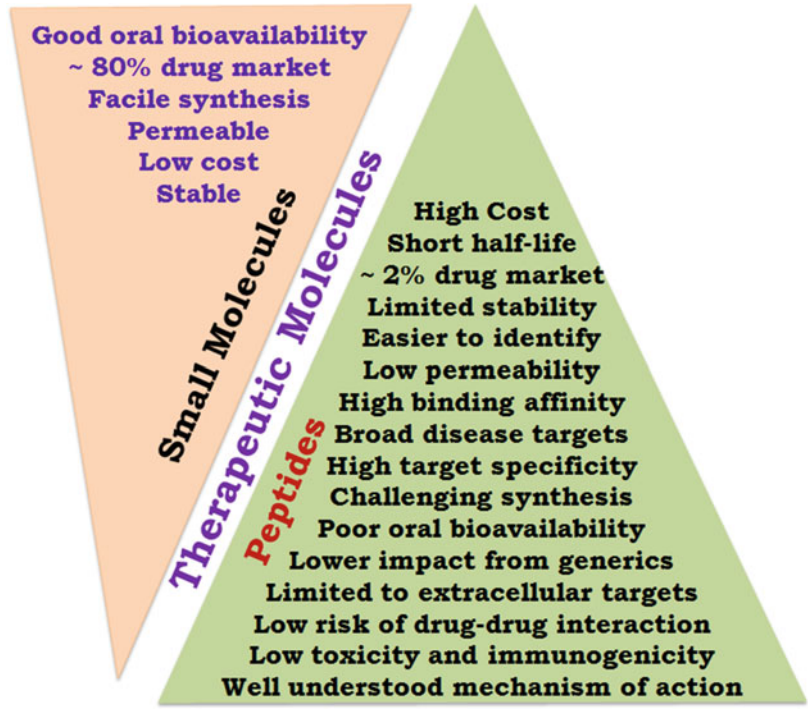

Fig. 10.8 Comparison of therapeutic properties and values of peptides and small molecules

peptide inhibits the replication of viral RNA. Mechanism of action is not widely known [37]. Alisporivir has been developed by Bebio Pharm in the brand name of *Debio-025*/*DEB-025* [99]. *Golotimod* (Fig. 10.9b) is used to treat hepatitis C by oral administration and developed by Apnoke Scientific Ltd. Golotimod can be also represented as SCV-07 (gamma-D-glutamyl-L-tryptophan) [100]. This is a dipeptide inhibitor that has D-glutamine and L-tryptophan connected by a gamma-glutamyl linkage. It has a role as a metabolite, an immunomodulator, an angiogenesis-modulating agent, and an antineoplastic agent [34]. Golotimod inhibits signal transducer and activator of transcription 3 (STAT3). This molecule is used for the treatment of various viral and bacterial infections [2]. This is also used in tuberculosis treatment [31]. *Oglufanide* (Fig. 10.9c) is a dipeptide composed of L-glutamic acid and L-tryptophan. It has a role as a metabolite, an immunomodulator, an angiogenesis-modulating agent, and an antineoplastic agent [48]. Oglufanide acts as a regulator of the body's immune response and is being given by intranasal administration to patients with chronic hepatitis C viral infection. *Telaprevir* (Fig. 10.9d) is an NS3/4a protease inhibitor [10]. NS3/4a protease is responsible for cleavage of viral proteins to mature proteins. This is used to inhibit hepatitis C virus replication [101, 102]. Telaprevir (VX-950) was developed in the brand name of *Incivek* and *Incivo* by Vertex Pharmaceuticals and Johnson & Johnson and administered orally. Approved in May 2011 by the FDA, *Incivek* was indicated for the treatment of HCV genotype 1 in combination with *ribavirin*, *peginterferon alfa-2a*, and *peginterferon alfa-2b*. *Incivek* has since been withdrawn from the market.

Atazanavir (Fig. 10.9e) is aza-peptidomimetic protease inhibitor for retrovirus [103]. It inhibits human immunodeficiency virus (HIV) type 1 aspartic protease [104]. Inhibition of this protease prevents the maturation process of the virus and reduces the formation of virions. The brand name of atazanavir is *Reyataz* (formally known as BMS-232632) developed by Bristol Myers Squibb [104]. This is administered in capsules, which is made available in the market. $Sar^{9},Met(O_2)^{11}$-*substance P* (Fig. 10.9f) is a peptidomimic

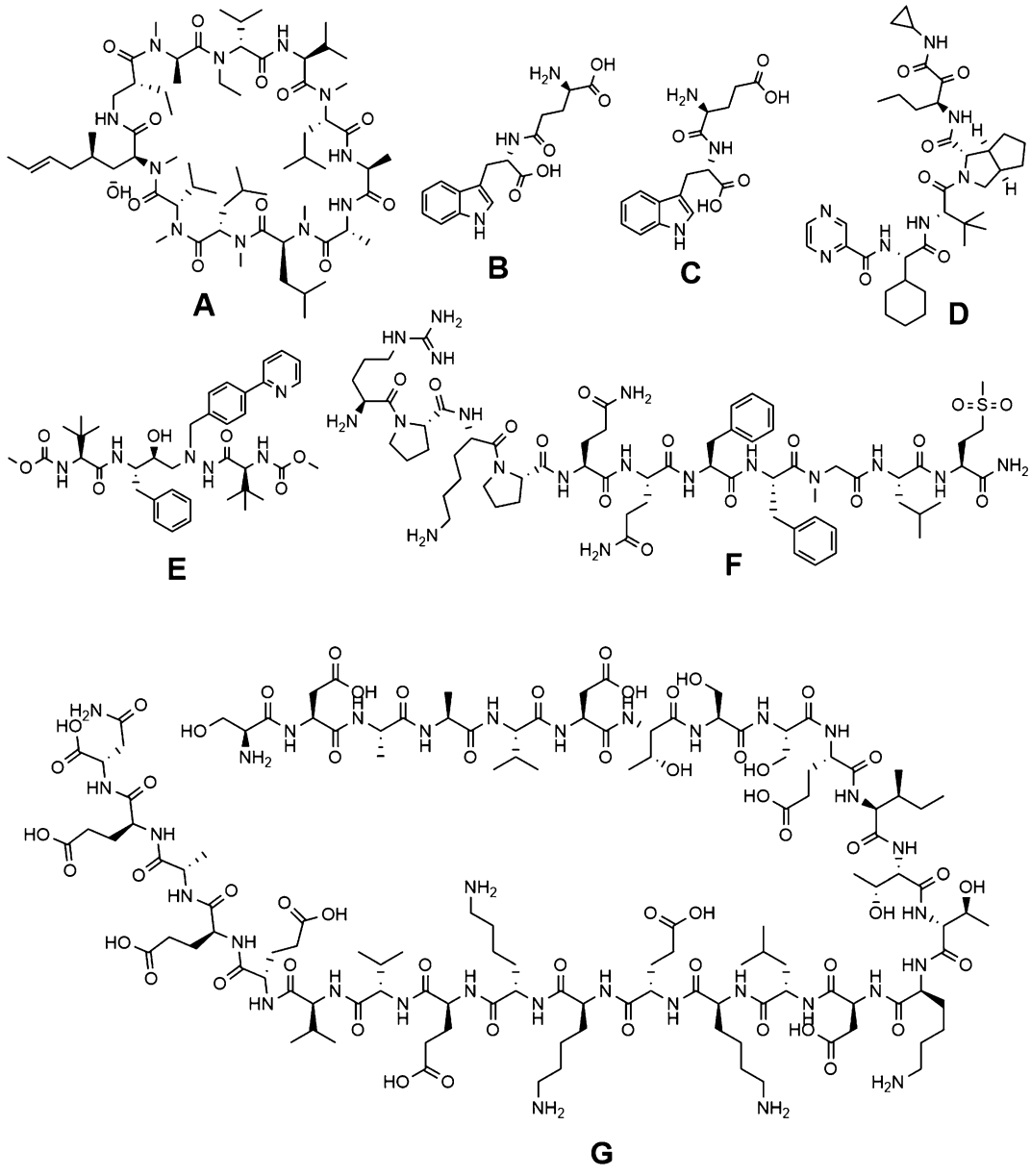

Fig. 10.9 Chemical structures of FDA-approved therapeutic peptides for viral diseases: (**a**) alisporivir, (**b**) golotimod, (**c**) oglufanide, (**d**) telaprevir, (**e**) atazanavir, (**f**) Sar^{9},$Met(O_2)^{11}$-substance P, and (**g**) thymalfasin

neurokinin-1 receptor agonist [64]. It is an analog of the naturally occurring human neuropeptide substance P, which is present throughout the body, particularly in the airways of humans. This is used in the treatment of acute respiratory distress syndrome (ARDS) and viral infection. *Thymalfasin* (Fig. 10.9g) is a synthetic analog of thymosin-alpha-1, a 28-amino acid peptide, derived from the precursor protein prothymosin-alpha [105]. Thymalfasin is shown to promote T-cell differentiation and maturation [106]. Mechanism of action is not clearly understood. The

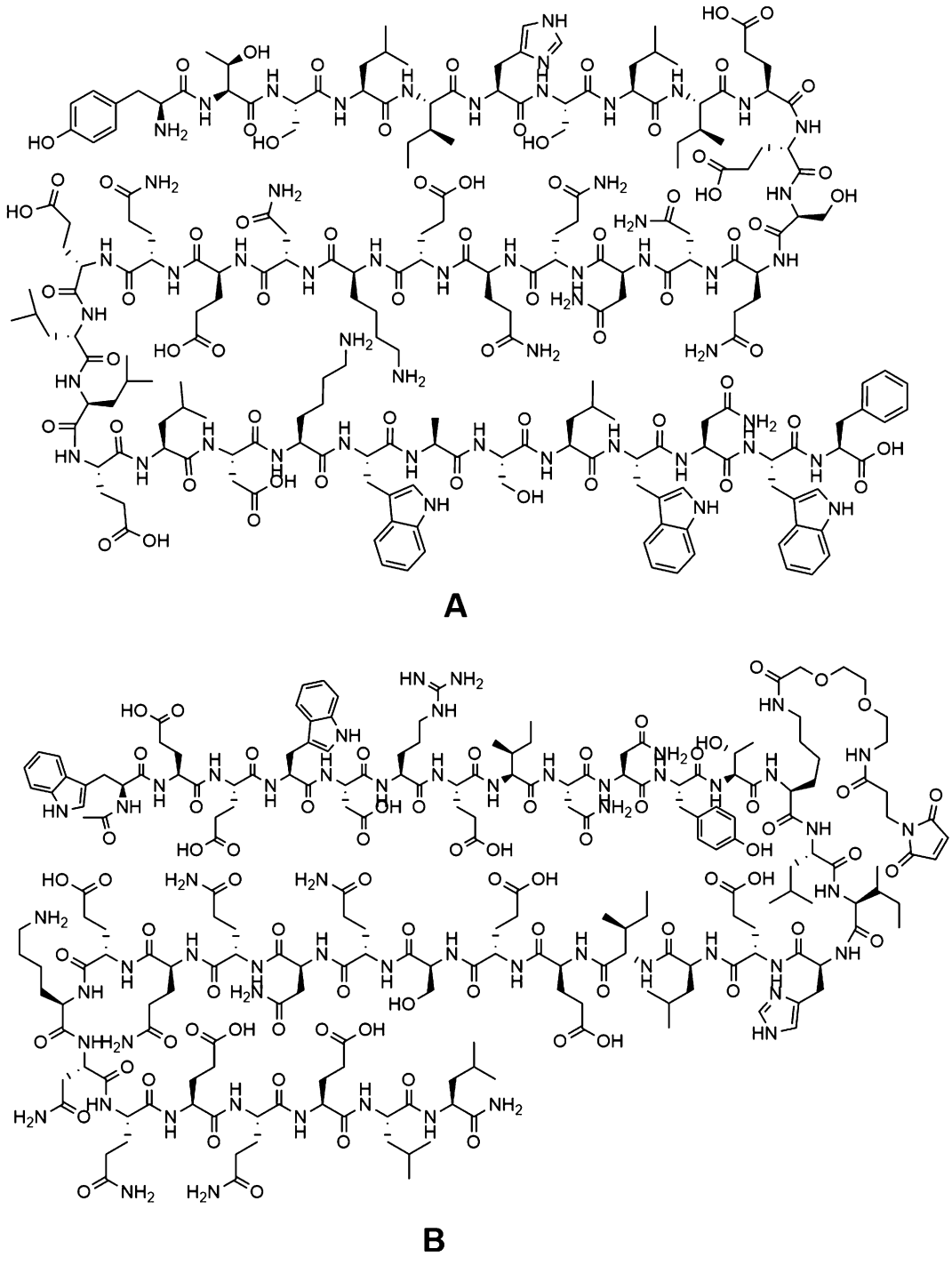

Fig. 10.10 Chemical structures of FDA-approved therapeutic peptides for viral diseases: (**a**) enfuvirtide and (**b**) albuvirtide

brand name of thymalfasin is *Zadaxin*. This was developed by SciClone Pharmaceuticals and available in the market to treat hepatitis B [107] and C [108]. Thymalfasin is administered via subcutaneous injection. *Enfuvirtide* (Fig. 10.10a) is a 36-mer-peptide; a first fusion inhibitor prevents the entry of HIV virus into the host CD4 cells [109]. This peptide binds with the heptad-repeat (HR1) in the gp41 subunit of the HIV virus. This disrupts the entry of viruses into the host cell [110]. This is administered subcutaneously. Roche develops this new class of antiretroviral drugs in the brand name of *Fuzeon*, and it is made available in the market. Though there are only a few peptides approved for antiviral therapeutics by FDA, the hope for the potential peptide-based drugs in the future is higher and brighter.

Albuvirtide (Fig. 10.10b) is a 3-maleimimidopropionic acid (MPA)-modified peptide HIV fusion inhibitor that can irreversibly conjugate to serum albumin [41]. Albuvirtide binds with the gp41 protein, which is an envelope protein present in HIV. As a result, the virus cannot enter into the human cell [111]. The drug is administered by intravenous mode, and it has been approved in China for the treatment of HIV.

10.12 Conclusions

Currently, more than 400 peptide drugs are under clinical trials, and FDA approved about 60 peptides. However, there are only a countable number of peptides for antiviral treatment. In spite of few potential limitations, peptides have several advantages compared to small molecule drugs (organics), and now strategies are developed to improve their drug-like properties such as permeability, oral bioavailability, and stability in blood and within cells. Presumably, in the future, peptides would play a central role in the development of drugs and vaccines for various infectious diseases. In this chapter, we threw light on various general features of therapeutic peptides. And we have discussed various antiviral peptides that are currently being developed and in the market.

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Covalent Antiviral Agents

11

Sako Mirzaie, Fatemeh Abdi, Amin GhavamiNejad, Brian Lu, and Xiao Yu Wu

Abstract

Nowadays, many viral infections have emerged and are taking a huge toll on human lives globally. Meanwhile, viral resistance to current drugs has drastically increased. Hence, there is a pressing need to design potent broad-spectrum antiviral agents to treat a variety of viral infections and overcome viral resistance. Covalent inhibitors have the potential to achieve both goals owing to their biochemical efficiency, prolonged duration of action, and the capability to inhibit shallow, solvent-exposed substrate-binding domains. In this chapter, we review the structures, activities, and inhibition mechanisms of covalent inhibitors against severe acute respiratory syndrome coronavirus 2, dengue virus, enterovirus, hepatitis C virus, human immunodeficiency virus, and influenza viruses. We also discuss the application of in silico study in covalent inhibitor design.

Keywords

Covalent antiviral agents · Structures · Mechanisms · Activities · In silico study

Abbreviations

Bz-nKRR-H	benzoyl-norleucine-Lys-Arg-Arg-aldehyde
DENVP	Dengue virus serine protease
DV	Dengue virus
EV71	Enterovirus 71
FDA	Food and drug administration
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTS	High-throughput screening
IC_{50}	The half maximal inhibitory concentration
K_i	Equilibrium inhibition constant
NCp7	Nucleocapsid protein (p7)
RT	Reverse transcriptase
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SP	Spike protein

11.1 Introduction

The prompt advent of resistance in viruses towards antiviral compounds and the manifestation of new viral pathogen together make a requirement for new antiviral methodologies with the potential for broad-spectrum activity [1]. The most fruitful molecules that form nearly all traditional drugs are small molecules [2]. These molecules can penetrate well into a variety of tissues and cells, can easily be

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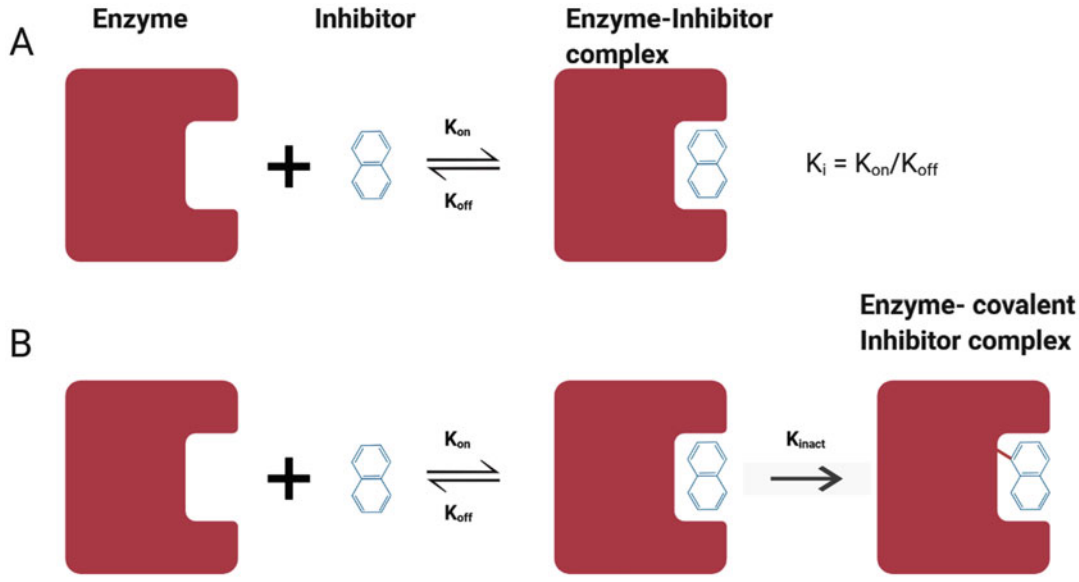


Fig. 11.1 Comparison of a noncovalent, traditional inhibitor (**a**) and nonconventional covalent inhibitor (**b**) interacting with an enzyme. Adopted from Ref. [3] with permission

combined with other medicines, and sometimes can inactivate their relevant targets rapidly.

Most small molecules are designed to inhibit their relevant targets noncovalently. In this strategy, the interaction between a small molecule and a target is a rapid and reversible process [3]. The intrinsic affinity between the ligand and biological target determines the ratio between the ligand-target complex to the free form of each component. To achieve the best results in the clinic, more efforts have been made to increase the strength of these noncovalent molecular interactions [3].

Along with this traditional approach of inhibition, nonconventional inhibitors, that is, covalent inhibitors, have been introduced and investigated. These inhibitors bind to their relevant targets through the formation of irreversible covalent linkages. The produced complex is durable, nearly eliminating its receptor from the life cycle. Based on the nature of inhibition, covalent inhibitors are not usually classified using standard and time-dependent IC_{50}. Instead, they are clustered based on the rate of inactivation of a target, or the rate constant of inactivation (k_{inact}). The activity of these inhibitors is usually expressed as k_{inact}/K_i ratio, where K_i is the equilibrium constant of binding (Fig. 11.1) [4, 5]. Studies have revealed that the most prevalent covalently modified targets are enzymes, and the most residues that undergo modification through covalent binding are cysteine and serine residues [6]. These polar residues have electron-rich atoms that make them suitable for electrophilic attack. The residues lysine, threonine, and a number of cofactors are in the next ranks [6].

Based on a study published in 2005, drug discovery efforts rarely tend to identify the covalent drugs with irreversible inhibition mechanism [7]. Researchers usually filter out the compounds with high and functional reactivity from the screening phase. The excluded molecules reveal noncompetitive kinetics. It should be noted that the indiscriminate reactivity can result in covalent binding to DNA, proteins, and glutathione, which can subsequently lead to undesirable toxicological outcomes [6]. However, it has been shown that 26% of the 71 targets investigated were covalently inhibited by the drugs approved by the United States Food and Drug Administration (FDA) [3]. Ibutinib, acalabrutinib, afatinib, and osimertinib are examples of US FDA-approved

targeted covalent inhibitors [8]. Despite opposition from pharmaceutical companies to covalent drugs development, SciFinder®-based statistical studies over the past 50 years showed that the manufacturing of these drugs has increased [9].

Prior studies revealed that the nucleophilic residues in the targets undergo covalent binding to inhibitors. In an ideal situation, a covalent inhibitor is not or poorly reactive in a buffer solution; but under physiological condition it can selectively react with a nucleophilic atom within the target protein [6]. An attractive increase in the potency and pharmacokinetics of a drug-like compound is to evoke the formation of a covalent bond. Compared with noncovalent inhibitors, the advantages of covalent compounds lie in the following aspects: higher potency, longer residence time, and decreased drug resistance [10].

All covalent inhibitors feature a warhead that enables them to make a covalent bond with their relevant residue target. The structures, activities, and inhibition mechanisms of covalent inhibitors against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), dengue virus (DV), enterovirus, hepatitis C virus (HCV), human immunodeficiency virus (HIV), and influenza viruses will be discussed in detail in the following part of the chapter. At the end of the chapter, the application of in silico studies in covalent inhibitor design and discovery is illustrated.

11.2 Classification of Covalent Inhibitors

Based on the nature of the inhibition mechanism, covalent antiviral agents are divided into two categories: reversible and irreversible inhibitors [10].

11.2.1 Covalent Reversible Inhibitors

In this type of inhibition, the inhibitor forms a covalent bond with an enzyme and inhibits its activity. However, this covalent bond can break down, and subsequently, the activity of the enzyme is recovered. The overall affinity is dependent on the formed covalent bond, and cleavage of this bond can lead to a decrease in the duration of inhibition and affinity to the enzyme. The "reversible" word refers to the formation of a covalent bond between the inhibitor and enzyme that can be reversed via cleavage. Dialysis or dilution of a solution containing enzyme-inhibitor complex can result in the dissociation of the unmodified inhibitor [11]. Peptide aldehydes synthesized by parallel solid-phase method are examples of covalent reversible inhibitors. In 2006, the Rademann group synthesized 25 peptide aldehydes by this method, and the inhibitory activity of these compounds against SARS-CoV M^{pro} were assayed. Peptides AcNSTSQ-H and AcESTLQ-H showed the lowest IC_{50} (7.5 μM) [12].

11.2.2 Covalent Irreversible Inhibitors

Covalent irreversible inhibitors are called inactivators as they result in an irretrievable loss of activity. The most important merit of this type of inhibitors is that even after the concentration of inhibitor in solution decreases, a certain population of enzymes could be irreversibly inactivated forever. Over time, the inhibited enzymes are accumulated progressively. Recently, Yang's group resolved the crystal structure of SARS-CoV-2 virus $3CL^{pro}$ in complex with an irreversible peptidomimetic inhibitor N3 (PDB code: 6 LU7) [13, 14]. The N3, a Michael acceptor inhibitor, was developed using computer-aided drug design. This inhibitor which is active against multiple coronaviruses, including SARS-CoV and MERS-CoV, showed inhibitory activity towards infectious bronchitis virus in an animal model [15]. Hexapeptidyl chloromethyl ketone, which inhibits SARS-CoV M^{pro}, is another example of a covalent irreversible inhibitor [16]. Crystallography data showed that a covalent bond is formed between the M^{pro} Sγ atom of Cys145 and the methylene group of the inhibitor (PDB ID 1P9U). Also, a network of hydrogen bonds stabilizes the inhibitor in the substrate binding site [16].

11.3 Covalent Inhibitors Against Viruses

Recently, covalent inhibitors against viruses have gained a lot of attention and has lived up to its success as an effective drug discovery option [17–19]. Numerous covalent inhibitors have been designed, synthesized, and evaluated against a wide range of viruses. For instance, antiviral agents were investigated to overcome the infections caused by SARS-CoV-2, dengue virus, enterovirus, HCV, HIV, and influenza viruses.

11.3.1 Covalent Inhibitors Against SARS-CoV-2

Coronavirus disease-19 (COVID-19) is a pandemic respiratory disease caused by the virus, SARS-CoV-2, and has infected over 34 million people and caused >1 million deaths globally as of early October 2020 [20–24]. Its widespread nature, fast transmission, and high death rate have triggered universal efforts to quickly develop vaccines and specific antiviral treatments [25, 26]. The rapid identification and publication of RNA sequence and protein structures of the virus have led to quick design of antiviral agents against spike protein (SP), RNA-dependent RNA-polymerase, non-structural protein 13, papain-like protease, (PL^{pro}), and chemotrypsin-like or main protease ($3CL^{pro}$, M^{pro}) [27–29]. The main protease (M^{pro}, $3CL^{pro}$) is a target in SARS-CoV-2 that has attracted great attention.

11.3.1.1 M^{pro}: The Most Important Target in SARS-CoV-2

M^{pro} has a pivotal role in viral replication by cleaving the pp1a and pp1ab polyproteins to functional proteins [28]. Since M^{pro} cleaves its substrates after a glutamine residue and human host cells lack proteases with such substrate specificity, this enzyme is considered as an ideal target for drug discovery [30–32]. Based on the superposition of M^{pro} from SARS-CoV-2 (PDB ID: 6Y2E), SARS-CoV (PDB ID: 2BX4), and MERS-CoV (PDB ID: 5C3N), a high degree of structural similarity and conservation of the active site have been revealed. So, the development of prior antiviral agents targeting SARS-CoV and MERS-CoV can be employed to design new M^{pro} inhibitors for SARS-CoV-2. M^{pro} is classified as a cysteine protease and forms a catalytic dyad (cysteine and histidine) in its active site (Fig. 11.2a) [28]. All other cysteine or serine proteases have a triad in their catalytic center, whereas in M^{pro}, a water molecule fills the third position [34–36]. M^{pro} is inactive in the monomeric state but active in the homodimer form [37]. Each protomer of M^{pro} consists of three domains (domain I-III) [14, 16, 32, 38]. The active site of M^{pro} is located between domains I and II [28]. Based on pairwise alignment, it has been revealed that the active site of M^{pro} in SARS-CoV and SARS-CoV-2 is highly conserved [28]. Figure 11.2b shows the possible enzyme-substrate complex of M^{pro} with a polyprotein, where "S" demonstrates the subsites (S5 to S5') on the enzyme, and "P" represents the point of cleavage in the substrate (P5 to P5'), based on the nomenclature of Schechter and Berger [39].

11.3.1.2 Diversity in the Structures of Covalent Inhibitors

Peptidomimetics and small molecules with affinity from micro- to nanomolar are two major classes of covalent inhibitors against M^{pro}. Different warheads lead to the synthesis of diverse covalent inhibitors. The warhead groups include Michael acceptors, aldehydes, epoxy ketones, or other ketones that are the reactive groups that covalently attack the catalytic cysteine residue in the M^{pro} active site [37]. The development of several compounds that targeted SARS-CoV or MERS-CoV has led to the identification of new covalent inhibitors against main protease in SARS-CoV-2 [14, 32, 33]. The peptidomimetics 1–3 shown in Fig. 11.3 mimic point of cleavage of the substrate from P1′ to P3 [32]. These inhibitors have α-ketoamide moieties and can covalently bind to Cys145 and form hemithioacetal. α-Ketoamide is a functional group in two FDA-approved anti-

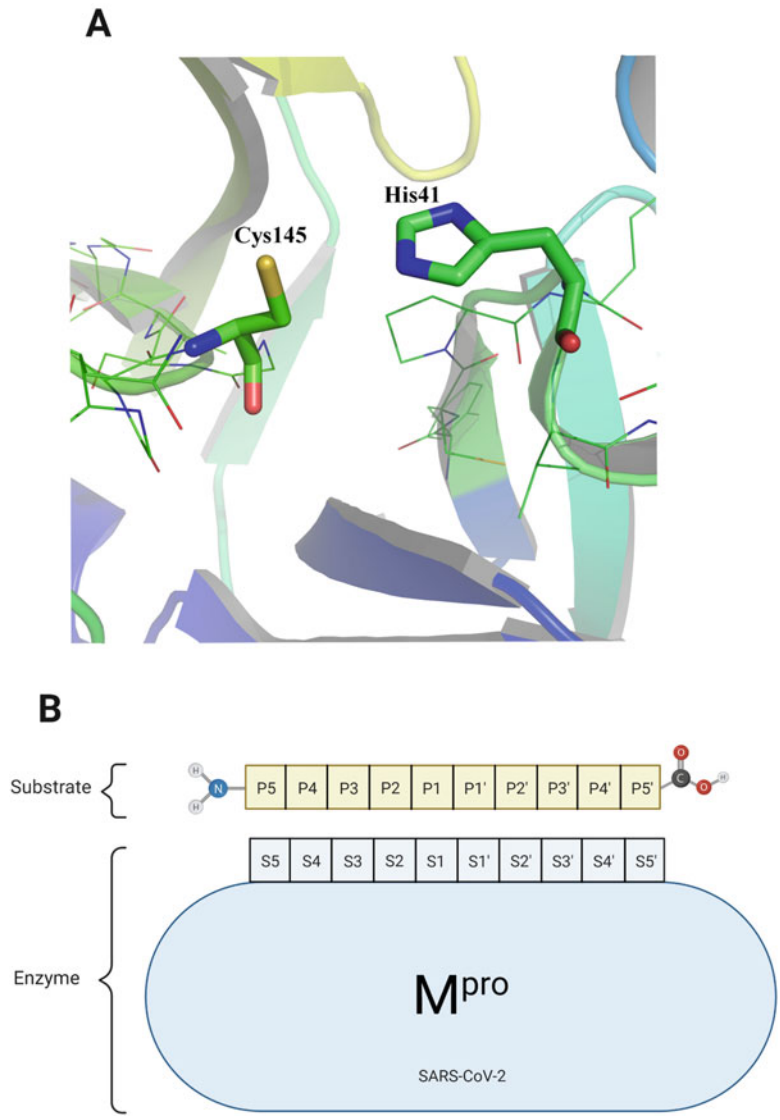


Fig. 11.2 Active site of SARS-CoV-2 M^{pro} showing the catalytic dyad (Cys and His). Schematic representation of M^{pro} subsites (S5 to S5') from SARS-CoV-2 with point of cleavage in polyprotein (P5 to P5'). Cleavage site is between P1 and P1' [33]

HCV drugs, telaprevir and boceprevir [40]. Compounds 1–3, in the position of P1, have a γ-lactam that imitates the glutamine residue. In position P2, usually nonpolar moiety is located that simulates the hydrophobic leucine residue in a natural substrate. Compounds 2 and 3 have pyridone rings and N-terminal Boc groups that increase thermodynamic solubility, plasma half-life, and kinetic solubility (Fig. 11.3). Compound 3 is active against SARS-CoV, MERS-CoV, and SARS-CoV-2 by inhibiting M^{pro}. Compound 4 is a Michael acceptor and can irreversibly modify Cys145. Compounds 5 and 6 have the lowest inhibition constants (two-digit nanomolar range) and contain an indole group and aldehyde warhead at the N-terminal (P3) and a C-terminal, respectively. An aldehyde is a reactive group that can covalently bind to Cys145 [33].

Based on enzyme assay and X-ray crystallography studies, aldehydes are considered as one of the strongest inhibitors against M^{pro} [12, 41]. High-throughput screening (HTS) of a library containing approved drugs and clinical

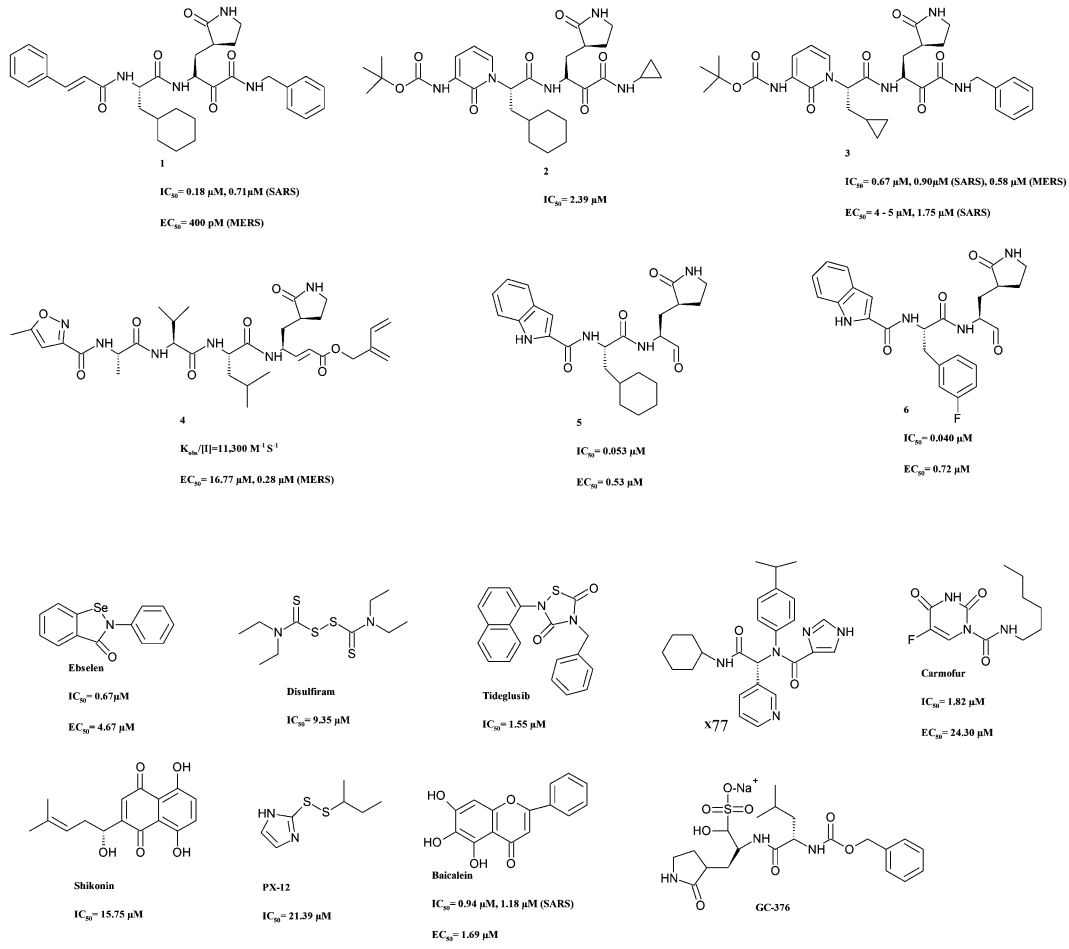


Fig. 11.3 Chemical structures and assayed inhibitory activities of peptidomimetics and other M^{pro} inhibitors in MERSCoV and SARS-CoV. Adopted from Ref. [28] with permission

candidates showed that six small molecules known as ebselen, disulfiram, carmofur, tideglusib, shikonin, and PX-12 are the inhibitors of SARS-CoV-2 M^{pro} [14]. Further experiments revealed that among them, ebselen, carmofur, and PX-12 can covalently bond to Cys145 [14]. These small molecules probably bind to various targets nonspecifically, which are called Pan-assay interference compounds [42]. Inhibitory activity of ebselen has been demonstrated in Vero cell line within micromolar range. Furthermore, X-ray crystallography study revealed that the fatty acid moiety ($C_7H_{14}NO$) of carmofur is connected to the Sγ atom of Cys145 from SARS-CoV-2 M^{pro} through a 1.8 Å covalent bond (PDB ID: 7BUY) [43]. GC-376 is another potent covalent inhibitor (IC_{50} of 0.030 ± 0.008 µM) with the aldehyde bisulfite as a warhead that binds to the Cys145 [44]. This inhibitor can adopt two different configurations, R and S, upon binding to the M^{pro} active site, and this fact may be the reason for its high activity and affinity [28].

11.3.2 Covalent Inhibitors Against Dengue Virus (DV)

DV, a single positive-stranded RNA virus, is transmitted to humans through the bite of an infected *Aedes* mosquito and annually infects

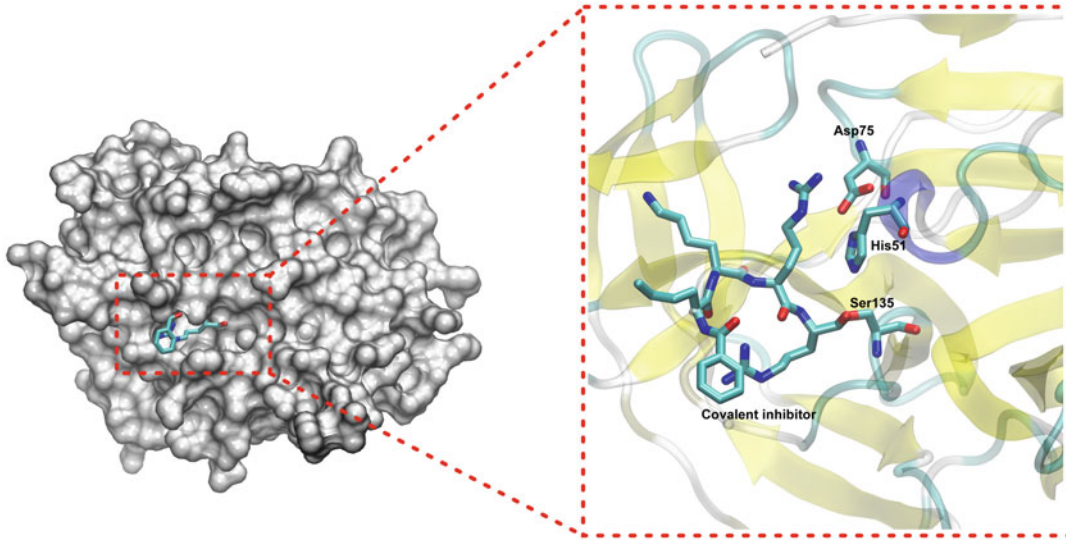


Fig. 11.4 Structure of DENVP bound to Bz-nKRR-H. (**a**) The structures of enzyme and inhibitor are shown in surface and stick representation, respectively. (**b**) Close view of catalytic triad and covalent binding of Bz-nKRR-H to Ser135 (PDB ID: 3u1i) [55]

50–100 million people worldwide [45]. Dengue fever, a disease caused by DV, is distinguished by high fever, headache, arthralgia, myalgia, rash, and stomach ache. Dengue fever can also lead to dengue shock syndrome with a mortality rate of 1–2.5% in proper medical care, and a fatality rate of more than 20% without proper treatment [46]. Due to the lack of vaccines or antiviral drugs against DV infection [45, 47], efforts to discover the covalent inhibitors are in high demand [1]. Reactive cysteine in the enzymes of DV is of increasing interest as drug targets due to the catalytic and structural importance of this residue in many proteins. Dengue virus serine protease (DENVP) is one of the attractive targets for drug design, yet up to now, no molecules or chemicals have reached clinical development [48]. This enzyme is a serine protease and has a vital role in the proteolytic processing of viral polyprotein, and finally, in viral replication [48, 49]. Several strategies including high-throughput screening (HTS) [50, 51], in silico docking [52], and peptidomimetics [53, 54] have been employed so far, but as mentioned above, none of them has been successfully approved by FDA as of yet.

11.3.2.1 Structure of Dengue Virus Serine Protease (DENVP)

The genome of DV encodes three structural (capsid, envelope, and membrane) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins that are translated as a single polypeptide chain. N-terminal and C-terminal of NS3 have two separate functions of the serine protease and RNA helicase, respectively. The protease domain forms a catalytic triad, which comprises of His51, Asp75, and Ser135 (Fig. 11.4a). However, NS2B is also needed for the catalytic activity as it helps the binding of NS2B-NS3 protease to the endoplasmic reticulum through its three transmembrane helices [56]. Also, NS2B adopts the β hairpin structure that directly interacts with the substrate [57–59]. A combination of host and viral NS2B-NS3 protease (DENVP) cleaves the viral polyprotein into the individual proteins [60, 61].

11.3.2.2 Structures and Mechanisms of Covalent Inhibitors against DENVP

Peptidomimetics are the most well-known covalent inhibitors that are designed and investigated

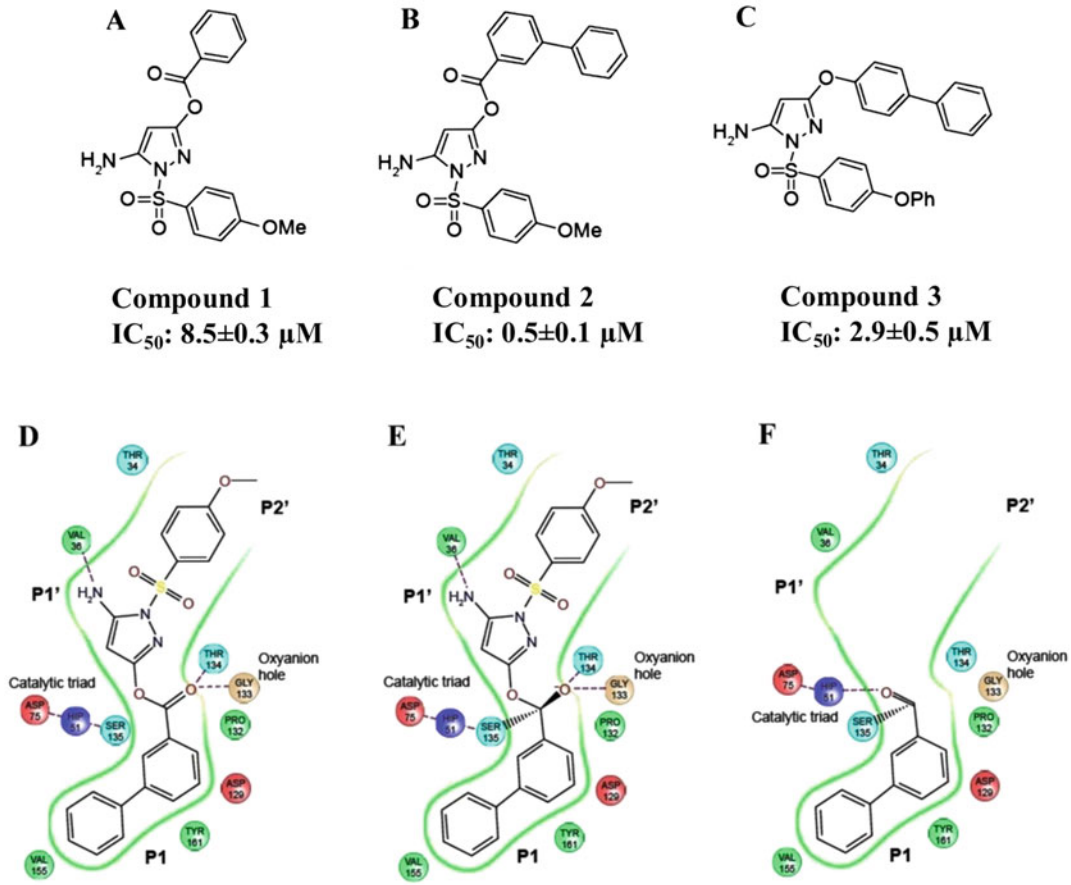


Fig. 11.5 Chemical structures of compounds 1 (**a**), 2 (**b**), and 3 (**c**). Schematic view of trans-esterification reaction between DENVP and compound 2 in steps 1 (**d**), 2 (**e**), and 3 (**f**). Adopted from Ref. [48] with permission

against DV. Bz-nKRR-H, a peptidomimetic inhibitor, inhibits DENVP with a K_i of 5.6 μM [54]. Bz-nKRR-H, with an aldehyde as a warhead, forms a covalent bond with Ser135 (Fig. 11.4b), and subsequently, the resulting hydroxyl of the inhibitor interacts with His51 of DENVP. The final produced structure is a tetrahedral hemiacetal that can result in either *S* or *R* stereochemistry [55]. Pyrazole ester derivatives are another class that is identified as potent inhibitors of West Nile virus and DV proteases. Figure 11.5a–c show three pyrazole ester derivatives that covalently bind to DENVP active site. Compound 1 contains a 5-amino-1-(phenyl) sulfonyl-pyrazol-3-yl core that is linked through the 3 position to a phenyl ester [62]. Compound 2, an improved form of compound 1, has the lowest IC_{50} [48]. Key steps of the trans-esterification reaction between DENVP and compound 2 have been described in Fig. 11.5d–e. At first, the oxygen atom of carbonyl from compound 2 makes hydrogen bonds with Gly133 and Thr134 in the oxyanion hole. Also, the amine group of the inhibitor forms an H-bond with Val36 from enzyme active site (Fig. 11.5d). Then, Ser135, a member of the catalytic triad, makes a nucleophilic attack on the carbon atom of carbonyl moiety from compound 2, and subsequently, a tetrahedral intermediate is formed (Fig. 11.5e). The deprotonated oxygen atom of compound 2 is stabilized by the oxyanion hole. In the last step, the pyrazole-phenylsulfonamide leaves the enzyme active site, whereas 3-phenyl

benzoyl remains covalently bound to Ser135 (Fig. 11.5f) [62].

In another study, a thousand-fold affinity was achieved by introducing a boronic acid group into the basic side containing dipeptidic inhibitors of the Zika, West Nile, and DV proteases [63]. However, the presence of boronic acid along with basic moieties in the inhibitor structure lead to decreased inhibitor passage through the membrane due to an increase in the molecule's polarity. Two solutions can be followed to overcome the polarity problem: first, boronic acid can be modified to become a prodrug, for instance, by esterification. Second, boronic acid may be replaced with other electrophilic reactive groups, for instance, a fluoromethyl ketone moiety with lower polarity [63].

In 2017, compound YKL-04-085, derived from QL47, was identified as a broad-spectrum antiviral agent [64]. QL47 is a potent and covalent inhibitor of bruton tyrosine kinase and other Tec family kinases [65]. Surprisingly, despite the lack of essential quinoline nitrogen required for hydrogen bonding to the kinases, YKL-04-085 is known as a potent antiviral agent. Preliminary mechanistic investigations showed that YKL-04-085 could inhibit DV through an effect on translation of the viral RNA genome. However, the exact molecular target of this compound is still under investigation [64].

11.3.3 Covalent Inhibitors Against Enterovirus 71 (EV71)

EV71, belonging to the family *Picornaviridae*, is an RNA virus that causes hand, foot, and mouth disease with a high rate of prevalence in the Asia-Pacific region including Australia, China, Malaysia, Singapore, Taiwan, Thailand, and Vietnam [66, 67]. Symptoms comprise of fever, vesicular rashes on the hands, vomiting, and oropharyngeal ulcers with self-limiting features [68]. However, some patients experience encephalitis, aseptic meningitis, and poliomyelitis-like flaccid paralysis, and even death [69, 70]. Two EV71 outbreaks took place in 2008 and 2011 and infected 0.49 and 1.6 million people, respectively [71]. Due to the lack of FDA-approved drugs against EV71, the development of antiviral agents against this virus is in high demand [71]. Of various targets in EV71, 3C cysteine protease is an interesting one for designing new antiviral agents.

11.3.3.1 Structure of 3C Cysteine Protease

As discussed previously, 3C cysteine protease, a member of the cysteine protease family, converts polyproteins into mature viral proteins through cleavage at the Gln-Gly peptide bond junction [72]. This enzyme also interacts and cleaves the host factors and proteins to suppress antiviral immunity [73, 74]. 3C cysteine protease from EV71 constitutes a chymotrypsin-like fold, which is a generic feature of all identified structures of 3C/3C-like proteases [16, 75–77]. This enzyme has 179 residues, from residues Leu4 to Glu182 and forming two domains. Domain I comprises of a 7-stranded β-barrel structure encircled by surface loops. Domain II features a dense barrel core, which is made of seven β-strands oriented in an antiparallel manner. His40, Glu71, and Cys147 form the catalytic triad in the active site of 3C cysteine protease [78].

11.3.3.2 Structures and Mechanisms of Covalent Inhibitors against EV71

Rupintrivir is a peptidomimetic inhibitor, including a lactam ring at P1, a fluorophenylalanine at P2, a valine residue at P3, a 5-methyl-3-isoxazole at P4, and an α, β-unsaturated ester at P1′ (Fig. 11.6). This inhibitor is placed in the substrate-binding groove and makes a covalent bond to the nucleophilic Cys147. This antiviral agent has an electrophilic ethyl propenoate Michael acceptor at its C-terminal, which reacts with the protease's nucleophilic Cys147 thiol via a Michael addition. Rupintrivir inhibits the 3C cysteine protease irreversibly with an IC_{50} of 7.3 ± 0.8 mM [79, 80]. Based on the structure of 3C cysteine protease derived from X-ray crystallography (PDB ID: 3SJO), the entire protease active site is nearly superficial except the S1 and S2 subsites [66].

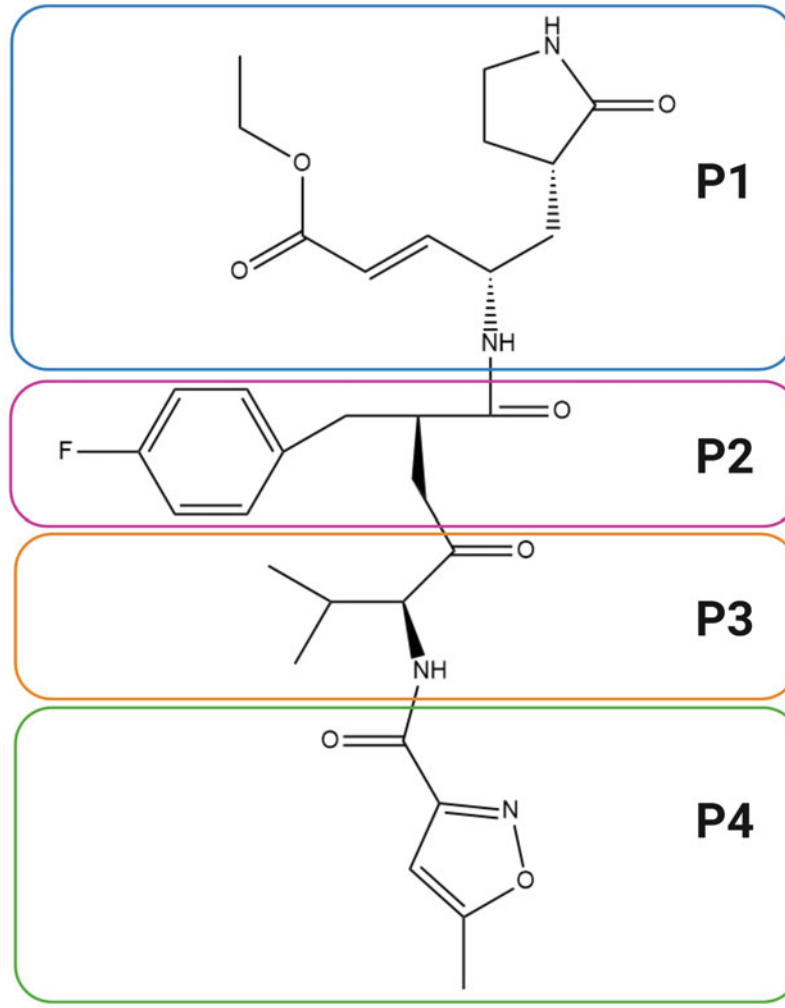


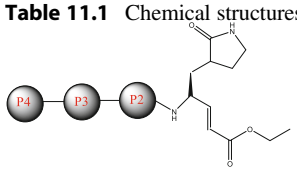
Fig. 11.6 Rupintrivir and P1, P2, P3, and P4 regions based on Schechter and Berger terminology

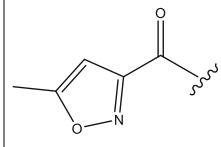
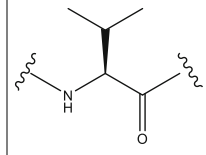
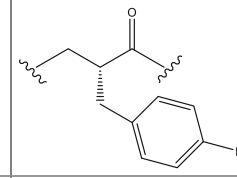
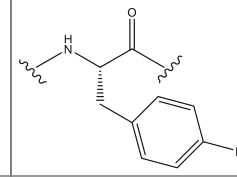
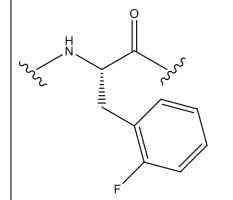
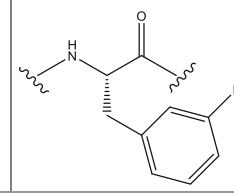
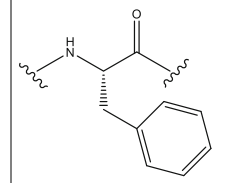
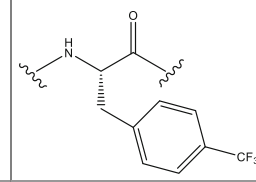
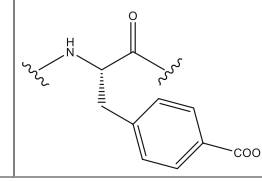
Also, residues Thr142 and His161 of the protease located in S1 interact with a five-membered lactam via hydrogen bonds. Furthermore, π–π interactions were seen between S2 subsite (His40) and P2 phenyl moiety, while aromatic para-F makes electrostatic interactions with the cationic Arg39 [66]. Table 11.1 shows the synthesized analogues of Rupintrivir. Based on this table, compound 9, a P2 ring-constrained phenylalanine analog, is the most potent inhibitor with an IC_{50} of 3.4 ± 0.4 μM with twofold more potency than Rupintrivir. Structure-activity relationship (SAR) study showed that employing geometrically constrained residues in P2 could be a noteworthy strategy to design the new potent inhibitor against 3C cysteine protease. However, one of the major problems with 3C cysteine protease inhibitors is their poor penetration into cells [67]. Therefore, along with the biochemical protease inhibition assay, antiviral activities of the inhibitors should be tested in cell culture. It has been shown that the nature of the P4 N-capping group could meaningfully affect the ability of a compound to penetrate the cell membrane [67].

11.3.4 Covalent Inhibitors against Hepatitis C Virus (HCV)

HCV, a hepatotropic positive-strand RNA virus, infected 71 million people worldwide and has emerged as a major health problem [81]. The

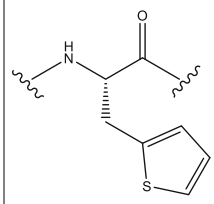
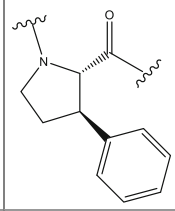
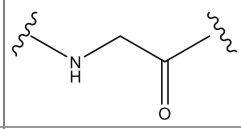
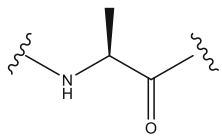
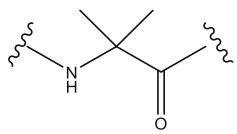
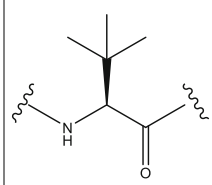
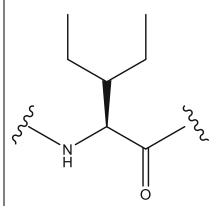
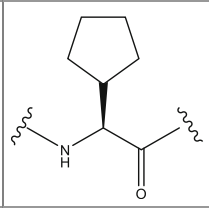
Table 11.1 Chemical structures and biological activities of Rupintrivir analogues against Enterovirus 71 protease [66]



Compound	P4	P3	P2	IC_{50} (μM)
Compound 1 (Rupintrivir)				7.3 ± 0.8
2				6.2 ± 0.6
3				14.9 ± 2.1
4				12.9 ± 1.5
5				13.0 ± 1.9
6				6.0 ± 0.8
7				>100

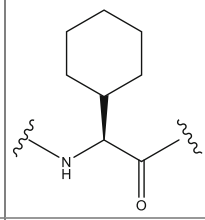
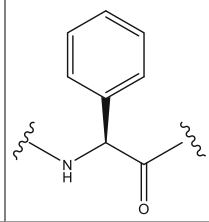
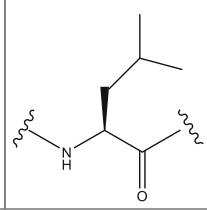
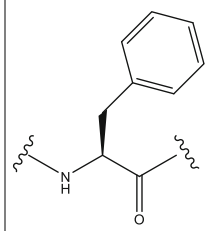
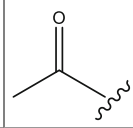
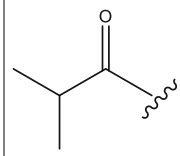
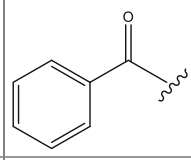
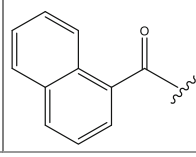
(continued)

Table 11.1 (continued)

Compound	P4	P3	P2	IC_{50} (μM)
8				7.5 ± 0.9
9				3.4 ± 0.4
10				32.8 ± 4.1
11				7.7 ± 0.5
12				13.4 ± 1.1
13				9.3 ± 0.8
14				12.7 ± 1.2
15				13.7 ± 1.1

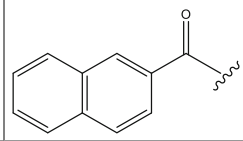
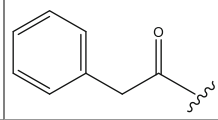
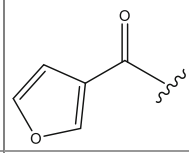
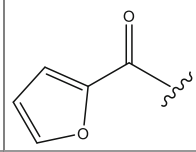
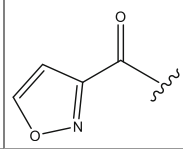
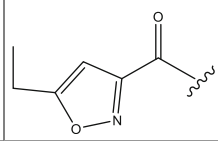
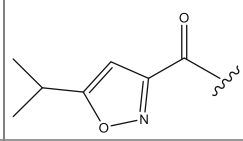
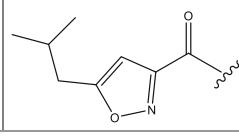
(continued)

Table 11.1 (continued)

Compound	P4	P3	P2	IC_{50} (μM)
16				18.3 ± 1.7
17				14.2 ± 1.3
18				10.5 ± 1.0
19				12.2 ± 0.9
20				9.5 ± 0.7
21				7.2 ± 0.6
22				15.7 ± 1.9
23				16.8 ± 2.7

(continued)

Table 11.1 (continued)

Compound	P4	P3	P2	IC_{50} (μM)
24				33.3 ± 3.7
25				12.9 ± 1.4
26				9.3 ± 0.7
27				16.1 ± 1.9
28				7.0 ± 0.9
29				8.5 ± 0.9
30				8.7 ± 1.5
31				8.3 ± 1.6

RNA genome of HCV encodes long polyprotein precursor of nearly 3000 amino acids. This polyprotein is cleaved into six structural and four nonstructural proteins. These nonstructural proteins are comprised of cysteine protease, RNA helicase, serine protease, and RNA-dependent RNA polymerase. Among them, serine protease and RNA-dependent RNA polymerase are the most interactive targets for drug design [82, 83].

11.3.4.1 Structure of NS3•4A Serine Protease

As mentioned for coronaviruses, NS3•4A serine protease is a bifunctional enzyme with a serine protease domain at the N-terminal domain and a helicase domain at the C-terminal [83]. In contrast to the well-protected, deep substrate-binding pocket for HIV protease, NS3•4A serine protease has a superficial, solvent-exposed substrate-

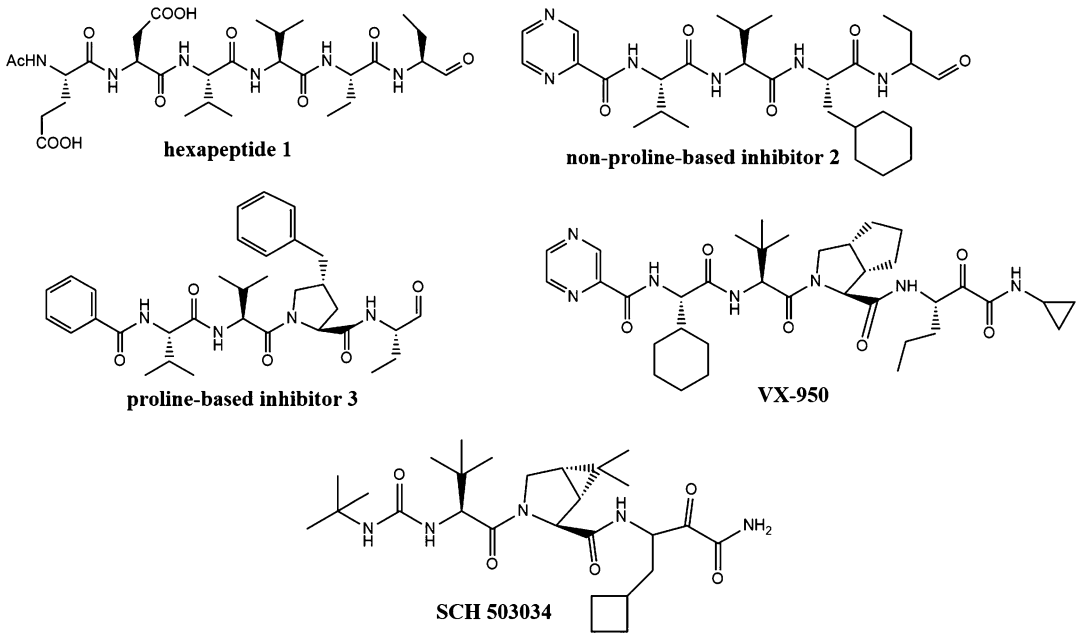


Fig. 11.7 Chemical structures of covalent inhibitors of HCV NS3•4A protease [83]

binding groove [84]. A wide range of electrophilic warheads from aldehydes to α-ketoamides have been employed to design the covalent, reversible HCV NS3•4A protease inhibitors.

11.3.4.2 Structures and Mechanisms of Covalent Inhibitors against HCV

Aldehydes, boronates, and α-keto groups are three important functional groups in covalent inhibitors of HCV. The following elaborates on how they form covalent bonds with NS3•4A serine protease.

Aldehydes

Aldehydes are the simplest reactive groups that can be engaged as an electrophilic warhead for covalent attachment to serine proteases. This functionality has been occupied to design the inhibitors against other serine proteases such as thrombin [85] or cathepsin K [86]. One of the disadvantages of aldehydes is that they are simply oxidized to their corresponding carboxylates. In the case of HCV NS3•4A serine protease, hexapeptide derivatives containing aldehyde warheads have been used extensively [87–89]. One of these inhibitors is hexapeptide 1 (Fig. 11.7) with IC_{50} of 5.5 μM. By eliminating the P5 (aspartic acid) and P6 (glutamic acid), the inhibitory property of the peptide is lost (enzyme $IC_{50} > 100$ μM) [87–89]. This result shows that, in addition to covalent binding of a warhead to catalytic serine, more binding interactions are required to inhibit the HCV protease. However, by increasing the size of P2, the loss of potency after hexapeptide truncation can be recovered. For example, a non-proline-based inhibitor 2 (inhibition constant $K_i = 13$ μM) and a proline-based inhibitor 3 ($K_i = 12$ μM) are two peptides with compensated inhibitory potency (Fig. 11.7).

Boronates

Boronate-containing molecules are potent covalent inhibitors of HCV serine protease. Bortezomib, or VELCADE is a boronate-containing agent that has been approved for the treatment of multiple myeloma by inhibiting the proteolytic activity of cellular proteasome [90, 91]. Up to now, penta- and hexa-peptide compounds containing boronate revealed good potency in enzyme assays [92, 93]. Pyridone-

based β-sheet mimetics are considered as non-peptide inhibitors of HCV serine protease [94–96].

α-Keto Inhibitors

Activated carbonyl compounds are classified as covalent, reversible inhibitors of the HCV NS3•4A protease. Some human serine protease inhibitors like fluoroalkyl ketones are weak inhibitors of the HCV protease ($K_i > 50$ μM) [97]. α-ketoacids, α-diketones, and α-ketoamides are three potent types of α-keto inhibitors of HCV NS3•4A protease [83]. VX-950 ($K_i = 0.04$ μM) and Scheme 503034 ($K_i = 14 \pm 1$ nM) are two covalent HCV NS3•4A protease inhibitors (Fig. 11.7). VX-950 (Telaprevir) was discovered through a structure-based drug design strategy and inhibits its target reversibly [97, 98]. This molecule was approved in 2011 but has been withdrawn from the market due to natural polymorphism resistance to it [99–101]. In addition, Scheme 503034 (Boceprevir) has been reported as a potent and irreversible ketoamide HCV NS3•4A protease inhibitor [102].

11.3.5 Covalent Inhibitors against Human Immunodeficiency Virus (HIV)

It has been more than 35 years since HIV was diagnosed, but this health issue still remains to be solved. This virus has infected 37 million people worldwide, and it continues to infect new people with a rate of more than ca. 2 million patients per year [103]. The major medical prescription regimen is based on a cocktail of drugs, known as combination antiretroviral therapy. However, this regimen has some limitations like drug resistance and side effects [104]. Atripla® (efavirenz + emtricitabine + tenofovir), Complera® (emtricitabine + rilpivirine + tenofovir), and Stribild® (elvitegravir + cobicistat + emtricitabine + tenofovir) are three new combination medicines that were introduced to overcome the problems mentioned above. Nevertheless, these cocktails also suffered from adverse drug-drug interaction. NCp7 is a multitasking protein with a preserved structure in all viral strains [104]. Reverse transcriptase (RT) is the second enzyme that could be a suitable target for covalent inhibitors. Furthermore, HIV-1 protease and viral capsid are noteworthy targets for covalent inhibitors.

11.3.5.1 Structure of NCp7

NCp7 is a member of the retrovirus nucleocapsid proteins family and has nucleic binding property. It is one of the products of viral polyprotein after the protease-induced breakdown. NCp7, in its mature form, has 55 residues where 27% of them have a basic character. The basic residues coordinate with two zinc finger motifs, each including 14 residues organized in a C-X2-C-X4-H-X4-C pattern [105]. Due to the presence of cysteine and histidine residues in NCp7, zinc finger motifs chelate zinc ions with high affinity (Ka $\sim 10^{12}$–10^{14} M^{-1}) [106]. The structure of NCp7 is entirely conserved among HIV viruses, and point mutations usually cause the production of fully imperfect virions [104, 105].

11.3.5.2 Structures and Mechanisms of Covalent Inhibitors against NCp7

For the first time, the irreversible covalent inhibitors of NCp7 were reported in the late 1990s. Up to now, several small molecule inhibitors have been discovered (Fig. 11.8), including azodicarbonamide (ADA, compound 2), dithiane (3), dithiobis(benzamides) (DIBAs, compounds 4–6), benzisothiazolone (BITAs, compound 7), pyridinioalkanoyl thioesters (PATEs, compounds 12–16), thiocarbamates (TICAs, compound 17 and 18), S-acyl 2-mercaptobenzamides (SAMTs, compounds 19–21), transition metal complexes (compounds 22–25), diselenobis (benzamides) (DISeBAs, compounds 26–29), and thioether prodrugs (compounds 30 and 31) [104]. All molecules shown in Fig. 11.8 are featured with a weak electrophilic group that is nucleophilically bound to the cysteine thiolate moieties of zinc finger motifs [107–114]. Following the formation of a covalent complex between inhibitor and NCp7, the native conformation of protein will

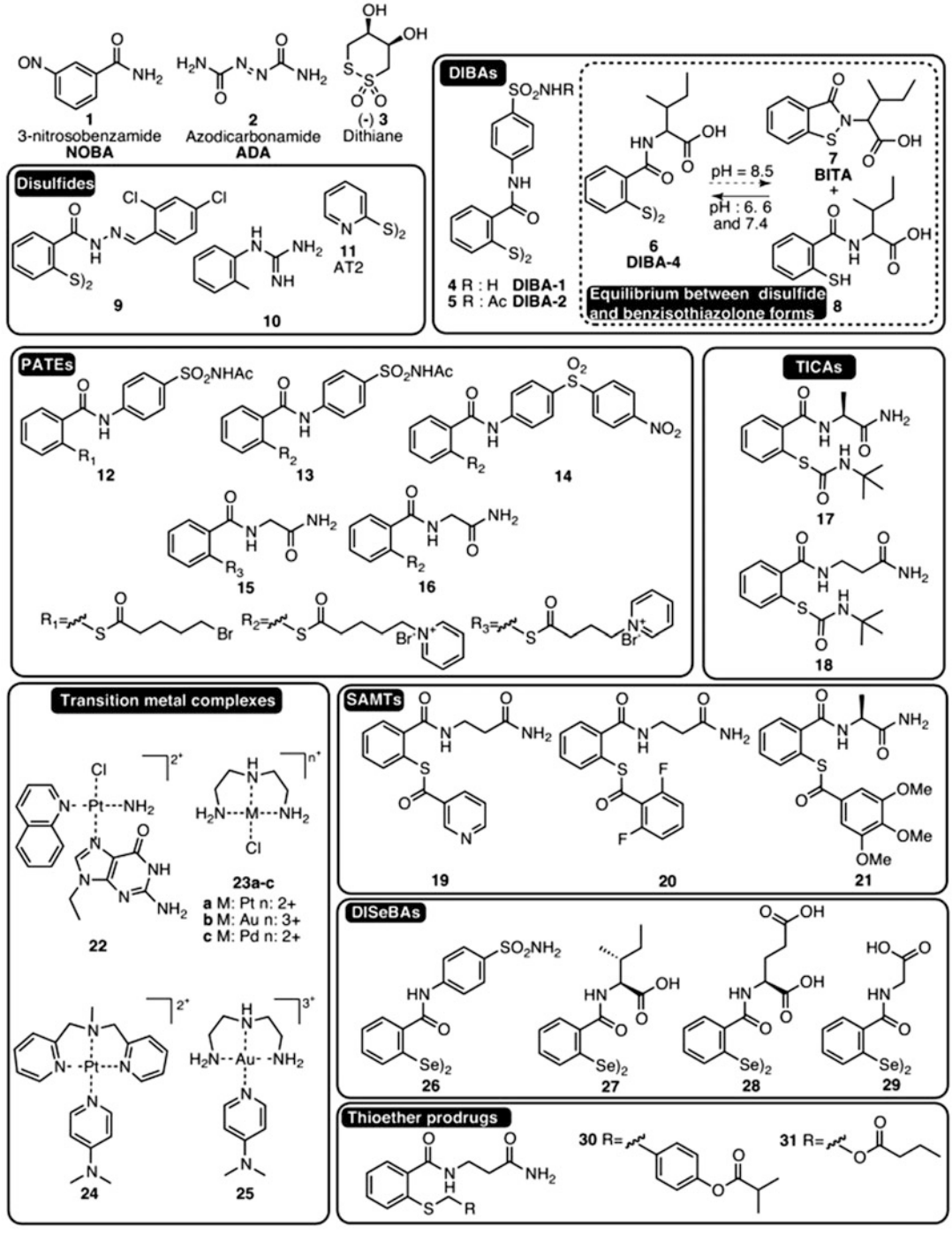


Fig. 11.8 Chemical structures of covalent inhibitors of NCp7. Reprinted with permission from Ref. [104]

be distorted, and consequently, the protein loses its activity [104].

11.3.5.3 Covalent Inhibitors against Reverse Transcriptase (RT)

RT is produced by the cleavage of a Gag-Pol polyprotein in HIV. The mature form of this enzyme consists of two large (p66, 560 amino acids) and small (p51, 440 amino acids) subunits [115]. Although numerous noncovalent inhibitors have been identified against RT [116], the emergence of resistance failed such treatment regimens [117, 118]. One of the most infamous mutations that is responsible for RT resistance is Y181C [117]. Hence, the new emerged residue, cysteine 181, has been explored as an attractive target for covalent inhibitor design. Compound 1, 2-naphthyl catechol diether binds to wild-type HIV-1 RT (Fig. 11.9a). Compounds 2–5 could react with the thiol moiety of Cys181 (Fig. 11.9b–d). Among them, compound 3 and 5 selectively alkylate the mutated residue, Cys181 without showing any toxicity in T-cell assay [119].

11.3.5.4 Covalent Inhibitors against HIV-1 Protease

A third protein that could be an intriguing target for covalent inhibitors is HIV-1 protease. In 2018, based on the 3D structure and mechanism of HIV-1 protease, the group of Raines, R. T. replaced the aniline group of darunavir (176) with phenylboronic acid moiety, which led to the identification of 177 with increased affinity with the protease by 20 times, and a high affinity for HIV-1 protease-resistant strain D30N (Fig. 11.9f) [120]. X-ray co-crystallization structure demonstrated that boronic acid group participated in triple hydrogen bonding, which was superior to the amino group in darunavir and other derivatives. The hydrogen bond distance between Asp30 (or Asn30) of protease and hydroxyl group of boronic acid was shorter than orthodox hydrogen bonding, which had a certain degree of covalence [120]. This is a reasonable explanation for high potency and remarkable anti-resistance profiles of boronic acid derivatives.

11.3.5.5 Covalent Inhibitors against HIV-1 Capsid

HIV-1 capsid plays critical roles in HIV-1 replication and, therefore, exhibits a distinguished drug target. The conical core of a mature HIV-1 comprises of 1500 capsid monomers assembled into 250 hexamers and 12 pentamers. To form the HIV-1 capsid, a 55-kDa Gag precursor proteolyzed into three folded proteins including matrix, capsid, and nucleocapsid and three small peptides (spacer peptides 1, spacer peptides 2 and p6) by viral protease. Inhibitors targeting viral capsid are capable of inhibiting HIV-1 replication at different steps of the viral life cycle. In 2016, a screening of a library containing 1280 compounds led to identification of ebselen [121]. This inhibitor covalently binds the C-terminal domain of HIV-1 capsid through a selenylsulfide linkage with active site Cys198 and Cys218 [121].

11.3.6 Covalent Inhibitors against Influenza

H5N1 avian influenza is a pathogenic virus that can be easily transmitted in mammals [122, 123]. Up to date, only a few drugs like zanamivir (Relenza) and oseltamivir (Tamiflu) have been discovered against the influenza virus (Fig. 11.10a) [124]. These drugs are designed to mimic the transition states of the natural substrate. Unfortunately, viral resistance to these drugs have led to the failure of treatment [125]. Therefore, finding new drugs is necessary to overcome the possible future influenza epidemics and pandemics. The genome of influenza A encodes 13 proteins [126]. Hemagglutinin, neuraminidase, and M2 ion channel are located on the surface of the virion, and hence, are entirely accessible targets for drug discovery [127, 128]. Among the three mentioned proteins, only neuraminidase is an enzyme and therefore, is the most prominent drug target against the influenza virus.

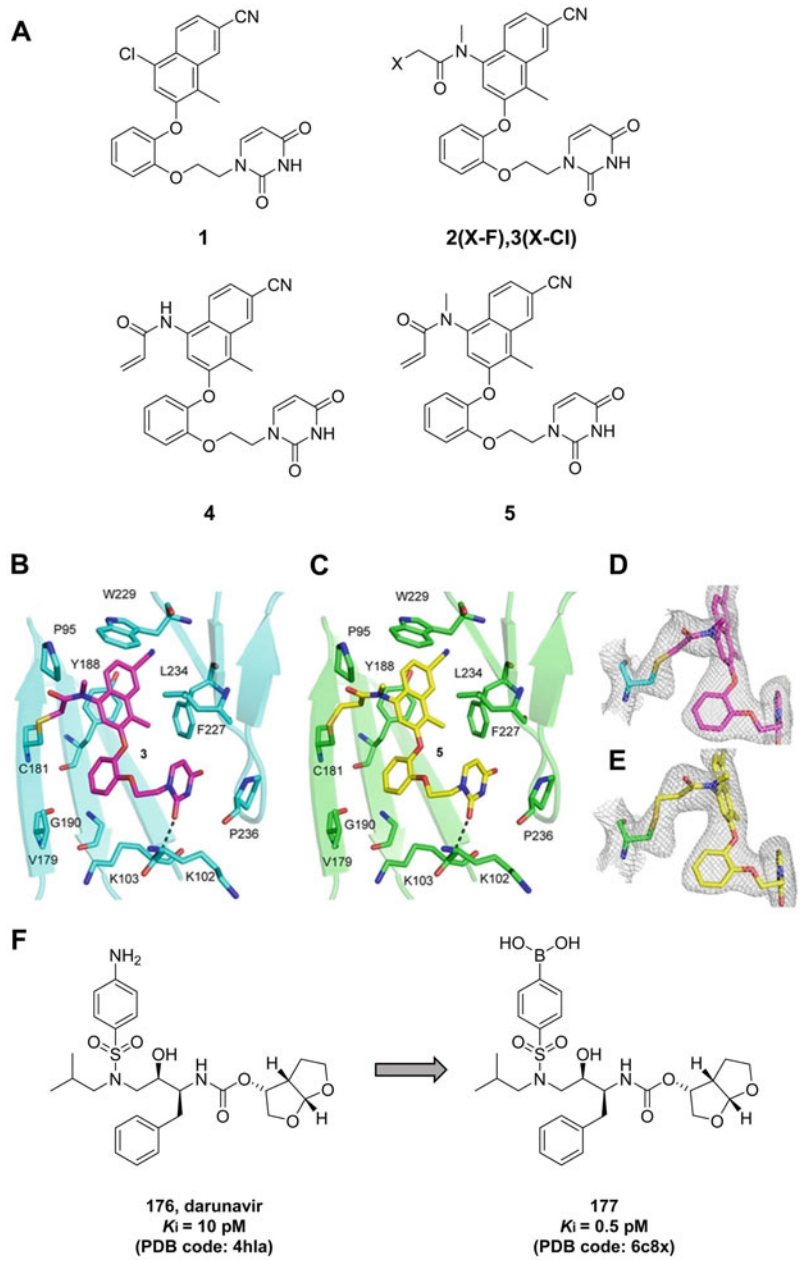


Fig. 11.9 Covalent inhibition of RT and HIV-1 protease. (**a**) Chemical formulae of covalent inhibitors of RT. (**b**) Stick model of compound 3, which forms a covalent bond with the thiol group of Cys181. (**c**) Stick model of compound 5, which forms a covalent bond with the thiol group of Cys181. (**d**) Continuous electron density map between compound 3 and Cys181. (**e**) Continuous electron density map between compound 5 and Cys181. Reprinted with permission from Ref. [119]. (**f**) Discovery of boronic acid-bearing sub-picomolar inhibitors of HIV-1 protease

11.3.6.1 Structure of Neuraminidase

Neuraminidase is a glycoprotein and it cleaves the α-ketosidic bond of terminally linked sialic acid. This enzyme, as glycosidase, selectively hydrolyzes N-acetylneuraminic acid, a predominant sialic acid in humans [129]. One of the most important residues is Tyr406 which is highly conserved in the active site of neuraminidase. This residue functions as a nucleophile in the catalysis [130–132]. Among the influenza neuraminidases, there is low similarity in their sequences. However, their active sites are highly conserved (100%), suggesting that they all possibly work in a similar manner.

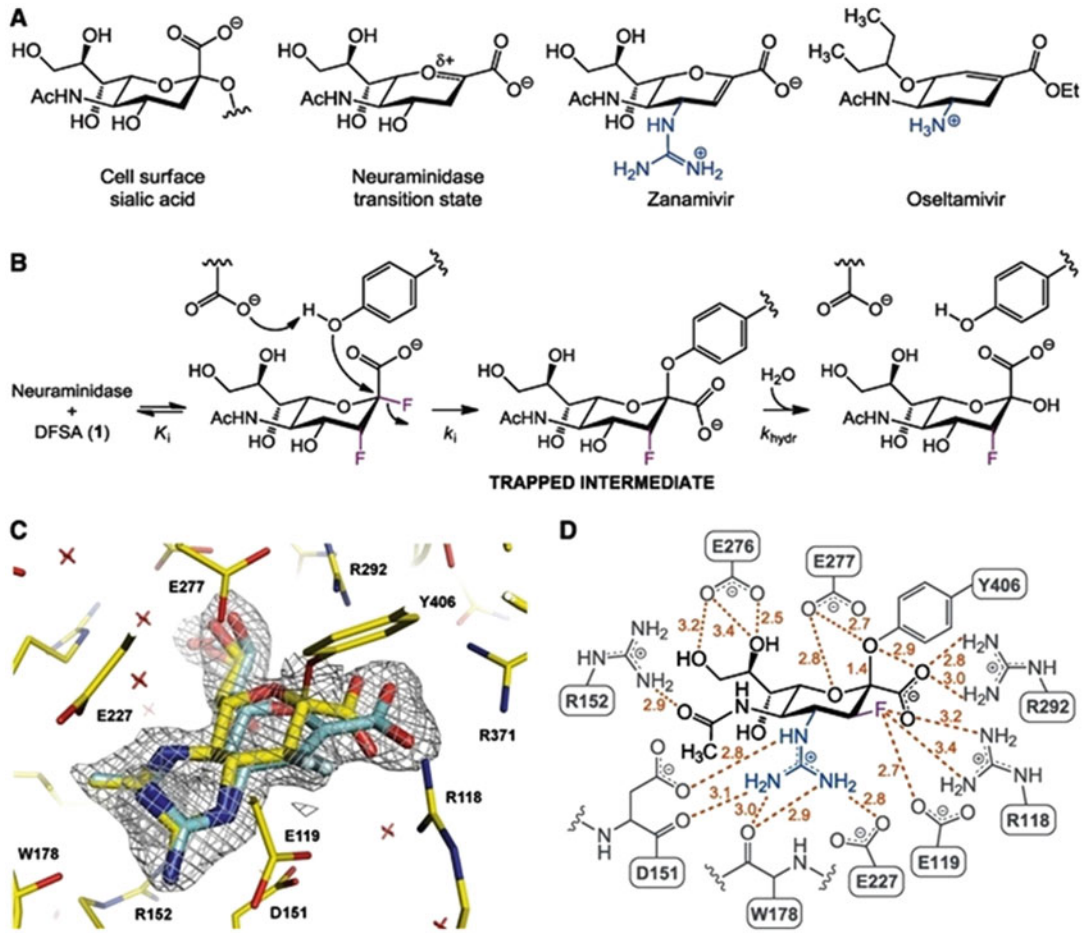


Fig. 11.10 Covalent inhibition of neuraminidase. (**a**) Chemical structures of sialic acid, neuraminidase transition state, zanamivir, and oseltamivir. (**b**) Mechanism of action of the 2,3-difluorosialic acid. (**c**) Electron density map of the active site of the neuraminidase trapped as its 3-fluoro(eq)-4-guanidino-sialyl-enzyme intermediate. The elimination product is shown in pale cyan. (**d**) The mode of interaction of sialic acid in the covalently inhibited enzyme

11.3.6.2 Structures and Mechanisms of Covalent Inhibitors against Neuraminidase

Fluorinated neuraminidases are similar to wild-type neuraminidase but have two fluorine atoms in their structures (Fig. 11.10a and b). These compounds have been studied for their anti- neuraminidase activity and are excellent choices for covalent influenza neuraminidase inhibitors [133–135]. In 2,3-difluorosialic acid, the electronegative fluorine atom at carbon 3 can destabilize the oxocarbenium ion-like transition states. On the other hand, fluorine atom at carbon 2 is a leaving moiety and causes the accumulation of the covalent intermediate (Fig. 11.10b). A covalent bond of 1.45 Å has been observed between carbon 2 of 3-fluoro sialic acid and the phenolic oxygen of Tyr406. The electron density map for this covalent bond is shown in Fig. 11.10c. The trapped covalent intermediate in the ionic residues of the neuraminidase active site is depicted in Fig. 11.10d [136].

11.4 In Silico Approaches in the Search for Covalent Inhibitors as Antiviral Agents

Before starting experiments in the lab, it is useful and important to employ a robust and accurate tool to model and design covalent inhibitors against pathogenic viruses. As seen in a variety of fields, using computers and computational software is a good approach as a starting point. The first step is to find the relevant target, which entirely depends on the virus to be eradicated. Several protein targets have been discussed in this chapter, which can be served as the targets for designing antiviral drugs. Moreover, one can search potential targets from RCSB protein data bank [137], a database containing more than 100,000 3D structures of proteins determined by X-ray diffraction, NMR, electron microscopy, hybrid, or other methods [138].

In the case that the 3D structure of a target is not available, homology modeling or protein threading (fold recognition) can be used to solve the problem [139]. The next step is to identify the binding site or active site of the desired target, which can be achieved by the crystallography data and literature review [140]. After that, the inhibitor can be designed against the target. Two strategies are usually used for inhibitor recognition and identification. The first strategy comprises the direct modification of published viral target inhibitors, through the addition of a covalent warhead by means of molecular modeling [140]. The second strategy encompasses virtual screening, to identify a set of noncovalent viral target inhibitor templates. Then, the templates will be converted to the covalent inhibitor by structural addition of the reactive warhead to a suitable position [137, 141]. In the virtual screening process, numerous ligands can be found from databases like ZINC [142], PubChem [143], ZincPharmer [144], ChemSpider [145], DrugBank [146], and ChEMBL [147]. To investigate the interaction mode and calculate the free energy of binding between a covalent inhibitor and viral target, covalent molecular docking can be performed using softwares like AutoDock [148], AutoDock Vina [149], CovalentDock [150], GOLD [151], Glide [152], CovDock [153], DOCKTITE [154]. An example of in silico application in antiviral covalent inhibitor design is discussed in detail below.

11.4.1 Covalent Inhibitor Identification against SARS-CoV-2 Main Protease by In Silico Study

In 2020, Alamri et al. employed the in silico studies to identify a covalent inhibitor against SARS-CoV-2 main protease [155]. As traditional methods for drug discovery are usually time-consuming and expensive, they used state-of-the-art simulation approaches [156, 157]. To figure out the evolutionarily conserved functional residues among SARS-CoV-2, SARS-CoV, and MERS-CoV, a multiple sequence and structure alignment analysis was accomplished. 3D structures and sequences of SARS-CoV-2 (PDB ID 6 LU7) [14], SARS-CoV (PDB ID 2A5I) [158], and MERS-CoV (PDB ID 5WKK) [159] main proteases were obtained from protein data bank. Alamri et al. utilized the commercial Asinex Focused Covalent library that consists of 1000 molecules (http://www.asinex.com/) to identify potential inhibitors having reactive warheads [155]. The SARS-CoV-2 main protease with PDB ID of 6 LU7 was used as target and prepared by "protein preparation" module [160] in the Maestro molecular modelling package. To screen the database, the covalent molecular docking was performed by a covalent docking (CovDock) tool implemented in the Maestro package.

In Alamri and his colleagues' study, catalytic residues His41 and His164 were set as δ-protonated, while His163 and His172 were assigned as protonated and double protonated, respectively. The compounds of library were prepared with "LigPrep" tool of Maestro. In addition to Michael addition, Alamri et al. assigned the other covalent reaction types implemented in

CovDock, including nucleophilic addition to a double and a triple bond, nucleophilic substitution, and aryl- and nitrile-activated conjugate addition to alkyne, and Cys145 was selected as the covalent bonding residue in the protein active site [155]. Molecular dynamics simulations and MM-GBSA calculations also were engaged to investigate the binding mode of inhibitors in the active site of viral main protease and calculate the free binding energy, respectively.

For docking validation, co-crystalized N3 peptide was re-docked toward the main protease by employing the Michael addition reaction [155]. All N3 peptide poses successfully formed a covalent bond with the thiol moiety of the catalytic cysteine. As a positive control and reference, MM-GBSA score and covalent docking affinity score of N3 peptide were − 79.8 and − 9.551 kcal/mol, respectively.

Usually, the covalent virtual screening approach is composed of two consecutive steps: 1) screening and selecting the best pose with its reactive group in close proximity to Cys145 and 2) the virtual formation of a stable chemical bond between the compound and the viral target residue [161, 162]. After virtual screening study, Compounds 51 (N-(benzo[d][1,3]dioxol-5-ylmethyl)-2-cyano-3-(1-(2-((2-methoxyethyl) amino)-2-oxoethyl)-1H-indol-3-yl)propanamide), 78 ((3S)-3-hydroxy-3-((4E,6E)-3-hydroxy-7-phenylhepta-4,6-dien-1-yl)-1-methylindolin-2-one), and 223 ((4R,5R)-1-(furan-2-ylmethyl)-5-(4-hydroxy-3-methoxyphenyl)-4-(3-methoxybenzoyl)-2-oxopyrrolidin-3-olate) were identified as potent covalent inhibitor against SARS-CoV-2 main protease [155].

Structural analysis of Alamri team revealed that compound 78 was the first rank from the "nucleophilic addition to double bond" screen based on the CovDock score, and from its β-Carbon of the indoline moiety formed a covalent bond with the Cys145 thiol group. In addition to the covalent bond, some noncovalent bonds were formed between compound 78 and the enzyme active site. For instance, the carbonyl carbon of the indoline formed a hydrogen bond with the binding site's His163. Compound 51 was a potential covalent inhibitor from the third reaction type, nitrile-activated conjugate addition to alkyne. A covalent bond (1.9 Å C-S distance) was formed between electrophilic β-C atom present between the indole moiety and the nitrile, and Cys145. Also, the indoline moiety of this inhibitor interacts with His41 via hydrophobic interaction. Furthermore, compound 51 made a hydrogen bond with Glu166 (2.51 Å) located at the S1 subsite. A third compound, compound 223 formed a covalent bond with the reactive nucleophilic thiol group of Cys145 (1.83 Å C-S distance) [155]. An H-bond also formed between the terminal furan ring and the side-chain nitrogen atom (NE) of His163 (at S1 subsite). The furan ring also makes van der Waals interactions with residues Glu166 and Phe140.

11.5 Conclusions and Future Perspectives

As new pathogens, such as SARS-CoV-2, continue to emerge and cases of drug resistance climb, the design and development of new drugs with high potency and specificity are highly demanded, especially when there is a lack of specific drugs and vaccines against the viral infection. Theoretically, covalent antiviral agents are more efficacious than noncovalent ones in viral eradication by halting the life cycle of target receptors. Therefore, up to now, numerous prospering examples of drugs acting through a covalent mode of action are on the market. However, possible nonspecific binding of covalent antiviral agents to proteins, nucleic acids, and other non-vital biomolecules raises a major concern about their toxicity. Potentially short half-lives of these molecules also have restricted the development of covalent drugs for a long time. These shortcomings of covalent antiviral agents may be mitigated by using nanoparticle drug delivery systems, which are able to prolong circulation time, cross biological barriers, and target to viruses or virus-infected cells, thereby reducing off-target toxicity while increasing therapeutic efficacy [8, 163–167].

Furthermore, to increase the specificity of binding with target sites and reduce off-target

toxicity, covalent enzyme inhibitors can be optimized to balance reactivity and selectivity. The covalent inhibitors can be modified from already optimized noncovalent inhibitors by introducing appropriate warheads. Almost in all cases, a warhead with electrophilic property forms a covalent bond with the nucleophilic atom, that is, an electron-rich atom, of a residue in the viral active site. Targeting the non-catalytic residue in the active site of viral proteins can increase the covalent inhibitor selectivity. Along with this, in silico approaches can be utilized to design and discover more selective inhibitors with potentially lower toxicity. An increase in the number of covalent therapeutics approved by the FDA indicates that more covalent inhibitors will be identified, developed, and enter clinical trials in the coming years.

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Safe-in-Man Broad Spectrum Antiviral Agents

12

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Abstract

Emerging and re-emerging viral diseases occur with regularity within the human population. The conventional 'one drug, one virus' paradigm for antivirals does not adequately allow for proper preparedness in the face of unknown future epidemics. In addition, drug developers lack the financial incentives to work on antiviral drug discovery, with most pharmaceutical companies choosing to focus on more profitable disease areas. Safe-in-man broad spectrum antiviral agents (BSAAs) can help meet the need for antiviral development by already having passed phase I clinical trials, requiring less time and money to develop, and having the capacity to work against many viruses, allowing for a speedy response when unforeseen epidemics arise. In this chapter, we discuss the benefits of repurposing existing drugs as BSAAs, describe the major steps in safe-in-man BSAA drug development from discovery through clinical trials, and list several database resources that are useful tools for antiviral drug repositioning.

Keywords

Broad spectrum antiviral agents · Drug repurposing · Drug discovery · Antivirals · Databases

Abbreviations

2D	2-dimensional
3D	3-dimensional
ADME	Absorption, distribution, metabolism, and excretion
BSAA	Broad-spectrum antiviral agents
CC50	Half maximal cytotoxic concentration
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CHIKV	Chikungunya virus
COVID-19	Coronavirus disease 2019
CPE	Cytopathic effect
CRISPR	Clustered regularly interspaced short palindromic repeats
DENV	Dengue virus
DMPK	Drug metabolism and pharmacokinetics

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dNTP	Deoxyribose nucleotide triphosphate
EBI	European Bioinformatics Institute
EC50	Half maximal effective concentration
ECM	Extracellular matrix
hAAV-2	Human adeno-associated virus 2
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV-1	Herpes simplex virus 1
iPSC	Induced pluripotent stem cells
LDH	Lactate dehydrogenase
MDCK	Madin-Darby Canine Kidney
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NHP	Non-human primate
NS5A	Non-structural protein 5A
NV	Norovirus
PD	Pharmacodynamics
PDB	Protein data bank
PK	Pharmacokinetics
PPi	Protein–protein interaction
RCSB	Research Collaboratory for structural bioinformatics
RdRp	RNA-dependent RNA polymerase
RPE	Retinal epithelial cells
RSV	Respiratory syncytial virus
SAR	Structure–activity relationship
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SI	Selectivity index
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

12.1 Introduction

Viruses are found ubiquitously in nature, infecting species from all five kingdoms of life. Despite being accepted and studied as a vector of human disease relatively recently, viruses make up for 75% of newly discovered pathogens [1]. This is primarily due to their intrinsically high rate of mutation, allowing them to expand genetically across many environments and hosts. Because of this ability to expand their reach, viruses with established, equilibrated relationships with hosts of one species can frequently gain the ability to infect another, previously unsusceptible species, causing emerging viral diseases [2]. To humans, this constant beat of viral emergence poses a threat to the global economy, public health, and lives.

Unlike bacteria that have highly conserved cell wall structures, enzymes for protein and nucleic acid synthesis, and intermediary metabolism, viruses have fewer unifying features to target. Because of this, antiviral development has classically followed a 'one drug, one virus' paradigm. Unlike bacterial infections that could be treated with a broad range of antibiotics, viral infections need to be targeted with specific antivirals, of which there exist only a handful, not nearly enough to cover the 259 known viral pathogens that infect humans [3, 4]. This leads to difficulties in combating viral infection, especially in emerging human diseases where inability to predict the next emerging virus can lead to epidemics and pandemics.

The rise of broad-spectrum antiviral agent (BSAA) development represents a shift in the 'one drug, one virus' paradigm. BSAAs act against a range of viruses by targeting conserved processes or structures, such as nucleic acid synthesis or viral protease. Alternatively, BSAAs can target commonly used host factors that assist with viral entry and replication [3]. BSAAs can lessen the impact of emerging diseases, because they can be leveraged as a potential treatment option for emerging viruses as soon as they surface.

Drug repurposing (also known as drug repositioning, drug reprofiling, drug redirecting, or drug re-tasking) is a strategy of drug development that focuses on finding new indications for existing drugs or drug candidates. This holds several advantages over de novo drug discovery, one of the most compelling being that many of

these drugs and drug candidates have already been demonstrated to be safe in humans by passing phase I clinical trials. While the first instances of drug repurposing have been based on serendipitous discoveries (such was the case for Viagra and Thalidomide), more systematic approaches have been developed as technological resources become more advanced. However, drug repurposing against viral diseases still represents a small percentage of total drug repurposing efforts [3].

Safe-in-man BSAAs are partially or fully developed compounds that have passed a minimum of phase I clinical trials and have shown antiviral activity across at least two different viral families [5]. By focusing on discovering and expanding the activity of drugs that have already been proven to be safe in humans and have broad antiviral activity, researchers can increase the activity spectrum of our available antiviral agents with less time, money, and resources than would be required of traditional drug discovery.

This chapter will highlight the case for the discovery and development of safe-in-man BSAAs and discuss major steps in the safe-in-man BSAA repositioning process. At the end of this chapter, several knowledge tools that are especially applicable to antiviral drug repositioning will be listed and described.

12.2 The Case for Safe-in-Man Drug Repurposing and Broad-Spectrum Agents

A hugely profitable industry, global pharmaceutical spending is predicted to surpass $1.5 trillion by 2023. For a chance to win a slice of this gargantuan pie, pharmaceutical companies spare no effort in the discovery and development of new drugs, only an average of 54 of which are predicted to be approved and launched per year from 2019 to 2023 [6]. With so much money at stake and a stunningly high failure rate, it should come as no surprise that any measures that reduce cost, time, and other resource use could be highly beneficial.

Conventionally, de novo drug development begins in the discovery phase, where targets are identified and lead compounds are selected and optimized. Following the discovery phase is the preclinical phase, where toxicology, DMPK (drug metabolism and pharmacokinetics), and ADME (absorption, distribution, metabolism, and excretion) studies are performed in animal models in preparation for the first human trials. Finally, phase I, phase II, and phase III clinical trials are conducted, establishing safety and efficacy in humans before it can finally be approved and launched as a new active substance. When a drug is finally approved and launched, it will have cost an average of $1.5 billion to develop and will have taken a bare minimum of 10 years to get from drug discovery to market [7].

Unfortunately, most drugs that undergo clinical trials never continue through to approval and launch. Although pitfalls exist along the entire drug development path, a sizeable portion of this failure lies with clinical trials. Most investigational drugs pass phase I clinical trials, with approximately 70% of drugs entering phase II. However, following phase I, only 8–10% of drugs will pass both phase II and III clinical trials and continue through to approval [8]. The most common reasons for failing these latter phases are safety concerns, disproportionate adverse effects, or lack of efficacy.

Many of the investigational drugs that have fallen off during clinical trials do so because of an inability to demonstrate sufficient efficacy. However, it is possible that some compounds in this ‘Valley of Death’ have several biological targets and exhibit more bioactivity than what was originally investigated. Indeed, many investigational anti-cancer drugs have been shown to exhibit potent antiviral activity in vitro and in vivo [9–15]. By re-directing drug discovery efforts into repurposing investigational drugs that have passed at least phase I of clinical trials, many efforts can be spared in the drug development process. Because these compounds have already been well-characterized in previous pre-clinical and clinical safety studies, these steps can be largely bypassed, allowing the price

tag for the development of these drugs to be decreased to as low as $8.4 million [16].

Viral diseases often disproportionately affect countries with lower income, and thus are overlooked by many pharmaceutical companies due to their low profit potential. However, antiviral drug development is nonetheless crucial to maintaining a functional society at today's population densities, as viruses continue to emerge from natural reservoirs and cause outbreaks among the human population. One barely needs to consider the overwhelming impact on public health, society, and the global economy brought on by the COVID-19 pandemic to understand why viral outbreak preparedness should have always been a foremost priority for the global community. The dramatically lowered price tag of drug repurposing allows researchers to overcome the often-insurmountable barriers to developing a drug from scratch, thus stimulating more participation and innovation in the field of antiviral research. Ultimately, this will translate to a greater number of therapeutic options against viral disease available to the general public.

In addition to the financial barriers discussed above, the nature of virus-borne disease often requires a level of preparedness against unknown or unspecified emergent viruses that may arise from natural reservoirs at random times. In the event that an emergent disease arises for which there is no effective drug to counter, drug developers must then be able to respond with a level of haste that is usually not required for other diseases. Currently, the conventional model for antiviral drug development fails to support either the requirement of preparedness or haste. However, development of broad-spectrum antivirals can increase pandemic preparedness because they target common biological functions within a wide range of viral families. In this way, they are much better positioned to be frontline drugs in the face of emerging viral diseases. Moreover, by repositioning phase I (or farther) drugs for these purposes, development time can be shortened by at least 5 years, allowing for a faster response to potentially life-threatening epidemics [7]. Importantly, if a potential antiviral agent is repositioned from an already-approved drug, doctors can legally prescribe them for off-label use immediately, thus allowing for the drug to be used even faster [17].

Finally, it is important to acknowledge the toll on other resources that can be spared when focusing on drug repurposing for broad-spectrum antiviral development. In addition to money and time, the drug development process is a major contributor to waste production, energy use, pollution, and CO_2 emissions. A study in 2019 estimates that the pharmaceutical industry pumps out 48.55 tons of CO_2 equivalents per million dollars, a value that is 55% greater than the automotive industry [18]. Part of this heavy environmental impact can be easily reduced by eliminating redundancies. Pivoting some drug discovery and development efforts towards drug repurposing and focusing on broad-spectrum agents is, therefore, one important way to reduce the strain on finite resources.

12.3 Methods for Safe-in-Man Broad Spectrum Antiviral Development

12.3.1 In Silico Methods for Drug Discovery

When searching for repurposed BSAA candidates, in silico methods are often used first due to their powerful, high-throughput, and low-cost nature. These techniques often centre on making novel connections between broad concepts like viruses, targets, and drugs, based on shared traits from the pool of existing data. Further experiments in vitro and in vivo are needed to further validate these results, as many of these discovery methods have high false-positive rates by nature. Moreover, these techniques rely on the aggregation and processing of existing data, and therefore may not be suitable for drug discovery endeavours for new drugs or new viruses for which no information is yet known. Common in silico methods include molecular docking studies, network-based modelling, and text mining (Fig. 12.1a).

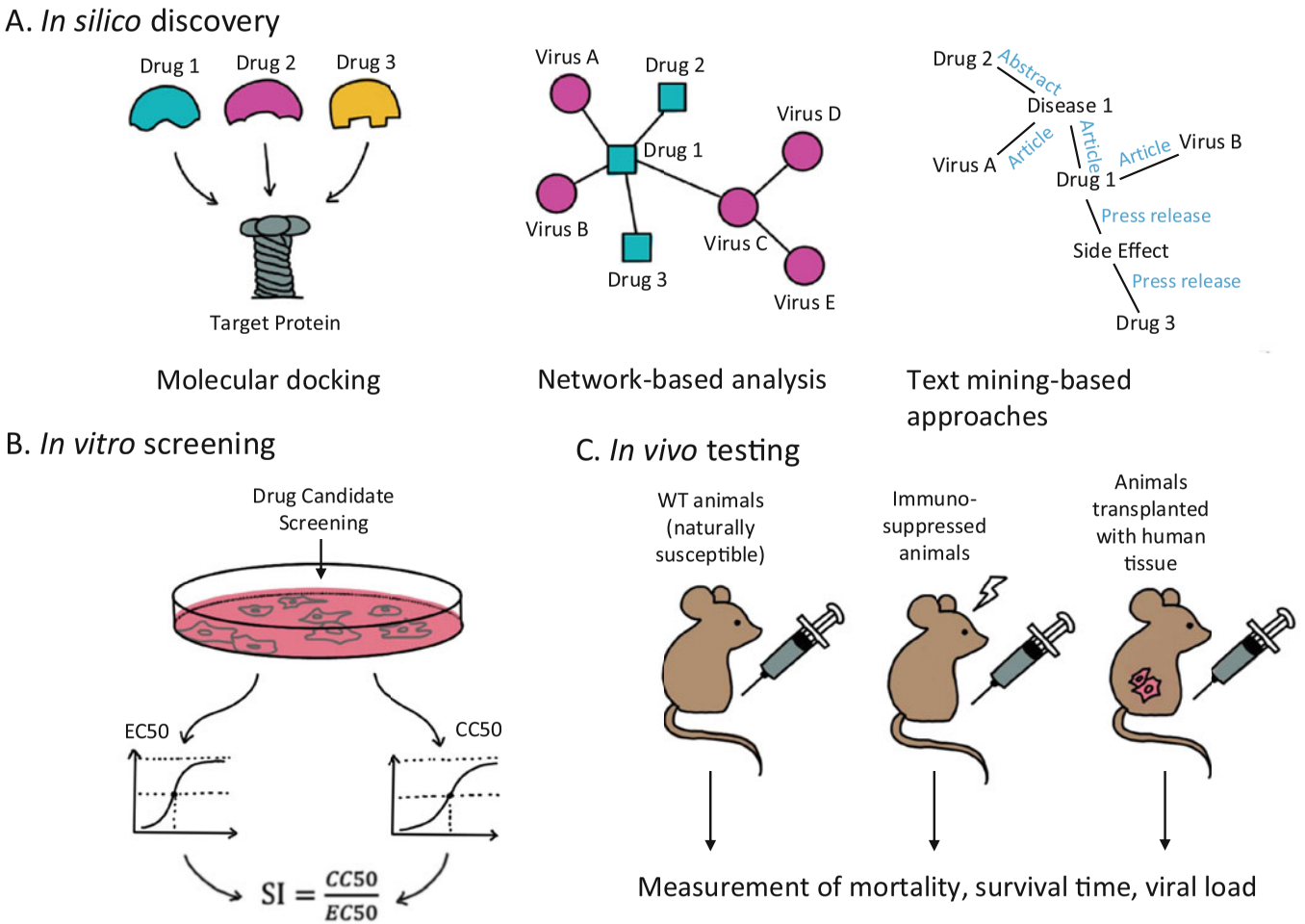

Fig. 12.1 The preclinical stages of BSAA repositioning. (A) In silico discovery of safe-in-man BSAAs. In molecular docking studies, drug and target structures are analyzed and interactions are simulated. In network-based analyses, networks are constructed of drugs, targets, or both. Drugs and viruses that are connected by few nodes are more likely to be connected themselves. For example, if Virus A is inhibited by Drug 1 and Drug 1 is structurally similar to Drug 2, then Drug 2 may have

Fig. 12.1 (continued) previously unknown activity against Virus A. Similarly, if Drug 1 is known to work against Virus C and Virus D and E are in the same family, then Drug 1 may have previously unknown activity against Virus D and E. In text mining–based approaches, networks are constructed from concepts taken from text documents such as research articles, abstracts, and press releases. Concepts are then connected in the network. For example, if Virus B is mentioned in connection with Drug 1, and both Drug 1 and Drug 3 are often mentioned in connection with a specific side effect, then Drug 3 may have previously unknown activity against Virus B. (B) In vitro screening of drug candidates. Cell lines, primary cells, or organoids are grown in cell culture and treated with virus. EC50 and CC50 values are determined and SI values are determined. (C) In vivo testing of final drug candidates. Infected animals are either naturally susceptible to the virus under evaluation, immunosuppressed, or transplanted with human tissue to become more susceptible to virus

12.3.1.1 Molecular Docking

Molecular docking is a technique that attempts to simulate interactions between a drug candidate and a drug target [19]. To accomplish this, 3D structural data for a potential drug target and a structurally characterized compound are extracted from a protein or drug database. Then, the interaction is simulated through a variety of docking algorithms. The top scoring drug-target pairs can then be selected for further testing in in vitro studies. This technique is commonly used in drug repurposing studies for all diseases, but is particularly applicable to antiviral drug discovery, where the small number of potential virus and host targets involved allows for a more exhaustive analysis without requiring additional time, computational power, or both.

Because of its applicability to antiviral discovery, molecular docking techniques have already been used extensively for antiviral-directed drug repositioning. For example, in an effort to combat re-emerging chikungunya epidemics, Tripathi et al. [20] screened an FDA-approved drug library for possible interaction with the nsP2, an essential protease in the CHIKV life cycle. From this screening, they identified telmisartan, an approved antihypertensive drug, and novobiocin, an approved antibiotic, for further studies for anti-CHIKV activity. Similarly, this screening strategy has played an important role in identifying drug candidates against SARS-CoV-2. Ribavirin, remdesivir, sofosbuvir, galidesivir, and tenofovir are all pre-existing drugs that have been identified as potential anti-SARS-CoV-2 agents through molecular docking against SARS-CoV-2 - RNA-dependent RNA polymerase (RdRp) [21]. Similarly, a molecular docking screen of over 10,000 compounds from the MolPort database against SARS-CoV-2 Main protease (M^{pro}) of returned nine naturally derived compounds for potential further antiviral development [22].

Antiviral screening can also be done by singling out a single compound and screening against a library of molecular targets. This method has been demonstrated successfully by Rizwana et al. [23], who used molecular docking to test interactions between the antiviral valacyclovir, an anti-herpes medication, against a library of viral proteins. Through this, they were able to identify a viral protein from DENV as a potential target of valacyclovir, suggesting that the previously specific antiviral may indeed have broad-spectrum antiviral qualities.

Several molecular docking tools exist in the literature. The two most widely used program packages are the Amber suite of biomolecular simulation programs [24] and the AutoDOCK suite [25]. Other tools include the AADS (Automated Active Site Detection, Docking, and Scoring) protocol, which combines an automated active site detection algorithm with an attached molecular docking protocol [26]. Additionally, several web-based molecular docking tools such as ParDOCK and Sanjeevini are freely accessible and can be used online [27, 28].

While a useful tool, molecular docking techniques have some limitations. First, molecular interaction simulations are computationally expensive, and large amounts of both computational power and time are required for performing screenings. This may serve has a barrier to entry for researchers without the computational resources to handle the large amount of data processing. Another limitation is the requirement for accurate and detailed structural information for drug targets, which can be difficult to obtain, even from protein structure databases. This lack of information will ultimately lead to high false positive rates, confounding the results of the study [29].

12.3.1.2 Network-Based Modelling

With the advent of network and systems biology, many more computer-based drug discovery methods have focused on the construction of networks. Network-based modelling consists of leveraging existing omics-level data to build and visualize networks that could be used to yield new drug/target connections. A network consists of nodes (representing an individual element such as a gene, protein, virus, or disease) and edges (representing a relationship between two or more nodes), which represent a subset of a biological system. These systems could be gene regulatory networks generated from transcriptomics data,

protein–protein interaction (PPi) networks generated from proteomics data, metabolic networks generated from metabolomics data, or other drug- or condition-specific datasets [30].

In general, network-based approaches follow a common framework: (1) gathering information from databases, (2) constructing the network, (3) derive new information from a starting data point, and (4) uncover new biological connections. To derive new information from the network, one can use clustering approaches, which takes advantage of the assumption that proteins or drugs in the same module of a network will likely have the same biological properties; or propagation approaches, in which the starting data point is assumed to be passed along to the connected nodes within the network. Although any kind of network can be used for drug discovery and repositioning, the field of BSAA repositioning derives most use from three main types of networks: PPi networks, drug–target interactions, and inter-drug connections.

Protein–protein interaction network modelling links proteins that are associated with each other and infers that associated proteins can be targeted similarly. For example, if one protein in the network is already a known drug target, other proteins connected to that node would become candidates for new drug target discovery. An innovative use of PPi network was done by Bösl et al. [31], who constructed a PPi network of human and virus proteins from existing databases. They then identified the host subnetworks most commonly targeted by viral proteins, thus returning a list of high-impact proteins that are likely to be antiviral drug targets that can inhibit a broad range of viruses.

Unlike PPi networks which have one type of node, drug–target interaction networks are constructed with two types of nodes: drugs and targets. A connection exists between a drug node and a target node if a drug is known to interact with a target, while a connection exists between two drugs or two targets if there is structural, chemical, or mechanistic similarities between them. The underlying assumption behind drug-target network modelling is that proteins targeted by the same drug may themselves be similar, and that drugs acting on the same protein may also be similar. Taking advantage of this assumption, Yi et al. [32] identified the structurally similar RdRps of DENV and NV as a potential target for BSAA development. Molecular docking was then used to identify entrecitinib, an approved anticancer medication, as a potential BSAA against DENV and NV.

Lastly, inter-drug networks are built with drugs that have shared similarities. For example, if two drugs have similar side effects or similar gene expression profiles, they would be connected. Two drugs could also be connected if their physical properties aligned with one another, such as if two drugs were both small and hydrophobic. These models use the assumption that compounds with similar properties would induce similar biological responses [33]. To cite an example in antiviral development, many amphiphilic drugs that were not originally developed as antivirals have been shown to exhibit antiviral activity towards a wide variety of human viruses, such as flaviviruses, filoviruses, herpesviruses, and coronaviruses [34].

The limitations of network-based modelling are similar to those of molecular docking: these discovery techniques rely on availability of accurate information. A starting target/drug pair is needed to extract information from the network, which means that this tool cannot uncover drug-target connections that is not linked by existing data already. In addition, network modelling only serves as guidance, as our current technology does not allow us to translate a network response into any kind of meaningful, qualitative prediction in vivo.

12.3.1.3 Text Mining–Based Approaches

If construction of networks from public databases leads to no results, text mining methods could also be used to identify potential drug-virus associations. Instead of using protein or drug databases, these techniques gather data from scientific literature databases, news articles, press releases, and other publicly accessible text documents. Like network-based approaches, most text mining approaches build ‘networks’ of

terms by connecting related concepts. Most of these methods are based on the Swanson 'ABC' model, which states that if two unconnected concepts ('A' and 'C') have a shared connection to a third concept ('B'), the original two may also be connected [35].

Especially due to new developments in natural language processing techniques, many drug repurposing efforts have been carried out with text mining methods [36, 37]. To accomplish these, several freely accessible, online-based text mining tools are available. Perhaps one the oldest resources for this is PolySearch, a biomedical text mining tool that returns associations between diseases, genes, drugs, toxins, metabolites, pathways, organs, tissues, organelles, clinical manifestations, drug mechanisms, Gene Ontology and MeSH terms, ICD-10 medical codes, and biological and chemical taxonomies mined from PubMed, Wikipedia, US patents, and text-rich knowledgebases [38]. Other publicly available biological text mining tools include DrugQuest, BEST, and KinderMiner [39–41].

12.3.2 In Vitro Studies

Once an existing drug has been identified for potential BSAA activity, in vitro experiments are performed to validate antiviral activity in a real biological setting (Fig. 12.1b). These studies are also often used to determine the potency of antiviral activity of a BSAA, as well as its cellular toxicity. This is often expressed as the concentration of a drug that is needed to achieve 50% of its maximal inhibitory activity (EC50) and the concentration of a drug that is needed to kill 50% of cells in culture (CC50). Strong drug candidates should have high potency (indicated by a low EC50 value) while having low toxicity (indicated by a high CC50 value). This selective ability of an antiviral to inhibit viral activity without harming cells is expressed as the selectivity index (SI = CC50/EC50). Thus, a compound with a higher selectivity value generally indicates a more effective compound.

In vitro studies can assess antiviral efficacy and potency in two main ways: measuring cell viability due to a virus' cytopathic effect (CPE) and measuring viral infection. To measure viability within a cell population, an indicator that often directly correlates with live or dead cells is used and quantified using colorimetry, chemiluminescence, or fluorescence. Mitochondrial reductase activity could be measured by the addition of resazurin or tetrazolium-based dyes such as MTT, MTS, and XTT, and cell viability could be quantified by a colour change of the dye once it is reduced. Alternatively, metabolic activity could be quantified by commercially available assays for esterase activity, ATP production, glycolytic flux, or oxygen consumption. Membrane integrity is also a commonly used correlate of cell viability, with membrane-impermeable dyes and extracellular LDH detection both being used as an indicator of cell death. Finally, apoptosis-related assays such as TUNEL (measuring DNA fragmentation), Annexin V (measuring phosphatidylserine externalization), and Caspase (detecting the levels of apoptotic caspase activity in cell lysates) can also be used to quantify cell viability.

Although cell viability can be an indicator of viral activity, the two are not perfect correlates. Cells infected by virus may not die immediately, and it is difficult to attribute cell death to viral infection instead of external factors. Because of this, quantifying viral infection could be an alternative approach to studying antiviral activity in vitro. This can be done by detecting parts of viruses using immunoassays, microarrays, or high-throughput sequencing techniques like RT-qPCR and RNA-Seq [42–46]. The standard method of determining virus titers is a plaque assay, which is often used to quantify the amount of infectious viral particles in a sample. To accomplish this, dilutions of a virus sample are incubated on a susceptible cell monolayer and covered with a gel. Then, the resultant patches of dead cells (plaques) are counted to determine the number of virus particles in a sample.

More versatile methods of viral quantification have also been developed, often using engineered viruses that express fluorescent proteins or luciferase, which allow for real-time surveillance and quantification of virus infection [47–52]. A similar reporter system can be set up for retroviral

infections, in which the host cell is made to express a reporter gene under the control of a promotor that is located on the virus, allowing for the continual detection of infected cells in which a virus is not actively replicating [53, 54]. Perhaps one of the most innovative additions to the antiviral research toolbox is the use of CRISPR-Cas technology to target viral RNA particles and subsequently cleave signal molecules from their quenchers, resulting in signal amplification and detection of very small amounts of virus [55, 56].

There are several different models to study antiviral activity in vitro, including cell lines, primary cells, stem cell-derived cells, and organoids. Typically, a variety of these models are needed to generate a convincing dataset for a drug candidate, and a complete published study of antiviral activity could include data from experiments on a combination of multiple cell lines, primary cells or iPSC-derived cells, and organoids [57].

12.3.2.1 Cell Lines

The first in vitro studies are often done in cell lines, because they are relatively easy to obtain and grow. However, different cell lines can vary in their susceptibility to viral infection, and care needs to be taken in selecting a cell line that will accurately reflect the infection cycle of the virus under investigation. One of the most broadly used cell lines in antiviral research is the Vero cell line and its derivatives, although many cell lines are used specifically for the virus model they are meant to study (to give an example, MDCK cells are a commonly used cell line in experiments involving influenza). It is important to note that large genomic differences exist between cell lines and human primary cells, which can lead to deviations in results between cell lines and primary cells. Moreover, many of the cell lines used for antiviral research originate from non-human species, possibly further confounding results. Therefore, experiments done on cell lines will almost always need further validation.

12.3.2.2 Primary Cells and Stem Cell-Derived Cells

Unlike immortalized cell lines, primary cells are harvested directly from tissues, are usually slower growing, and have a finite lifespan. Additionally, they are often highly differentiated, and can better represent an infected organ system to more accurately reflect a cellular response to infection. For these reasons, they are often used to validate data obtained from cells lines [58–61]. Importantly, factors such as age, sex, and genetic differences between donors must be considered when choosing primary cells to work with, as these small changes can play a huge role in a cell's response to viral infection [62, 63]. Primary cells also have their limitations. Like cell lines, not all primary cells are susceptible to all viruses. Moreover, primary cells often have more stringent growing conditions, may require specialized media, have a slower division time, and need to be regularly harvested, all of which increases the cost and effort needed to maintain them.

If primary cells are difficult to obtain, differentiated cell types may also be generated from induced pluripotent stem cells (iPSCs). iPSCs are often derived from an easily obtainable origin, such as blood or skin cells, and are reprogrammed into a pluripotent state. From there, the iPSCs can be supplied with differentiation factors to redirect its differentiation to a cell type of choice. However, generation of iPSC-derived cells can be time-consuming and labor-intensive, and they may respond to viral or drug treatment in unpredictable ways. For example, thymidine kinase of Herpes simplex virus type 1 (HSV-1) is commonly inserted into cells to create an inducible cell death system triggered by ganciclovir. However, Iwasawa et al. [64] reported that expression of thymidine kinase itself was toxic to iPSCs, irrespective of the addition of ganciclovir, suggesting that iPSCs have a markedly different response to small changes in dNTP levels from other cells. Nonetheless, iPSC-derived cells are often used in antiviral studies when attention needs to be focused on a specific tissue type, such as in the case of studying

Hepatitis B virus in hepatocytes, or Zika virus in neuronal cells [65–67].

12.3.2.3 3D Cultures and Organoids

One further method to mimic true physiological conditions in vitro is by experimenting on 3D cell cultures. It is often criticized that cells grown in 2D monolayers do not sufficiently mimic the complex intercellular interactions that occurs in the body. To alleviate these differences, ECMs and other hydrogels are often used as scaffolds to encourage 3D attachments and geometries in cell cultures [68]. In 2011, Straub et al. demonstrated the first successful in vitro culture of NV was achieved by infecting them in a 3D-cultured colorectal cell line [69]. In addition, keratinocyte raft cultures have been used to study both human papillomavirus (HPV) and human adeno-associated virus-2 (hAAV-2), while more complex 3D cultures of keratinocytes have been used to study Poxviruses [70–72].

Organoids are a form of 3D cell culture that is derived from the progenitor cells of a certain organs. They are self-organizing, and therefore can have more complicated structures than man-made 3D scaffolds. Organoids are important models for viral infections that affect whole organ systems, such as Zika virus on brain organoids [67, 73, 74] or rotavirus on intestinal organoids [75–79].

12.3.3 In Vivo Studies

After proof of concept is established in cell culture, in vivo studies are performed to gauge the effect of a drug in animal models (Fig. 12.1c). Animals are an attractive model due to the presence of multiple, complete biological systems that are analogous to human biology, allowing researchers to investigate the effect of a drug in a more complete biological environment. Unfortunately, many human-infecting viruses do not readily infect non-human animals, although some cross-species infections exist [80–83]. To help with infectivity, many animals are immunocompromised through genetic or chemical manipulation [84]. Additionally, some animals need to be genetically altered to more resemble human physiology or need to be 'humanized' though transplantation with human tissue before successful infection can take place [85]. Other animal studies employ species-adapted versions of virus that can infect the species of interest, thus circumventing a need for developing special animals [86, 87].

Although animal models provide a good perspective of drug–virus interactions in the context of a living organism, there are still large differences between animal and human biology that make animals an imperfect model. Thus, caution should be exercised when interpreting results, and potency demonstrated in animals may not translate to potency in humans [88, 89].

12.3.3.1 Mice

Mice are an oft-used animal model in all aspects of human medicine research, and the same is true for antiviral research. Mice are widely used in research on a wide variety of viruses, although different strains must be used due to unpredictable and varied susceptibility to different viruses. For example, it has been established that mice are susceptible to Zika virus infections [90]. However, it has been observed that while the SJL strain of mouse exhibits striking similarity to human response to infections with Zika-infected mothers bearing microcephalic pups, the same microcephaly does not present at all in C57BL/6 pups [91]. Other flaviviruses have also been shown to infect mice to varying degrees of success, with inbred mice being easily infected and wild mice being resistant [92]. A similar phenomenon is seen with influenza, with laboratory strains easily infected and wild mice exhibiting resistance [93]. Additionally, murine cytomegalovirus (a herpesvirus endemic to mice) is known to affect BALB/c mice severely and C57BL/6 mice mildly, whereas Sindbis virus (a togavirus causing fever in humans) is known to affect C57BL/6 mice severely while BALB/c mice are largely resistant [94].

The main shortcoming of the mouse model is the degree of its divergence from human biology, particularly with respect to immunology [95]. This variation often leads to differences in

observed symptoms and may lead to discovery of mechanisms that are fundamentally inapplicable to human medicine. To illustrate, influenza infection often causes fever and a rise in body temperature in humans, but the same infection in mice can lead to hypothermia [96]. The fundamental difference between human and mouse virology can be illustrated with CEACAM1, a glycoprotein that is expressed in both species. It has been shown that mice expressing CEACAM1a are susceptible to murine coronavirus, while those that do not express CEACAM1a are not susceptible [97]. This might have been taken as an indication that human CEACAM1 could be a possible antiviral target for antivirals against coronaviruses; unfortunately, human coronaviruses do not interact at all with human CEACAM1. For this reason, the use of mouse models in antiviral research should be carried out with caution, as well as a clear understanding of the differences between mouse and human biology.

12.3.3.2 Non-human Primates

Non-human primates (NHPs) are similar to humans in their adaptive immune system, have high sequence homology for immune genes, and follow the same ABO blood groups that humans have. They represent a very reliable model for antiviral research due to this similarity, although it is associated with serious ethical concerns as well as high maintenance costs.

Much of research on Zika virus has taken place in NHPs. This is in large part due to the fact that the virus was first isolated in rhesus macaques, a common NHP model [98]. Moreover, other NHPs have been known to carry Zika virus in the wild [99]. Because of the similar gestational process, placental barrier, and fetal development between NHPs and humans, these models are often used to study the effect of Zika on gestational development. Indeed, rhesus macaques, cynomolgus macaques, and pigtail macaques have all been used as animal models in the study of Zika virus [100–108].

Another area of virus research that has taken advantage of NHP models due to its origin is respiratory syncytial virus (RSV). RSV was first isolated in chimpanzees, and subsequent studies of RSV infection were carried out in chimpanzee models [109]. A wide variety of primate species have been used to study RSV, including owl monkeys, baboons, capuchin monkeys, African green monkeys, rhesus macaques, bonnet monkeys, and cynomolgus macaques [110].

12.3.4 Clinical Trials

In order for a drug to become approved for a new use, it must first pass three phases of clinical trials (Fig. 12.2). Phase I trials are usually limited to under 100 human patients and have the purpose of establishing safety and dosage. To be considered safe in humans, a therapeutic agent must have already passed phase I clinical trials. Because of this, these can often be skipped in the development of existing safe-in-man BSAAs.

Phase II clinical trials follow phase I. These can involve several hundreds of patients and last up to 2 years, with the intention of evaluating efficacy and possible adverse side effects. Because study population sizes still may be too small to effectively determine efficacy, a large portion of phase II trial failure is due to safety concerns. Many BSAAs are drug candidates that either have not yet passed phase II trials or have failed a previous phase II trial. Unsurprisingly, failure to pass an earlier phase II trial raises safety concerns, especially if this failure is attributed to adverse effects. However, these drug candidates may be assessed again if the new patient population is disparate from the original population and there is evidence to suggest that the same safety concerns could be dismissed in the new population.

After passing phase II trials, phase III clinical trials can begin. Phase III trials incorporate more patients and take longer than phase II trials, thus allowing drug developers to monitor longer-term adverse effects and evaluate efficacy with greater statistical power against the current standard of care. Compounds may fail phase III trials due to inability to perform comparably or better than contemporary standard treatment, despite an established safety profile. Compounds that either

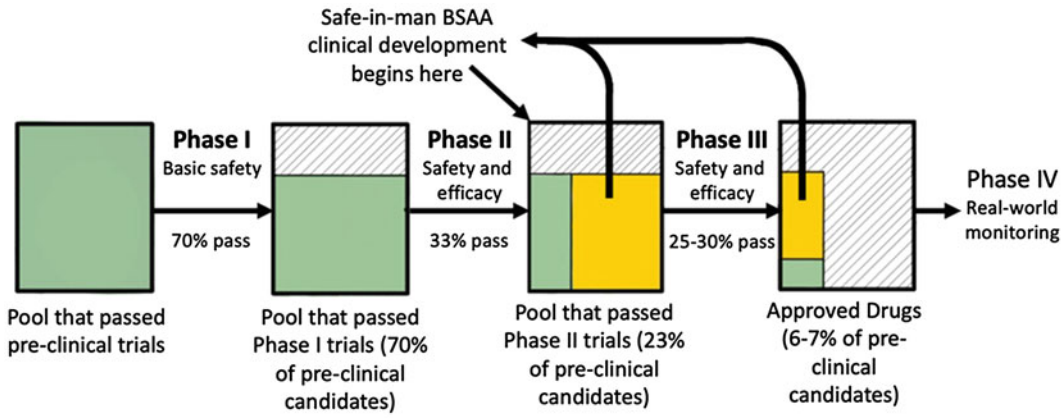

Fig. 12.2 The clinical stages of BSAA repositioning versus conventional drug development. Green-shaded areas represent the average proportion of viable drug candidates that remain after each round of trials, while yellow-shaded regions indicate the pool of drug candidates available for repurposing. Safe-in-man BSAAs can often skip phase I clinical trials. Moreover, failed drug candidates from previous trials can often be repurposed for new indications

have not yet passed phase III trials or have failed phase III trials due to inability to demonstrate the proper efficacy provide a promising pool of potential safe-in-man BSAAs due to the more stringent safety requirements defined by passing phase II. All repositioned BSAAs, including those agents that have already been approved, must still undergo a phase III trial to demonstrate sufficient efficacy before being approved for use for a new condition.

Once phase III trials have been passed, the drug can finally be approved for use. Upon being approved, many drugs must continue to be subject to continual monitoring to address any further long-term safety concerns in a long-term, phase IV trial. However, BSAAs that have been redirected from already-established drugs which have spent a considerable time on the market will have already undergone some levels of this post-market surveillance, providing a level of security and knowledge before phase IV studies begin.

12.4 Structure–Activity Relationships

As is the case with many drugs, structure–activity relationships (SARs) exist among BSAAs. This is not surprising, as structurally similar compounds are more likely to have similar targets. Because of this, antiviral discovery has often relied on the use of SARs, both to synthesize new antiviral agents and to improve upon existing ones [111, 112].

Interestingly, the breadth of antiviral activity seems to be related to chemical structure. Figure 12.3 shows the structure–activity relationships of known BSAAs. Compounds close to one another on the tree are structurally similar, while the number of viruses targeted by each compound is indicated by a bubble next to the name of the compound. The size of the bubble corresponds to the number of viruses for which antiviral activity has been established for the compound. This relationship between structure and breadth of activity is illustrated clearly by the structurally similar nucleotide and nucleoside analogs favipiravir, brincodofovir, cidofovir, and gemcitabine, all of which have a noticeably broader spectrum of antiviral activity compared to other BSAAs with structurally divergent neighbours.

This relationship can be used to broaden the antiviral activity of known BSAAs. For example, nitazoxanide, an antiparasitic agent and BSAA that possesses proven antiviral activity against 17 pathogenic human viruses, is structurally

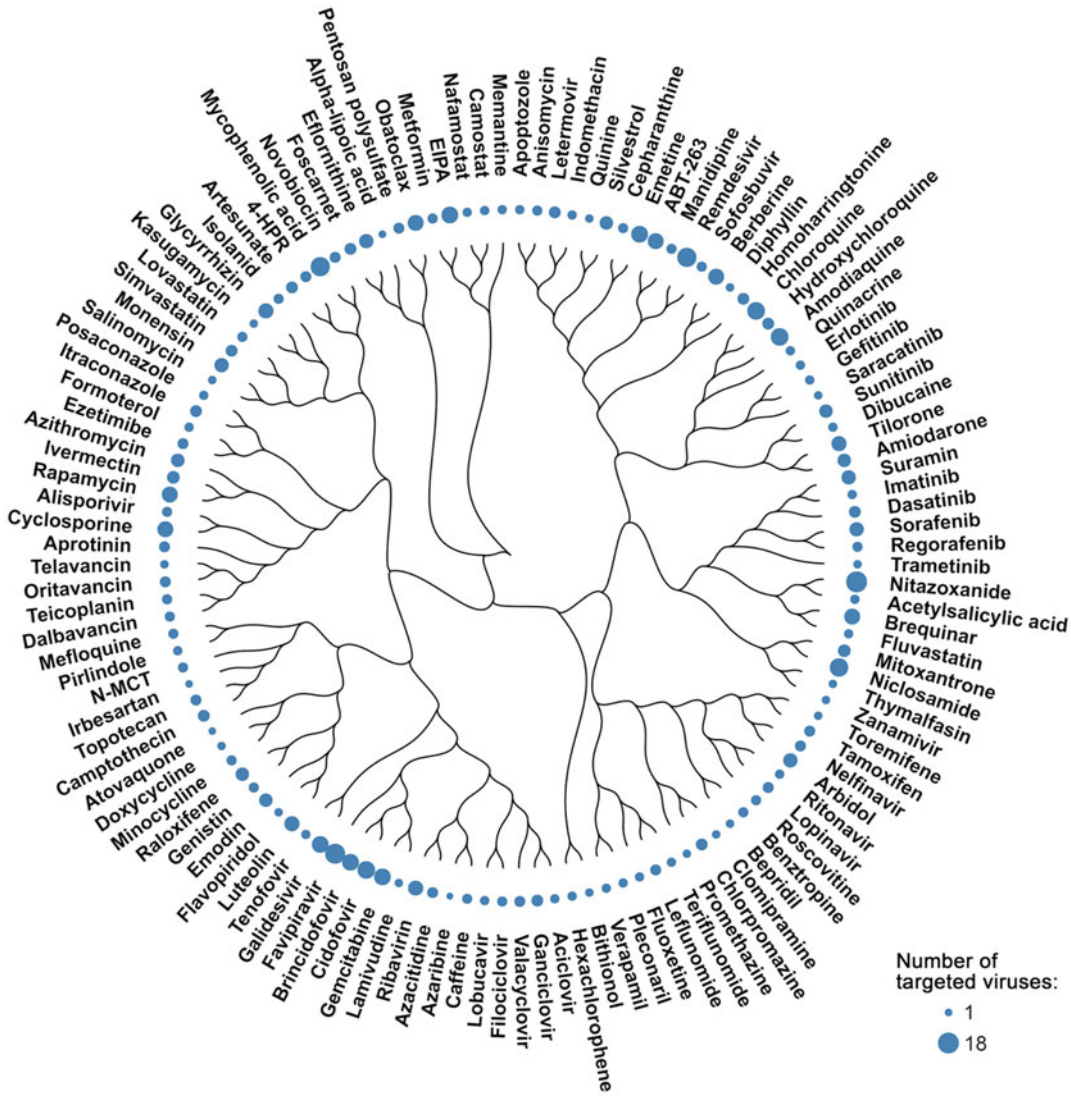

Fig. 12.3 Structure–activity relationship of known BSAAs. Compounds were clustered based on structural similarity calculated by ECPF4 fingerprints and visualized using the D3 JavaScript library. The broad-spectrum antiviral activities of the compounds are shown as bubbles, with larger bubbles corresponding to a larger number of targeted viruses

related to acetylsalicylic acid, which has only shown antiviral activity in 4 viruses [31, 79, 113–125]. Because of their structural similarity, it would then be more likely that acetylsalicylic acid could possess previously uncovered, broad antiviral activity as well. Likewise, ementine, remdesivir, and ABT-263 are structurally related BSAAs that have been demonstrated to possess relatively broad antiviral activity, while other structurally related drugs like cepharathine, manidipine, and sofosbuvir have relatively narrow demonstrated antiviral activity. These structural similarities could then pave the way for drugs like cepharanthine, manidipine, and sofosbuvir to be investigated for potential expansion into other viral diseases.

12.5 BSAA Combinations

One Achilles' heel of antiviral agents lies in the speed and ease by which viruses replicate, leading to a high evolution rate. Since viruses are constantly mutating and evolving, monotherapy treatment using a single antiviral agent can often lead to development of resistance [126]. To combat this, combination therapy can be used to treat viral infections. Combination therapies may be more effective because viruses that are able to evade the mechanism of action of one antiviral agent will likely be inhibited by a second agent with a different mechanism of action, thus requiring two specific mutations in order for a virus to develop any lasting resistance.

Indeed, current treatments for rapidly evolving viruses such as HIV and HCV often involve a combination of antivirals. These include Triumeq (abacavir/dolutegravir/lamivudine – a combination of two viral reverse transcriptase inhibitors and one viral integrase inhibitor) for the treatment of HIV and Harvoni (ledipasvir/sofosbuvir, a combination of an HCV NS5A inhibitor and an HCV RdRp inhibitor) for the treatment of HCV [127, 128]. Other common treatments include Symtuza (darunavir/cobicistat/emtricitabine/tenofovir) and Kaletra (lopinavir/ritonavir) for HIV, as well as Epclusa (sofosbuvir/velpatasvir) for HCV.

Administration of antiviral agents in combination may lead to additive effects, synergism, or antagonism. Additive effects occur when the antiviral activity of a combination is equal to the expected effect of the two separate drugs added together. Synergism occurs when the combined antiviral activity is greater than that of the two individual drugs added together. Synergism between antivirals is advantageous because it can lead to the use of smaller quantities of drug, thereby reducing toxicity decreasing chances for adverse side effects. Finally, antagonism is likely to occur if two antiviral agents have redundant mechanisms of action or interact with each other in a non-productive way. This results in combined antiviral activity that is weaker than that of the two individual drugs combined. Generally, antiviral drugs that have shown antagonistic activity should not be used with each other.

The efficacy of several drug combinations against viral infection has been investigated in clinical trials. Recent clinical trials against COVID-19 have tested combinations of danoprevir/ritonavir (clinicaltrials.gov ID: NCT04291729) and favipirair/tocilizumab (Chinese Clinical Trials Register ID: ChiCTR2000030894). Other clinical trial investigations have included the use of elbasvir/gazoprevir (clinicaltrials.gov ID: NCT03111108), sofosbuvir/ledipasvir (clinicaltrials.gov ID: NCT02480166), and peginterferon-alfa-2b/ribavirin (clinicaltrials.gov ID: NCT00383064 and NCT01045278) against HCV, as well as use of entecavir/adefovir (clinicaltrials.gov ID: NCT01023217) and peginterferon-alfa-2a/adefovir or peginterferon-alfa-2a/entecavir (clinicaltrials.gov ID: NCT00922207) against HBV.

In addition to clinical trials, several pre-clinical research endeavours have also revealed synergistic antiviral activity against a broad range of viruses. For example, in vitro studies have shown that a combination of pimodivir and gemcitabine was shown to exhibit synergism against Influenza A [129]. Similar in vitro studies have also shown that obatoclax and saliphenylhalamide have synergistic activity against Zika virus infection in RPE cells [130]. Most recently, it was demonstrated by Ianevski et al. that salinomycin, amodiaquine, obatoclax, emetine, and homoharringtonine all show synergistic activity when administered with nelfinavir against SARS-CoV-2 infections in cell culture [131]. Interestingly, it was noted that all synergistic combinations that were identified involved one host-directed drug and one virus-directed drug, while most combinations involving two host-directed drugs proved to be antagonistic. This finding suggests that synergism among antivirals may be more likely to exist if a host-directed agent is combined with a virus-directed agent.

When administered correctly, combination antiviral therapies can improve efficacy by simultaneously exhibiting synergistic effects and

preventing development of antiviral resistance. This can be especially useful for repositioned BSAAs, because they may inhibit different viruses with different degrees of efficacy. In these cases, the boost in efficacy given by drug synergism can be a valuable tool for antiviral repurposing. Moreover, several BSAAs can be purposefully combined in one formulation to cover the broadest set of viruses possible, which could then be used as a frontline solution to treat emergent viral diseases [132]. For these reasons, the use of BSAA combinations can be a useful tool to maximize the antiviral activity of existing drugs.

12.6 Useful Tools for Antiviral Drug Repurposing Studies

BSAA repositioning strategies are often considered low risk and high reward because initial drug discovery costs are low and chances for success are high. A large part of the reason for this low-risk/high-reward profile is because this strategy benefits from large amounts of pre-existing data that have already been collected from years of previous research. Traditionally, extensive literature reviews were required to collect this data; however, recent acknowledgement by the research community for the need of integrated, comprehensive knowledgebases has led to the establishment of several openly accessible tools that shorten the drug discovery process further. Several useful databases for antiviral repositioning are discussed below and summarized in Table 12.1.

12.6.1 Pharmacological Databases

12.6.1.1 DrugBank

DrugBank is an openly accessible database that contains over 13,000 approved, investigational, experimental, illicit, withdrawn, and nutraceutical drug entries [133, 134]. It contains both chemical information about drugs such as molecular weight and structure, as well as biological information on drug targets such as biological pathways of the target, mechanism of action, PK/PD, and toxicology data. Additionally, information is included on drug–drug interactions, as well as links to source material such as research articles and clinical trials. The database can also be queried using targets, pathways, or indications. Importantly for BSAA research, DrugBank aggregates a list of 151 drugs categorized as antiviral agents along with 690 associated drug targets, which can be browsed or searched individually. This resource can be accessed at: https://www.drugbank.ca/.

12.6.1.2 DrugCentral Database

DrugCentral database is a manually curated online compendium of approved therapeutic agents [135]. It monitors information about drug approvals, as well as collects information on dosage, reported adverse events, indications, and drug formulations. The database can both be browsed from a web browser or downloaded directly for high-throughput analysis. This resource can be accessed at: http://drugcentral.org/.

12.6.1.3 Pharmacogenomic Knowledgebase (PharmGKB)

PharmGKB is an interactive online tool that allows users to query by genes, variants, drugs, diseases, and pathways, focusing on associations between drug phenotype and genetic variants [136]. It is also annotated with clinical and prescription information. A built-in text mining system provides automated annotations of predicted linkages between drugs and genetic variants from PubMed and PubMed Central, and retrieves the sentence from which the information is mined. Each entry also contains links to other molecules, genes, or diseases in the database, with which the entry has some association. This resource can be accessed at: https://www.pharmgkb.org/.

12.6.1.4 DrugVirus Database

The DrugVirus database is a resource specifically focusing on safe-in-man BSAAs [5]. It

Table 12.1 Publicly accessible resources for BSAA repositioning

Type of resource	Resource name	Area of focus	Web address
Pharmacological databases	DrugBank	Drug and drug candidates	https://www.drugbank.ca/
	DrugCentral	Drug approvals, indications, and adverse events	http://drugcentral.org/
	PharmGKB	Drug phenotypes, disease variants	https://www.pharmgkb.org/
	DrugVirus	BSAAs, drug repositioning	https://drugvirus.info/
Proteomics databases	RCSB-PDB	Experimental protein structures	https://www.rcsb.org/
	Proteopedia	Protein structure and function wiki	http://proteopedia.org/
	UniProt	Protein sequences, broad protein information	https://www.uniprot.org/
Chemical structure databases	PubChem	Bioactive compounds (editable)	https://pubchem.ncbi.nlm.nih.gov/
	ChEMBL	Bioactive compounds (manual curation)	https://www.ebi.ac.uk/chembl/
	ChemDB	Commercially available compounds	http://cdb.ics.uci.edu/
Viral databases	ViPR	Viral bioinformatics	http://www.viprbrc.org/
	ViralZone	Viral molecular biology	https://viralzone.expasy.org/

summarizes and tracks the antiviral activity and developmental status of drugs and drug candidates that have passed at least phase I clinical trials and have shown antiviral activity in at least two viral families. The database includes 816 unique drug-virus combinations, covering 118 safe-in-man drugs that target 83 human viruses. Of these drug-virus combinations, 592 have demonstrated efficacy in cell lines, 14 have demonstrated efficacy in primary cells or organoids, 69 have demonstrated efficacy in an animal model, 18 are currently in phase I clinical trials, 27 are in phase II clinical trials, 28 are in phase III clinical trials, 49 have been approved for use for the virus in question, and 19 are being monitored in phase 4 clinical trials (Fig. 12.4). The graphic interface of the database also highlights BSAA-virus combinations that have not yet been explored. Drug–virus interactions are visualized on a heatmap, which displays viruses of the same family and classification close to one another. Thus, if a drug has been shown to work against one virus in a larger family of viruses, it is more likely that it will have some antiviral activity against the surrounding viruses on the heatmap as well. The DrugVirus database can be accessed at: https://drugvirus.info/.

12.6.2 Proteomics Databases

12.6.2.1 The RCSB Protein Data Bank

Another useful protein database is the RCSB Protein Data Bank (RCSB-PDB) [137]. This database collects 3D structural information for proteins, nucleic acids, and assemblies of both, along with annotations about structural features, function, and links to published articles. The structures can be searched and narrowed down by species, taxonomy, method for structural determination, structural resolution of the entry, release date, protein classification, and symmetry. The RCSB-PDB is a useful resource for molecular docking studies, as it contains comprehensive 3D structural information for candidate drug targets. It can be accessed at: https://www.rcsb.org/.

12.6.2.2 Proteopedia

Similar to other crowd-sourced knowledgebases like Wikipedia, Proteopedia is an editable protein wiki that contains information about structure and function [138]. Like the RCSB-PDB, the main goal of Proteopedia is to provide insight into 3D structure and function. Each protein entry also contains a 3D structural representation, as well

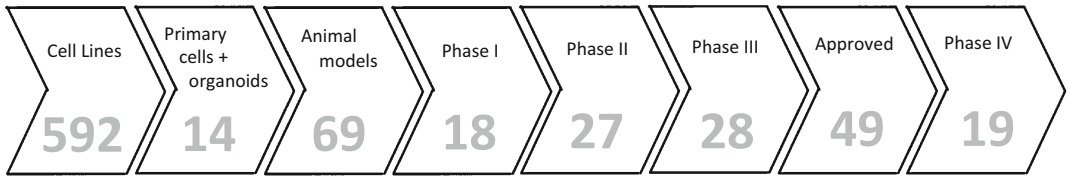

Fig. 12.4 Summary of drug-virus combination statuses on DrugVirus.info

as links to literature and associated protein entries within the database. This resource can be accessed at: http://proteopedia.org/.

12.6.2.3 UniProt Knowledgebase

UniProt was developed to be a universal protein resource, consisting of information on protein sequences obtained from nucleic acid sequencing data, functions, and structure [139]. To eliminate redundancy, double entries are merged based on sufficiently redundant protein sequences. The database is divided into two sections: TrEMBL, which contains automatically annotated information, and Swiss-Prot, which contains only manually curated information for each entry. In addition to the knowledgebase itself, UniProt contains the UniProt archive (UniParc), a non-redundant archive of all publicly available sequences, as well as UniProt reference clusters (UniRef), which clusters entries based on sequence similarity. This resource can be accessed at: https://www.uniprot.org/.

12.6.3 Chemical Structure Databases

12.6.3.1 PubChem

PubChem is an editable online repository of bioactive chemicals and substances from the National Institutes of Health [140, 141]. Although it is mainly a chemical database, it includes some large biological macromolecules such as antibodies. It contains physical and chemical information about substances such as 2D, 3D and crystal structure, conformation, boiling point, melting point, density, and solubility. It also includes a wealth of relevant biological information such as proteins, pathways, and bioassay data, patent information, and links to literature. PubChem can be accessed at: https://pubchem.ncbi.nlm.nih.gov/.

12.6.3.2 ChEMBL

A bioinformatic resource provided by the European Bioinformatics Institute (EBI), ChEMBL is a database of bioactive molecules and drugs [142]. It is manually curated to reduce the error rate of information extraction and contains over 1.9 million molecular compounds targeting over 13,000 proteins. The entries include binding and functional information, as well as pharmacological and clinical data. This resource can be accessed at: https://www.ebi.ac.uk/chembl/.

12.6.3.3 ChemDB

ChemDB is a purely chemical database, containing over 5 million commercially available small molecules [143]. This database collects a broad range of chemical compounds, not just focusing on bioactive molecules. However, it provides an extensive resource for experimentally determined physiochemical properties not often covered by other drug discovery-adapted databases. The database also curates annotations from the vendors of the compounds, and allows for searching by these annotations. This resource can be found at: http://cdb.ics.uci.edu/.

12.6.4 Viral Databases

12.6.4.1 Virus Pathogen Resource (ViPR)

ViPR is a virus database funded by the National Institutes of Health [144, 145]. It collects data on known viral pathogens, with focus on genetic sequences, immune epitopes, host factor data, 3D structures, and associated antiviral drugs. In

addition to its database, ViPR also provides bioinformatics tools such as phylogenetic tree building, sequence alignment, primer design, and gene annotation for analysis of novel data. Finally, ViPR allows for community contributions and data sharing, which assists in the constant growth of the database. This resource can be accessed at: http://www.viprbrc.org/.

12.6.4.2 ViralZone

ViralZone is a web-based resource from the Swiss Institute of Bioinformatics [146]. It includes information on virions, virus molecular biology, reference sequences, and a downloadable virus thesaurus (virosaurus), which includes complete and partial sequence datasets for eukaryotic and plant viruses. As of June 2020, ViralZone contains 918 pages, covering 128 virus families, 567 virus genera, 7 individual species, and 216 resources on viral molecular biology. The ViralZone resource can be found at: https://viralzone.expasy.org/.

12.7 Conclusion and Future Perspectives

The repositioning of safe-in-man BSAAs represents a paradigm shift from a conventional model of drug development to one that is cheaper, faster, and more efficient. Antiviral research is uniquely positioned to benefit from this strategy, because development of safe-in-man agents can alleviate the lack of financial incentives and funding required to find new antiviral drugs. In the same manner, BSAA repositioning can also provide an avenue for antiviral developers with less funding to take advantage of the vast collection of resources generated by more well-funded areas of drug development, such as cancer research.

There are still hurdles to safe-in-man BSAA development. For instance, while it is both faster and cheaper to develop pre-existing drugs, many research institutions and pharmaceutical companies are less likely to do so when they cannot patent the molecule. This is somewhat mitigated by the both the US and European patent systems, which now both consider sufficiently inventive applications of existing compounds for intellectual property protection [147]. However, achieving 'sufficiently inventive applications' may be a hurdle in itself, especially when considering repurposing an existing antiviral for use against another, if different, virus. Patents can also be issued for existing drugs if the new indication requires a different formulation from its original indication. Again, this stipulation does not apply well to repositioning of safe-in-man BSAAs because the goal is to have an antiviral agent with one formulation that can act broadly, much like the case with antibiotics against a broad range of bacteria. Moreover, because it is legal for doctors to prescribe existing drugs for off-label uses, it would be difficult to deter doctors from prescribing the existing or generic drug in that capacity, even if a patent were obtained for a new indication.

Because of these hurdles, interest in drug repositioning studies is falling more and more to pharmaceutical companies with existing drug patents that want to broaden the scope of their own portfolio as much as possible. Even this can prove to be an endeavour unworthy of pursuit because by the time a company successfully identifies a new indication for their drug, the remaining patent time for the drug will likely be too short to make a significant profit [148, 149]. This, in addition to the relative lack of profitability of antivirals, leads to little incentive for many to pursue the study of antiviral repositioning.

However, it is becoming more evident that antiviral development cannot be ignored, and that safe-in-man BSAA can save valuable time and money when an emergency arises. Indeed, three of the most promising antiviral agents against SARS-CoV-2 (remdesivir, favipiravir, and ivermectin) are previously existing, safe-in-man agents that have shown broad-spectrum antiviral activities before the beginning of the COVID-19 global pandemic [150–154].

Focus on discovery and development of safe-in-man BSAAs allows adequate preparedness in the face of constant viral threats and has the capacity to save millions of lives in the process. However, it is imperative that several shifts need to occur in the drug development sphere,

including the reworking of pharmaceutical patent systems that have previously focused on the 'one drug, one disease' paradigm. In addition, increased funding for antiviral research with special focus on drug repurposing and broadly acting agents, as well as collaborations between academic research and the private sector, are necessary to increase incentives and entice researchers to participate.

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Exploiting Ubiquitin Ligases for Induced Target Degradation as an Antiviral Strategy

13

Rati Verma

Abstract

Posttranslational modifications of targeted substrates alter their cellular fate. Ubiquitin is a highly conserved and ubiquitous covalent modifier protein that tags substrates with a single molecule or with a polyubiquitin chain. Monoubiquitination affects trafficking and signaling patterns of modified proteins. In contrast, polyubiquitination, particularly K48-linked polyubiquitination, targets the protein for degradation by the Ubiquitin-Proteasome System (UPS) resulting in a committed fate through irreversible inactivation of substrate. Given the diversity of cellular functions impacted by ubiquitination, it is no surprise that the wily pathogenic viruses have co-opted the UPS in myriad ways to ensure their survival. In this review, I describe viral exploitation of nondegradative ubiquitin signaling pathways to effect entry, replication, and egress. Additionally, viruses also harness the UPS to degrade antiviral cellular host factors. Finally, I describe how we can exploit the same proteolytic machinery to enable PROTACs (Proteolysis-Targeting Chimeras) to degrade essential viral proteins. Successful implementation of this modality will add to the arsenal of emerging antiviral therapies.

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Keywords

Antivirals · PROTACs · Ubiquitin · Ubiquitin ligases · Degraders · Proteasome · Antiviral therapeutics

Abbreviations

AdV	Adenovirus
ARIH2	Protein Ariadne-2 homolog
ART	Arrestin-related trafficking proteins
bTRCP	Beta-transducin repeat containing
CARDs	Caspase activation and recruitment domains
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CRBN	Cereblon
CRL	Cullin-RING ligase
DDB1	DNA damage-binding protein 1
DUBs	Deubiquitinases
EBOV	Ebola virus
EBV	Epstein barr virus
ESCRT	Endosomal sorting complex required for transport
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HDAC6	Histone deacetylase 6
HECT	Homologous to E6-AP Carboxyl Terminus
HEXIM1	Hexamethylene bisacetamide inducible 1

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HIV-1	Human immunodeficiency virus type 1
HPV	Human papillomavirus
HRSV	Human respiratory syncytial virus
HSV-1	Herpes simplex virus 1
HTLV-1	Human T lymphotropic virus type 1
IAV	Influenza A virus
IFN	Interferon
IMiD	Immunomodulatory
IRF3	IFN-regulatory factor 3
ISG15	Interferon stimulated gene 15
KSHV	Kaposi's Sarcoma herpes virus
LUBAC	Linear Ub chain assembly complex
MAVS	Mitochondrial antiviral signaling protein
MHC	Major histocompatibility complex
NEDD4	Neural precursor cell expressed developmentally down-regulated
PAMPs	Pathogen-associated molecular patterns
PLPro	Papain-like protease
PML-NB	Promyelocytic Leukemia Protein-Nuclear Body
PPIs	Protein:protein interaction interface
PROTACs	Proteolysis targeting chimeras
PRR	Pattern recognition receptors
RBR	RING-between-ring
RIG-1	Retinoic acid-inducible gene 1
RING	Really interesting new gene
RNP	Ribonucleoprotein particle
RSV	Rous sarcoma virus
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SM	Small molecule
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAK1	TGFb-activated kinase 1
TBK1	TANK-binding kinase-1
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIM	Tripartite motif
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
VHL	Von Hippel Lindau
VIF	Viral infectivity factor
ZIKV	Zika virus

13.1 The Ubiquitin-Proteasome System (UPS)

Ubiquitin is an evolutionarily conserved eukaryotic protein that shares 96% sequence conservation between baker's yeast and humans. Structurally, this small 76 amino acid protein adopts the highly versatile β-grasp fold that is also found in prokaryotes and terminates in a signature diglycine sequence. Reflective of the strong selective pressure across the whole molecule, it comes as no surprise then that ubiquitin (Ub) plays an essential role in cellular processes as diverse as cell division, apoptosis, and autophagy [1, 2]. Ub is covalently conjugated to substrate through the formation of an iso-peptide bond between the ε amino group of acceptor lysine and C-terminal glycine of Ub. Ub itself has seven lysine residues as well as a free amino end. Poly-Ub chains that are formed via lysine 63 usually confer signaling functions on substrates. Chains formed via lysines 11, 29, or 48 (or branches thereof) generally result in substrate being targeted to the 26S proteasome for degradation, with homo-polymeric K48 chains predominating [3]. In addition to the canonical isopeptide-linked conjugation, N-terminal methionine-linked linear ubiquitination characterized by a head-to-tail linkage has been shown to play a prominent role in immunity [4]. Ub conjugation also serves as a tag for the autophagic pathway whereby cytosolic cargo is delivered to the lysosome for destruction [5]. The lysosomal pathway can be distinguished from the UPS in that degradation is ATP-independent and it is confined to the cytosol.

Ub is covalently attached to substrate by a sequential cascade of Ub-activating (E1), Ub-conjugating (E2), and Ub-ligase (E3) enzymes (Fig. 13.1). Mammalian cells express two E1s, 40 E2s, and approximately 600 E3s. Ub, which is relatively inert, is first activated by binding to E1 in an ATP-dependent

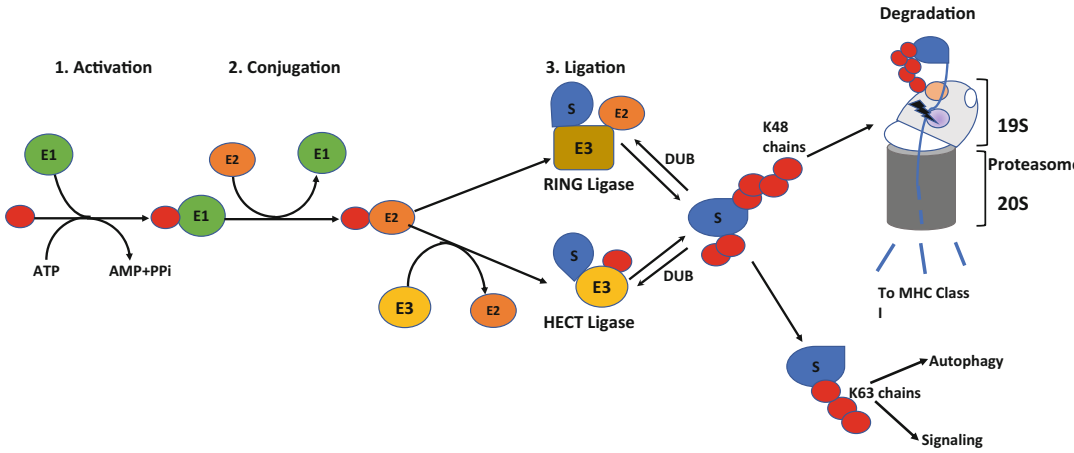

Fig. 13.1 Schematic of the ubiquitin cycle. The E1 enzyme activates ubiquitin (Ub) (red circles) to form a thioester bond with its active site cysteine and the carboxy terminus of Ub. The thioesterified Ub is then transferred to the active site cysteine of a Ub-conjugating enzyme E2. E3 Ub ligases bind to both substrates (S) and E2 ~ Ub thioesters, which results in the transfer of Ub to substrate. There are two classes of ligases: HECT (Homologous to E6-AP Carboxyl Terminus) ligases which undergo an additional trans-thioesterification at active site cysteines, and RING (Really Interesting New Gene) ligases which only enable Ub transfer but are not themselves covalently modified. Approximately 100 Deubiquitinase enzymes (DUBs) can stabilize substrates by removal or trimming of Ub chains. Ubiquitinated substrates are escorted to the 26S proteasome or bound by intrinsic receptors (e.g., RPN10/S5a, orange circle in schematic of 19S) present in the 19S regulatory cap of the 26S proteasome. The substrate is translocated into the 20S peptidase core of the proteasome by the 19S ATPases concomitantly with the removal of the Ub chain en bloc by the intrinsic deubiquitinating enzyme RPN11/POH1 (purple circle in 19S schematic). The proteolytic core degrades the substrate to generate peptides that are processed before presentation by MHC-class I molecules. Ub is recycled by additional DUBs that cleave within chains to release monomeric Ub. Prolonged inhibition of the proteasome results in the depletion of the intracellular pool of free Ub

manner. Activated Ub is then transferred to the active site cysteine of an E2, generating a high-energy E2 ~ Ub thioester [6]. E3 Ub ligases bind to both substrates (S) and E2 ~ Ub thioesters, which results in the transfer of Ub to substrate. Ligases can be classified very broadly into two classes: HECT (Homologous to E6-AP Carboxyl Terminus) ligases which undergo an additional trans-thioesterification at an active site cysteine, and RING (Really Interesting New Gene) ligases which do not [7]. About 28 HECT ligases are known. The conserved HECT domain containing the catalytic cysteine is invariably present at the C-terminus whereas the N-terminal lobe bears the E2 binding site [8, 9]. The NEDD4 family members (nine in total) comprises the largest group within this class of ligases. The domain organization of this family is shown in Fig. 13.2a. The C2 domain serves as a lipid-binding, membrane-tethering domain. Substrates containing a PPXY motif, the so-called degron motif [10], are recognized by the central WW domains of the ligases. As will be discussed below, the NEDD4 ligases play a critical role in egress of infectious virions that contain the PPXY motif.

The >600 RING ligases catalyze the turnover of the bulk of known UPS substrates [11, 12]. The conserved RING domain is typically 40–60 aa in length and contains the C_3HC_4 motif (seven cysteines and one histidine arranged nonconsecutively) that binds two zinc cations in a cross-braced arrangement. Since this module binds to the Ub-charged E2, it constitutes the catalytic arm of the ligase, and is typically found in the C-terminal end of the ligase. In some cases, the RING ligase is encoded by a single polypeptide (e.g., CBL and MDM2) but the largest subclass is the multi-subunit CRLs (Cullin-RING Ligase Family). The Cullins provide a banana-shaped

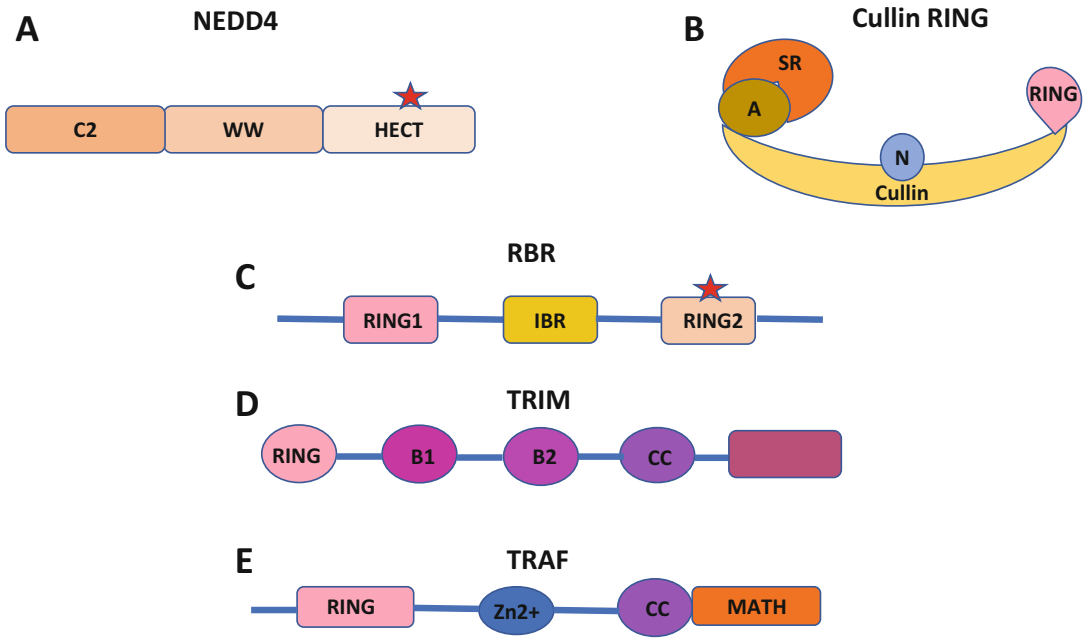

Fig. 13.2 Domain organization of the major subclasses of ubiquitin ligases. (**a**) The domain organization of the HECT ligases is exemplified by the NEDD4 family members (9 total) that are deployed by enveloped retroviruses for viral egress. All HECT ligases contain the catalytic HECT domain at the C-terminus. The red star in the figure denotes a catalytic cysteine that is covalently modified following Ub transfer from E2. The N-terminus contains the membrane-tethering C2 domain followed by the central WW domains (2–4). These are the substrate-binding domains that recognize the PPXY motif in targets including L domains in viruses. (**b**) The Cullin family of RING finger ligases (CRLs) comprise at least 250 family members. There are seven Cullins: CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7. At the catalytic C-terminus, each Cullin is bound to the RING finger protein RBX1, with CUL5 unique in binding to RBX2. The RING interacts with the E2 enzyme, thereby conferring Ub-transferring catalytic activity. The N-terminus is bound by the Adaptor-Receptor (AR) module that recognizes substrates. There are three classes of modules. (i) The modules for CUL3 are single BTB (Broad complex, Tramtrack, Bric-a-brac) proteins that contain a BTB fold that interacts with the N-terminus of CUL3 and an additional protein-interaction domain that binds to substrates. (ii) The AR modules for CUL1, CUL2, CUL5, and CUL7 use one of two BTB-fold proteins (SKP1 or ELOC) to interact with the N-terminus of their respective Cullin. SKP1 and ELOC contain additional sequence elements that associate with specific classes of substrate-binding receptor proteins, for example, F-box proteins for SKP1-CRL1. (iii) The AR modules for CUL4A and CUL4B are composed of DDB1 and members of the DCAF family of substrate receptors. (**c**) The RBR ligases are RING-HECT hybrids. As described for the canonical RINGs in (**b**) above, the RING1 domain binds Ub-loaded E2s. RING2s contain an essential active-site cysteine that receives Ub from an E2 ~ Ub to generate a covalent E3 ~ Ub intermediate. There are 14 RBR ligases including Parkin, HHARI, TRIAD1, and the LUBAC complex ligases with each member expressing unique N-and C-terminal domains. (**d**) The TRIM ligases possess an N-terminal RING finger, one or two B-boxes (B1, B2), and a coiled coil (CC) domain. Family members are classified into 11 subfamilies (C-I to C-XI) based on the C-termini. For example, C-I and C-IV are characterized by the presence of C-terminal SPRY and PRY domains. These domains bind substrates. (**e**) All TRAF ligase family members except for TRAF1 contain the canonical RING domain at the N terminus followed by a variable number of zinc finger domains. The C-terminus contains the TRAF domain (~200 amino acid residues) with the exception of TRAF7. It is divided into two subdomains: the TRAF-N domain, which is a coiled-coil (CC) domain, and the TRAF-C domain (MATH domain) which forms an eight-stranded beta-sandwich that forms a mushroom-shaped trimer that interacts with receptors and underlies the receptor-adaptor signaling function of TRAFs

scaffold on which assembles the RING domain containing Rbx1/2 at the C-terminus (Fig. 13.2b). Cullin N-termini bind, via adaptors, to a collection of substrate receptor modules that confer

specificity in substrate selection. For each receptor family, between 20 to 100 specific receptors have been identified. Thus, although there are only seven Cullins, the multitude of substrate receptors appended to them combine to give them a modular combinatorial diversity which as we shall see below, the viruses have exploited very effectively. Most CRLs exist in an inactive state and have to be activated by a small Ub-like molecule called NEDD8 which binds to an activation domain within Cullins [13].

Variants of the above class of ligases include members that contain the U-box which exhibits the RING domain fold but does not bind Zn^{2+} (e.g., CHIP, UFD2/E4B). The RBR (RING-between-RING) family is a hybrid between the HECT and RING ligases [14]. Within this family, RING1 binds to Ub charged E2, whereas RING2 contains an active site cysteine that forms the obligatory Ub ~ thioester as also observed for HECT ligases (Fig. 13.2c). RBR ligases exist typically in an autoinhibited state and have to be activated via binding to neddylated Cullins (e.g., ARH1) or phosphoUb (Parkin). An emerging and relatively large new class of RING ligases comprised of approximately 70 family members is the TRIM (Tripartite Motif) family [15]. In these ligases, an N-terminal RING domain is followed by one or two cysteine/histidine-rich B-boxes followed by a coiled-coil domain, hence the additional moniker of RBCC domain ligases (Fig. 13.2d). The RING and B-boxes all coordinate Zn^{2+}, and the family members are further classified as CI to CXI based on the variable C-terminal extensions. These ligases remained relatively uncharacterized despite the presence of a RING domain because they are only found in metazoans but play no less a varied role in cellular pathways as CRLs. TRIM ligases serve as regulators of innate immunity via PRRs (Pattern Recognition Receptors) such as Toll-like Receptor and RIG-1 (Retinoic acid-Inducible Gene 1) which detect evolutionarily conserved motifs on pathogens termed PAMPs (Pathogen-Associated Molecular Patterns). These ligases by their diverse abilities to catalyze both K48- and K63-linked polyubiquitination serve as hubs that control IFN and TNF-α production and response. As such, they play key roles in antiviral immunity and have also been exploited by viruses to evade host defense pathways [16]. Finally, the TRAF (TNF receptor-associated factor) RING ligases are characterized by their conserved C-terminal TRAF domain that binds to signaling receptors and transmits these signals to downstream effectors (Fig. 13.2e) [17]. They will be discussed further below.

While ligases append the Ub tag on substrates and are thus WRITERS of the Ub code, ubiquitinated proteins are recognized and bound by READERS that can display exquisite specificity for Ub chain linkage and topology [18]. Ubiquitinated substrates are escorted to the 26S proteasome by shuttling receptors such as HHR23A which contain the Ub-binding UBA domain or are recognized by intrinsic UIM-containing receptors within the 26S proteasome (e.g., RPN10/S5a). Finally, the Ub cycle (Fig. 13.1) is terminated by a class of enzymes known as ERASERs or DUBs (Deubiquitinases) [19]. Approximately 100 DUBs belonging to seven distinct classes are known. DUBs can remove Ub chains *en bloc* as exemplified by the essential proteasomal RPN11/POH1 [20, 21], or in linkage, and position-specific fashion. The bulk are nonspecific cysteine proteases that can stabilize substrates by removal or trimming of Ub chains. DUBs can be found in specific compartments of the cell, and a new class of specific DUB inhibitors are emerging that have therapeutic potential [22].

Two ubiquitin-like modifier proteins are worth mentioning here. The first, SUMO, is conjugated to acceptor lysines contained within the conserved motif ψKxE (where ψ is a hydrophobic residue and x any residue) in target substrates. The process of sumoylation utilizes its own unique E1 (AOS1/UBA2), E2 (UBC9), and E3 enzymes. Like Ub, SUMO plays diverse roles in the cell, and some of these may be mediated by effectors via noncovalent SIMS (SUMO-interaction motifs). Notable cellular pathways modulated include nuclear transport through alterations of the NPC (Nuclear Pore Complex), regulation of transcription and maintenance of

genomic integrity. SUMO also contributes to the formation of PML-NB (Promyelocytic Leukemia protein-Nuclear Body), also known as ND10 (Nuclear Domain 10). The major protein component of these bodies, PML, is both sumoylated and contains a SIM which contributes to an elaborate 3D structure by recruitment of approximately 100 proteins. The ND10 is a major target during successful infection by DNA viruses [23]. SUMO also plays a role in the signaling pathway governing type I IFN production which is manipulated by RNA viruses [24].

The second ubiquitin-like modifier protein is ISG15 (Interferon Stimulated Gene 15). As the name implies, expression of ISG15 is stimulated by interferon treatment or viral infection [25]. The 15 kDa protein consists of two Ub-like moieties linked by a short hinge. Again, like ubiquitination and sumoylation, ISGylation is a three-step process involving E1 (UBE1L), E2 (UBCH8), and the E3 (HERC5). Since a large percentage of HERC5 is ribosome-associated [26], newly translated viral capsid proteins are ISGylated, and this has been speculated to negatively impact viral assembly. PLPro (Papain-like Protease) from SARS-CoV-2 has recently been shown to predominantly cleave ISG15 off from host proteins such as IRF3 (Interferon Responsive Factor 3), blocking its nuclear translocation, and thereby reducing type I interferon responses [27].

A number of viruses encode components of the UPS in their genomes, particularly Ub ligases and DUBs, that they put into good use in disarming cellular host restriction factors. These are summarized in Table 13.1 and their roles are further reviewed in [28]. It is envisioned that SM (Small Molecule) inhibitors designed for these class of UPS enzymes could be repurposed as antivirals if host pathways are not too severely affected. Alternatively, selectively could be engineered.

13.2 Role of Ubiquitin in the Life Cycle of Viruses

Given the all-encompassing role of Ub in cellular pathways, it follows that viruses co-opt this covalent modifier for all aspects of their life cycle. Below I discuss a few well-studied examples.

13.2.1 Viral Entry

Viruses employ different routes of entry into the cell. Some viruses inject their nucleic acids by direct penetration or membrane fusion. However, the most common route is endocytosis which could be clathrin, caveolar, lipid-raft, or micropinocytosis-mediated. Typically, extracellular ligands are recognized by plasma membrane-embedded receptors that are phosphorylated at key tyrosine residues. One way signaling is attenuated is by internalization of these receptors following ubiquitination of the receptor by Ub ligases. CBL is a RING ligase that helps internalize varied immune and growth factor receptors via endocytosis [29]. In a well-studied example, very early post KSHV infection, CBL is activated by viral-induced phosphorylation. CBL monoubiquitinates the integrins which together with the amino acid transporter protein x-CT translocates KSHV into lipid rafts [30]. Interestingly, knockdown of the ligase blocked macropinocytosis and receptor translocation. Instead, KSHV was diverted to a clathrin-lysosomal noninfectious pathway. In another example, CBL forms a complex with the HSV-1 entry receptor Nectin-1 by forming a ternary complex with the viral glycoprotein D that is then internalized. This process promotes entry of the virus in uninfected cells [31].

Although HECT ligases are usually co-opted by viruses during release as mature virions, through interactions of their WW domains with PPXY motifs in the L domains of viruses (see below), there are some examples of HECT ligases also promoting entry. AdV is a non-enveloped dsDNA virus that enters cells by receptor-mediated endocytosis. The WWP1/2 and ITCH HECT ligases can all bind to the N-terminal PPXY motif present in the AdV capsid penton base protein and mediate internalization [32]. Additionally, the internal capsid VI protein is exposed after internalization. The PPXY motif

Table 13.1 Examples of ubiquitin ligases and deubiquitinases/deISGylases encoded by viruses

Virus	Gene/Protein	Function
HSV-1	ICP0 RING ligase	Inactivates host ND10 and p53 responses; blocks IRF3/7 activation of ISGs
RV	NSP RING ligase	Targets RIG-1, beta-TrCP, and IRF3/5/7/9 for proteasomal degradation
KSHV	K3, K5-RING CH ligases	Downregulates MHC-I antigens by lysosomal degradation
KSHV	RTA ligase	Regulates latent-lytic switch; degrades the repressor HEY1 and sumoylated proteins
hHSV-1	$UL36^{USP}$-DUB	TRAF3, IκBα
EBV	BPLF1-DUB	PCNA, TRAF6
KSV	KSVORF64-DUB	RIG-1
SARS-CoV	SCoV-PLpro-DUB	RIG-I, TRAF3, STING, TBK1, IRF3
SARS-CoV-2	SCoV2-PLpro-deISGylase	Cleaves ISG15 from IRF3 and dampens type I interferon responses

in this protein is recognized by NEDD4 which aids in microtubule-dependent trafficking to the nucleus [33] and reduced autophagic sequestration [34].

Another class of ligases, the TRIM ligases, are better known for their antiviral roles. But in a striking example of usurping host ligases for viral entry, the ZIKV exploits TRIM7 to assemble non-degradative K63-linked chains on the envelope E protein [35]. Ubiquitinated E binds to the host cell receptor TIM1 which helps confer tropism selectivity, leading to infection of tissues such as brain but not liver. In this seminal study, the key acceptor lysine residues on E were identified as K38 and K281, and mutagenesis confirmed the importance of these residues in vivo. Strikingly, it was determined that a monoclonal anti-K63 antibody could lead to viral neutralization, a somewhat surprising result given that K63-linked ubiquitination plays a significant role in host innate immunity pathways (see below). ZIKV belongs to the *Flaviviridae* family but unlike other family members can cause congenital neurological disease and can replicate in reproductive tissue.

13.2.2 Viral Uncoating

Following entry, the viral envelope has to be proteolyzed and capsid disassembled to release viral nucleic acids for replication. The M1 protein of the internalized IAV in the late endosomal compartment is ubiquitinated by the HECT Ligase ITCH, and this process is essential for IAV escape from late endosomes and transport to the nucleus [36]. For capsid disassembly, the virus utilizes the host aggresome processing machinery. The viral core containing unassembled Ub chains is exposed to the cytosol resulting in recruitment and activation of HDAC6 [37]. HDAC6 is a microtubule-associated deacetylase involved in processing aggregates derived from ubiquitinated misfolded proteins. In a process that requires the Ub-binding domain of HDAC6, the virus is uncoated following binding to Ub and matrix protein M1. Shearing forces of the cytoskeletal motors disassemble capsid, and vRNPs are imported into the nucleus [38]. In contrast to IAV, the incoming AdV virions attach to the NPC. The RING ligase MIB1 (Mind Bomb 1) is essential for AdV uncoating, rupture and delivery of vDNA into the nucleus [39].

13.2.3 Viral Transcription and Replication

Ub pathways play an oversized role in the viral replicative cycle. For example, IAV vRNP complexes contain IAV RNA segments packaged together with RNA polymerase and viral nucleoprotein NP. NP is ubiquitinated by host RING ligase CNOT4 (CCR4-NOT transcription complex subunit 4) at lysine residues K184, K227,

and K273 within the RNA binding groove of NP. Mutations of these sites to arginine, or deubiquitination by USP11 reduces viral RNA replication [40].

The highly pathogenic EBOV genome encodes VP35, a viral protein that is an essential cofactor of the viral polymerase as well as a strong antagonist of the host antiviral IFN-I. VP35 binds to TRIM6 which promotes both viral replication and antagonizes the IFN response through mechanisms that are not yet understood [41]. The HIV-1 transcription factor TAT, on the other hand, has been researched for decades and its role in promoting viral transcription is better understood. Many genes in eukaryotes contain stably paused RNA Pol II downstream of transcription start sites. The dimeric kinase P-TEFb (Positive Transcription Elongation Factor b), composed of the subunits CCNT1 (cyclin T1) and CDK9 (Cyclin-Dependent Kinase 9) releases paused polymerase into a productive elongating form. P-TEFb activity is regulated by sequestration into the inhibitory 7SK snRNP (Small Nuclear Ribonucleoprotein) complex at promoters including the HIV-1 promoter [42]. HEXIM1 (Hexamethylene bisacetamide inducible 1) inhibits the kinase activity of CDK9 in a 7SK snRNA-dependent manner, and TAT can activate transcription by displacing the inhibitory subunit [43]. Additionally, TAT recruits cytoplasmic UBE20 Ub ligase to ubiquitinate HEXIM1, thereby revealing for the first time a cytoplasmic pool of the inhibitory complex. Following ubiquitination, HEXIM1 is sequestered in the cytoplasm while P-TEFb is released from the snRNP and transported into the nucleus [44].

13.2.4 Viral Egress

Late assembly (L) domains encoded by enveloped retroviruses promote the separation of nascent virus from infected cell. Pioneering studies that identified PPXY motifs in viral L domains and host WW domain-containing proteins that recognize this peptidic degron soon led to the identification of the HECT family of ligases as playing a key role in viral egress [45]. The first example discovered was the PPXY degron located in the N-terminus of p2b of the RSV GAG protein that binds to NEDD4 [46, 47]. It was soon established thereafter that several retroviruses deployed the NEDD4 family for viral budding. Monoubiquitination of viral matrix proteins allows them to engage the host ESCRT (Endosomal Sorting Complex Required for Transport) pathway which is normally used to sort monoubiquitinated cargo into resculpted multivesicular bodies that eventually target cargo to the lysosome for degradation [48]. Viral budding leading to exit of mature virions from cell plasma membrane can essentially be viewed as topologically inverted clathrin-mediated endocytosis. Viral proteins such as HIV-1 GAG1 that lack the PPXY degron are still dependent on NEDD4 for exit because in this case, NEDD4 ubiquitinates an ESCRT protein TSG101 [49]. Additionally, the ART (Arrestin-Related Trafficking Proteins) proteins such as ARRDC1 can be recruited to sites of viral budding and may themselves be ubiquitinated, thus functioning as adaptors between HECT ligases and PPXY-L domain viruses [50, 51].

13.3 Role of Ubiquitin in Antiviral Immunity

Following viral entry, penetration and uncoating, the release of viral nucleic acids triggers the host innate immune response [52]. This is initiated by PRRs. There are three major classes of PRRs: (1) the cytosolic RLRs (RIG-I-like receptors) that sense viral RNA produced during both RNA and DNA virus infections; (2) the membrane-bound TLRs (Toll-Like Receptors) that detect viral RNA or DNA in endolysosomes; and (3) a group of viral DNA sensors, exemplified by cGAS (cyclic GMP-AMP Synthase). The Ub dependence of these pathways is described briefly here only for well-studied pathways. A comprehensive review on the topic is provided in [53].

RIG-1 belongs to the DExD/H box containing RNA helicase family and contains two N-terminal CARDs (Caspase Activation and

Recruitment Domains). Following vRNA binding by the helicase domain, a conformational change ensues in RIG-1, relieving autoinhibition and freeing up the CARD domains. TRIM25, RIPLET/RNF135, and TRIM4 ligases can all induce 63-linked ubiquitination of RIG-1. Notably, TRIM25 ubiquitinates CARD at lysine-172 which induces RIG-1 oligomerization that allows for association with the downstream signaling adaptor MAVS (Mitochondrial Antiviral Signaling protein). These activating ubiquitinating marks can be reversed by DUBs such as CYLD, USP3 and USP21. Additionally, RIG-1 can be degraded by the 26S proteasome following K48-linked ubiquitination by RING ligases RNF122 and 125. Viral proteins can also block RIG-I activation. The NS1 protein of IAV and HRSV targets TRIM25 to block K63-linked ubiquitination of RIG-1 [54, 55].

Activated MAVS propagate their signal to TRAF6 (TNF Receptor-Associated Factor 6) and TRAF3 culminating in the activation of the transcription factors NFkB and IRF3/7 respectively that turn on the expression of proinflammatory cytokines and Type I IFNs [56]. The K63-linked polyUb chains formed via TRAF6 and TRAF3 autoubiquitination creates a scaffold hub for downstream signaling kinases to interact [17]. Ubiquitinated TRAF6 interacts with TAK1/TAB1/2/3 complex via the Ub-binding domain of TAB2 leading to activation of TAK1 (TGFβ-Activated Kinase 1) kinase. TAK1 activates the downstream trimeric IKK kinase complex by phosphorylating the catalytic subunits IKKβ/IKKα, while their essential regulatory subunit IKKγ/NEMO promotes their dimerization by binding to linear Ub chains via its UBAN domain [57]. NEMO is also ubiquitinated by the trimeric LUBAC (Linear Ub chain Assembly Complex) [58]. IKK phosphorylation of IκBα creates a phosphodegron that is recognized by β-TRCP-CRL1, leading to its ubiquitination and degradation. The released NFκB heterodimer is translocated to the nucleus and activates transcription. The second arm of the K63-linked Ub signaling pathway arising from autoubiquitinated TRAF3 leads to activation of the noncanonical IKKε and TBK1 (TANK-binding kinase-1) which phosphorylates the transcription factor IRF3 (IFN-Regulatory Factor 3) leading to its dimerization and translocation into the nucleus [56].

Cytoplasmic viral DNA is sensed by cGAS and other PRRs. TRIM56 monoubiquitinates cGAS on lysine 335 promoting dimer formation, enhanced DNA-binding and cGAMP production [59]. Other activating ligases that have the same output are TRIM41 and RNF185. The latter ubiquitinates cGAS at K173 and K384 [60]. Pathogenic viruses evade cGAS by promoting its degradation or inhibiting its enzymatic activity. Examples include UL31 and pp65 of HCMV, VP22 of HSV-1, and ORF52 of KSHV [61–63]. cGAMP activates STING (Stimulator of Interferon Genes), an ER-resident enzyme [64]. The TRIM ligases 32 and 56, as well as the mitochondrial E3 MUL1 append K63-linked Ub chains on STING which facilitate its trafficking to the Golgi compartment leading to its palmitoylation and subsequent downstream activation of the IRF3 signaling pathways [65, 66]. STING is also ubiquitinated by the RING ligase AMFR (Autocrine Motility Factor Receptor) an E3 localized at the ER. In response to cytoplasmic DNA stimulation, AMFR, together with INSIG-1 interacts with STING and conjugates K27-linked polyUb chains on STING. The K27-linked Ub chains promote trafficking to perinuclear microsomes and provide a scaffold for TBK1 kinase which phosphorylates and activates IRF3 [67].

In addition to the above pathways, PRRs also turn on several mitogen-activated protein kinases that activate AP-1 (Activator Protein-1). A convergence of transcriptional programs driven by IRF-3/7, NF-κB, and AP-1 thus aids in establishing antiviral immunity.

13.4 Viruses Hijack Ub Ligases to Circumvent Cellular Defenses

Viral proteins subvert the host proteostasis network to create neo-complexes designed for their survival [68, 69]. Human HPV-16 and HPV-18

are associated with cervical cancer. These viruses encode two oncoproteins, E6 and E7, which are expressed in HPV-positive cancers. E6 protein hijacks the cellular E6AP (E6-associated protein) and directs it to ubiquitinate and degrade the tumor suppressor p53 thus ensuring a growth advantage for its host cells (Fig. 13.3a). This viral pathway of transformation led to the identification of the HECT (homologous to the E6 carboxy terminus) class of ubiquitin ligases, of which E6AP is the founding member [70].

Another virus that exploits the UPS is HIV-1, a canny pathogen that resisted taming for years. The viral-specific accessory protein VPU is an integral ER membrane phosphoprotein that induces the degradation of newly synthesized host membrane proteins such as the viral receptor CD4 and MHC (Major Histocompatibility Complex) class I molecules. Two conserved phosphoserine residues in the cytoplasmic domain of VPU trigger CD4 degradation (Fig. 13.3b). The viral phosphoserines are essential for the recruitment of the substrate receptor $\beta - TRCP1$. A ternary complex comprising CD4, VPU, and β-TRCP-CRL1 is formed, and proximity to the ligase triggers ubiquitination and proteasomal degradation of CD4 [71]. As will be discussed in the next section, induced proximity to Ub ligases is the guiding principle for developing PROTACs (Proteolysis Targeting Chimeras).

Two additional examples merit description because they exemplify how host innate and adaptive immunity pathways are neutralized by viruses via the harnessing of Ub ligases. The HIV protein VIF (Viral Infectivity Factor) is required for viral replication in nonpermissive T cells and macrophages but not in permissive cells such as epithelial cells. The nonpermissive cells express an intrinsic immune modulator: APOBEC3G, a cellular cytidine deaminase. APOBEC3G is normally encapsulated into virions, which when released, wreaks havoc with viral replication by inducing cytidine to uracil mutations in newly synthesized viral DNA. However, VIF masquerades as a substrate receptor of the CRL5 ring family via a SOCS box motif that interacts with the Elongin C subunit of CRL5 as well as the CUL5 scaffold (Fig. 13.3d). It recruits ABOBEC3G into the ligase complex leading to its polyubiquitination and proteasomal degradation [72–75]. It was subsequently found that VIF additionally hijacks the transcriptional cofactor CBF-β leading to a stable pentameric ligase complex whose structure could then be elucidated engendering hope that the VIF:Ligase PPIs (Protein:Protein Interaction interface) can eventually be targeted by SM inhibitors [76–78]. Amazingly, this complex continues to illuminate both ligase and viral biology when it was further discovered that the pentameric ligase complex recruits the RBR ligase ARIH2 (protein Ariadne-2 Homolog) to prime APOBEC3G with monoubiquitin before Ub chain elongation by CRL5 [79].

Mirroring VIF, the SV5-V protein also adopts the guise of a substrate receptor by hijacking yet another ligase-CRL4 (Fig. 13.3c). DDB1 is the adaptor for this ligase and is structurally unique amongst all the CRL adaptors. It is formed by a cluster of three WD40 beta propellers of which two (BPA and BPC) are tightly coupled with a large pocket in between whereas the third propeller (BPB) is attached flexibly on the side to the CUL4 scaffold [80]. CRL4 ligase is interchangeably bound to about 25 DCAF (DDB1-CUL4 Associated Factors) substrate receptors via an alpha helical fold that inserts into the pocket of the double BPA-BPC propellers of DDB1. The viral V protein of paramyxoviruses mimics the DCAFs and similarly inserts an entire alpha helix into the pocket (Fig. 13.3). As substrate receptor of CRL4, SV5-V recruits the transactivators STAT1/2 (Signal Transducer and Activator of Transcription) leading to their degradation and an effective block in interferon signaling [81]. Interestingly, the viral HBx protein, despite sharing no sequence conservation with SV5-V, utilizes the same alpha helical motif to deploy CRL4 [82]. This motif is essential for HBx infection. The CRL4-HBx complex degrades the SMC (Structural Maintenance of Chromosomes) complex proteins SMC5 and 6, which act as host restriction factors by limiting HBV expression [83]. Remarkably, three independent phenotypic screens for cytotoxic SMs identified inhibitors of CDK12 that induced degradation of the

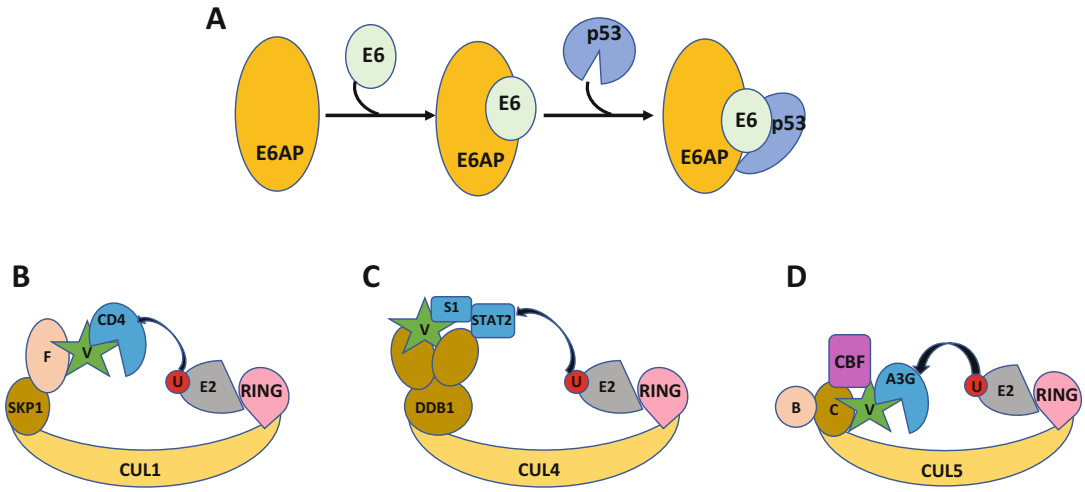

Fig. 13.3 Key examples of viral deployment of host cellular Ub ligases. (**a**) Binding of HPV E6 protein to HECT ligase E6AP induces a conformational change in the ligase that results in recruitment of p53 near the catalytic center of E6AP. (**b**) CRL1 hijacked by HIV VPU that forms a ternary complex with native ligase substrate receptor β-TRCP and recruited neosubstrate CD4. Viral protein is depicted as a green star. (**c**) CRL4 deployment by SV5 V that inserts itself into the DDB1 propeller pocket mimicking native DCAF receptors to recruit STAT1/2 for degradation. (**d**) CRL5 hijacking by HIV-1 VIF forcing reassembly of ligase complex to target neosubstrate APOBEC3G. The additional cofactor ARIH2 for this ligase is described in the text but not shown here for simplicity

associated CCNK (Cyclin K). The SM allows CDK12 to adopt the same alpha helical fold that inserts CDK12 into the DDB1 propeller pocket, leading to degradation of the bound cyclin which occupies the substrate space [84–86]. These screens suggest that the DDB1:Viral PPI could similarly be amenable to SM inhibitors.

13.5 PROTACs: Chimeric Molecules that Degrade and Inactivate Targets by Inducing their Proximity to a Ub Ligase

The potential of exploiting Ub ligases to degrade mutant as well as wild-type proteins to create conditional knockouts in genetically intractable organisms presents a huge opportunity in both basic research and the therapeutic landscape. As proof of principle, the well-characterized β-TRCP-CRL1 was selected. The F-box substrate receptor β − TRCP is bound to a ten aa phosphopeptide contained within the substrate IκBα [87, 88]. Accordingly, a chimeric molecule was synthesized containing the phosphopeptide linked to ovalicin, an angiogenesis inhibitor that covalently bound to the target MetAP-2-(methionine aminopeptidase-2). Amazingly, MetAP-2, which was not known to be a UPS substrate, was ubiquitinated in a CRL1-dependent manner and degraded [89].

A second milestone in the development of CRL-based PROTACs was the discovery of the SM inhibitor VHL-1 (VHL ligand 1), which disrupted interaction of the CRL2 receptor VHL (Von Hippel Lindau) and its substrate HIF1α [90, 91]. A combination of fragment-based screening and computational simulations that were guided by crystal structures of intermediates led to VHL-1. It binds VHL with sub-micromolar affinity and degraded reporter substrates such as HaloTagGFP with nM potency with >90% of the protein degraded [92, 93].

Contemporaneously, thalidomide, introduced as a sedative in the 1960s but retired in disgrace because of teratogenic side effects had been

repurposed successfully for treatment of multiple myeloma. Using a new affinity technique coupled to mass spectrometry, Ito et al. identified CRBN (Cereblon), a substrate receptor of the CRL4, as the primary target of thalidomide [94]. Newer analogues such as lenalidomide and pomalidomide, classified as IMiD (immunomodulatory) drugs also bound CRBN and promoted the recruitment and degradation of neosubstrates such as the transcription factors IKZF1 (Ikaros), IKZF3 (Aiolos), and casein kinase 1α [95–99]. The IMiDs function more like "molecular glues" stapling target and ligase together and obviating the necessity for a linker [100]. However, they cannot be synthesized prospectively, having been discovered more through phenotypic screens or serendipitously, although one prospective screen has been reported [101]. Nonetheless, their discovery led to the development of PROTACs that recruited CRBN-CRL4 as the ligase enforcing destruction.

As earlier reviews have emphasized, PROTACs offer the potential to target any protein-of-interest, as long as a ligand is available, and structural studies can guide optimization [102–104]. Target binders need not be high affinity binders. Ternary complex between Target-PROTAC-Ligase has been deemed necessary but not sufficient, since not all acceptor lysines in target may be oriented correctly for Ub transfer from E2-E3s. As opposed to monovalent inhibitors of enzymatic activity that have to achieve high target occupancy, PROTACs are catalytic and able to function sub-stoichiometrically. They are regenerated following substrate degradation, and sustained effects have been observed after washout of the PROTAC. The rate of resynthesis of target may define efficacy but PROTACs have outperformed inhibitors in several tumor cell models [105]. Another advantage with using PROTACs versus inhibitors is the degree of selectivity achieved amongst paralogs. The amino acid sequence identity is usually high around the active site of enzymes making it a herculean task to identify homolog-selective inhibitors. However, this kind of selectivity has been achieved with degraders. In one example, selective degradation of CDK6 among all CDK family members allowed for a precise delineation of its role in cell cycle signaling [106]. And, in another example, the intricacies of ternary complex formation contributing to specific degradation of either p38alpha or p38delta among the p38 MAPK family members were illuminated [107]. Following the culmination of decades of groundbreaking research, the case has been made for PROTACs in cellular models and in vivo animal studies as versatile chimeras available to target the undruggable 80% of the proteome. PROTACS have now entered the clinic. The first oral PROTACs (ARV-110) targeting AR (Androgen Receptor) and ARV-471 targeting the ER (Estrogen Receptor) have been dosed into patients suffering from metastatic CRPC and breast cancer respectively in clinical trials initiated by Arvinas, Inc.

Although the entry of oral PROTACs into the clinic is encouraging, challenges remain with this therapeutic modality. Since the molecules are heterobifunctionals, there is contribution in molecular weight from ligands for both target and ligase. The linker conjugating the two is typically comprised of alkyl or ethylene glycol repeating units of variable length determined empirically and adds to the total size. These features result in PROTACs occupying a chemical space that lies outside the classical Lipinski "Rule of 5"creating hurdles in membrane permeability [108]. As the targeted protein degradation field matures further, the "ideal" physicochemical properties of PROTACs including molecular weight, rotatable bonds, lipophilicity, and topological polar surface area are being elucidated such that some design principles are emerging [109]. Moreover, since PROTACs are catalytic and high target occupancy is neither necessary nor desired (due to potential toxicity), efficacy may be driven more by the rate of synthesis of target rather than how much gets internalized. This concept was illustrated by a recent in vivo study of PROTACs in rats targeting RIPK2 (Receptor-Interacting Serine/Threonine Protein Kinase 2), which has a half-life greater than 50 hours. It was observed that RIPK2 degradation persisted beyond the detectable pharmacokinetic

presence of PROTAC prompting hopes of infrequent low-dose regimens in humans [110]. Obviously, more will be learnt about pharmacodynamic efficacy as additional PROTACs enter the clinic.

PROTACs can degrade intracellular cytosolic and nuclear proteins predominantly, although a few membrane-bound targets encoding receptor tyrosine kinases have indeed been described [111, 112]. The newly described LYTACs represent a more broadly applicable approach for targeting extracellular and membrane-anchored proteins [113]. In their first iteration, LYTACs consist of a small molecule or antibody fused to glycopeptide ligands for cell surface lysosome-targeting receptors. These have been shown to target epidermal growth factor receptor and Programmed Death-Ligand 1 to the lysosome for degradation. It is anticipated that this platform will grow, as will the newly described AUTACs (Autophagy Targeting Chimeras). Guanine derivatives serve as degradation tags that are linked to target warheads leading to K63-linked polyubiquitination and clearance of target by selective autophagy [114]. The authors demonstrated clearance of both proteins and organelles, thus enhancing the scope of this process beyond xenophagy or antibacterial autophagy.

13.6 PROTACs as Emergent Antiviral Therapeutics

PROTACs have been shown to degrade targets as varied as epigenetic readers, transcription factors, and kinases. The question that can be posited now is if they can demonstrate efficacy as SM antiviral therapeutics? An early report tested this idea using a peptidic version of PROTACs. The X-protein of HBV is essential for viral replication, and WHO data indicates that a percentage of people chronically infected with HBV will eventually die of hepatocellular carcinoma. This is because the X protein promiscuously transactivates genes that aid in cellular proliferation. The X protein is a native substrate of the UPS and has a C-terminal instability domain that is most likely the site of binding by ligases and hence contains an intrinsic degron. Additionally, the N-terminus of X promotes oligomerization of the protein. Accordingly, a minimal peptide was identified that enhanced binding to the oligomerization domain which was fused to the c-terminal instability domain. This fusion construct, designated PROTAC, was able to promote degradation of ectopically expressed protein X, either full length, or a truncated form [115]. Interestingly, this somewhat "nontraditional" PROTAC worked as well as the more traditional PROTAC where the instability domain of Protein X was replaced with the ODD (Oxygen-Dependent Degradation) domain of HIF-1α, a degron motif recognized by VHL-CRL2 [90, 91].

A more recent study describes an antiviral PROTAC that exemplifies the potential of this modality [116]. Chronic HCV infections lead to liver cirrhosis, hepatitis, and hepatocellular carcinoma. HCV encodes a single polyprotein that is cleaved by cellular and viral proteases to produce ten structural and nonstructural proteins. The multifunctional HCV NS3 protein encodes a C-terminal helicase that is essential for viral replication. The N-terminus, together with its cofactor NS4, constitutes a serine protease which cleaves the HCV polyprotein and host proteins as well. Telaprevir is a peptidomimetic protease inhibitor that was approved in 2011. However, it was withdrawn from the market because of the emergence of resistance mutants after patient exposure. To establish proof-of-concept, the authors chose this drug as a well-validated HCV ligand. A crystal structure of drug bound to protease was used to deduce the solvent-exposed moiety of the SM and attach a linker (Fig. 13.4). The linker was conjugated to IMiD ligands for the CRBN-CRL4 ligase. The best PROTAC DGY-08-097 was effective in degrading NS3 protease with a DC_{50} (concentration at which 50% of target has been degraded) of 50 nM. Quantitative mass spectrometric proteomic analysis of transiently transfected cells confirmed that NS3 protease was the only significantly depleted protein amongst a total of >8000 proteins characterized. In functional assays monitoring HCV infectious units, it was confirmed that

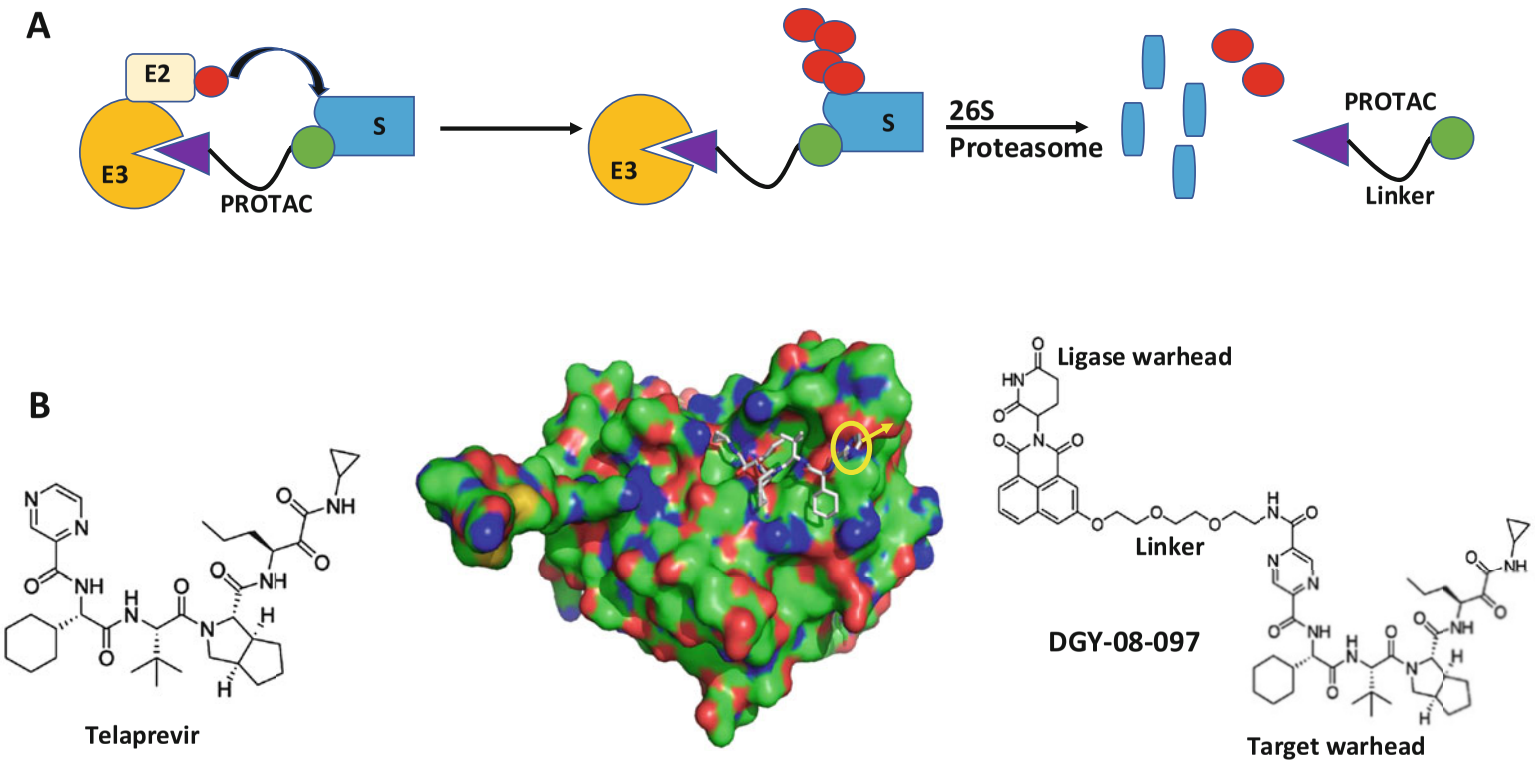

Fig. 13.4 PROTACs. (**a**) Schematic of a general PROTAC-induced degradation cycle. Ligase and target warheads (purple and green, respectively) are conjugated via a linker to yield a PROTAC. The PROTAC-induced proximity of target to ligase results in polyubiquitination and proteasomal degradation of target. (**b**) Telaprevir and the crystal structure of NS3/4A protease in complex with Telaprevir (PDB # 3SV6) (left and middle panels). The yellow circle highlights site of linker attachment. Right Panel: structure of DGY-08-097, a degrader molecule that recruits CRBN-CRL4 ligase to degrade NS3/4A protease

PROTACs display antiviral activity. Most importantly, the PROTAC also degraded the drug-resistant variants of NS3. This key result demonstrates that although high-affinity binding of Telaprevir is lost contributing to loss of occupancy and thereby efficacy, sufficient target engagement is provided by the PROTAC (which is catalytic) to promote degradation and inactivation of HCV-NS3/4A.

The seminal study described above lays the groundwork for PROTACs to be deployed more broadly by researchers in areas other than oncology, where PROTACs have garnered the most attention and development in inactivating oncogenes. In a recent editorial in *Drug Discovery Today*, Martinez-Ortiz and Zhou [117] invoke PROTACs as antiviral therapeutics against SARS-CoV-2 to combat the coronavirus disease 2019 pandemic. The authors hypothesize that a PROTAC against SARS-CoV-2 envelope protein E would demonstrate efficacy based on prior data targeting envelope proteins. Apart from the technical advantages of being small and unglycosylated, earlier studies have shown that inactivation of the SARS-CoV E protein attenuates viral replication and infectivity in vivo [118, 119]. The E protein contains a transmembrane domain that forms pentameric alpha helical bundles that likely form an ion channel. Channel activity has been demonstrated in vitro and can be inhibited by hexamethylene amiloride [120]. Homology modeling suggests that the SARS-CoV-2 E protein is potentially also a gated ion channel and screening of a repurposed library of ion channel blockers yielded Gliclazide and Memantine [121, 122]. Identification of chemical matter for protein E eliminates the first challenge in synthesizing PROTACs. Lending further impetus are two separate studies documenting that (1) PROTACs induce the presentation of novel MHC class I peptides [123] and (2) increased ubiquitination of HIV-1 GAG protein leads to enhanced MHC class I presentation of a GAG-derived epitope [124]. It follows, therefore, that host T-cell antiviral activities could potentially be enhanced by PROTAC-induced degradation of protein E.

It is envisioned that antiviral therapeutics of the future will harness the proteolytic power of the proteasome or the lysosome to inactivate pathogenic viruses. A multipronged approach leading to inhibition of several proteins essential for the infectious cycle would be more foolproof to resistance mechanisms acquired by the rapidly adapting viruses. Moreover, the ability to target structural proteins with PROTACs, not just the traditional active sites of viral polymerases and proteases, expands the tool kit available to the virologist.

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