Antiviral Drugs Against Herpesviruses

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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

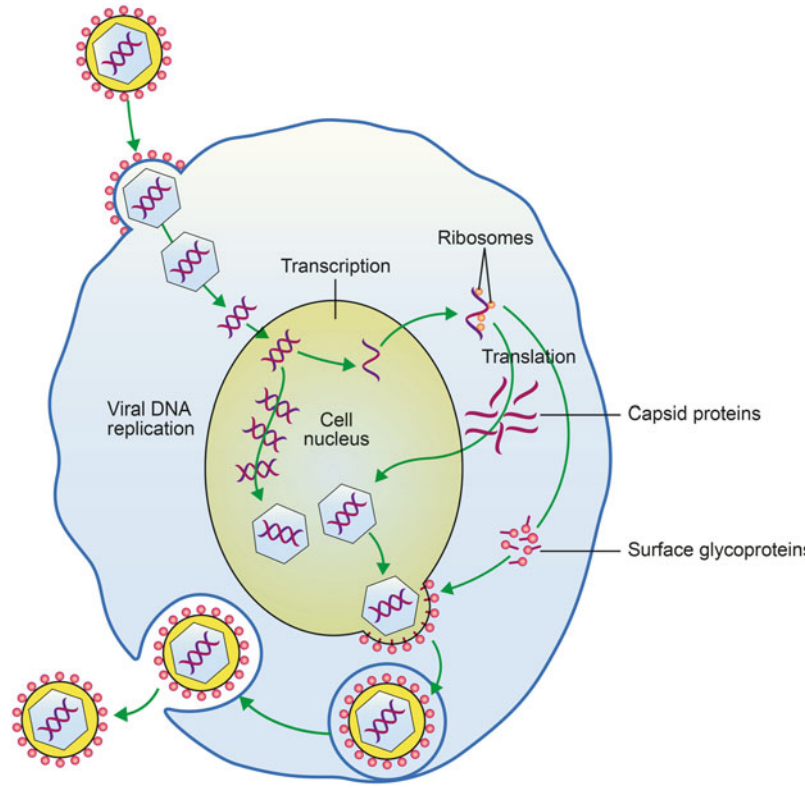

Fig. 1.1 Replicative cycle of herpesviruses

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

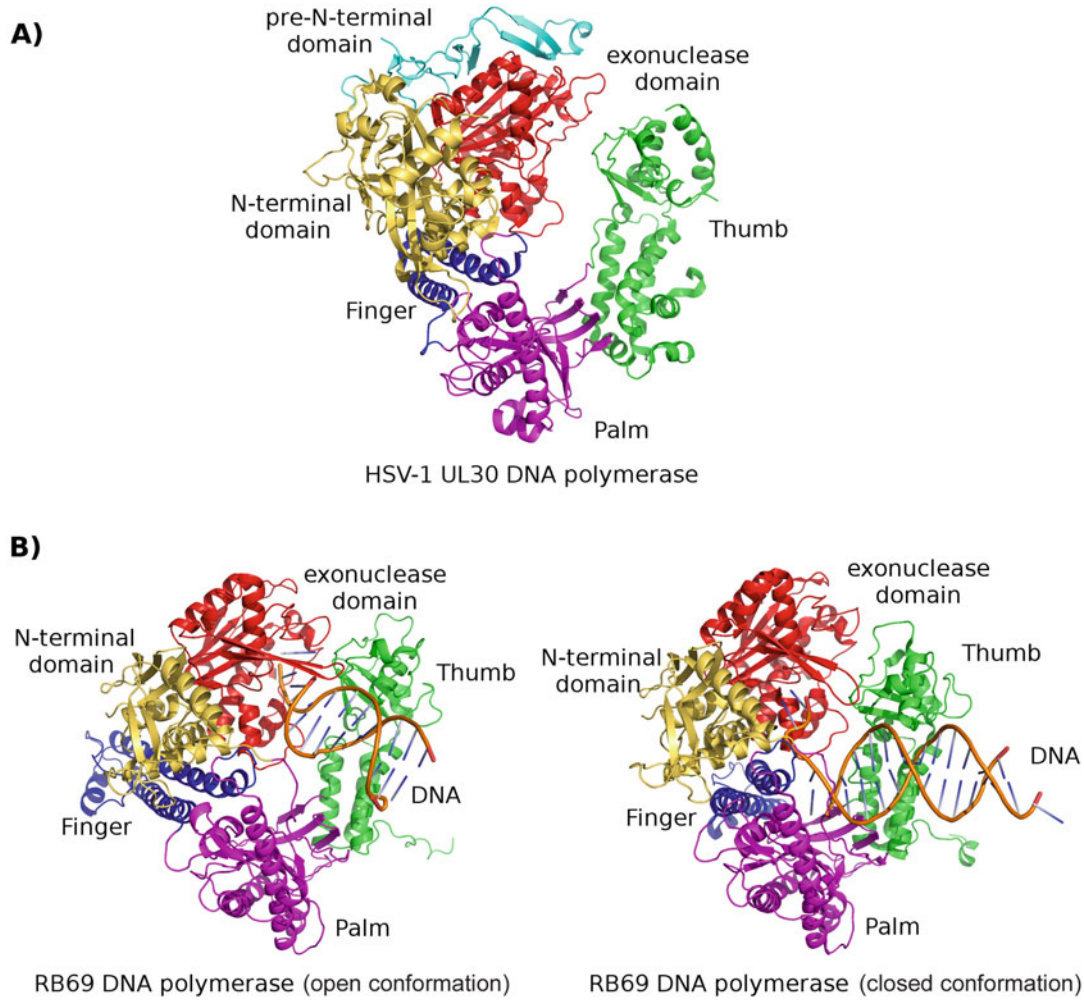

Fig. 1.2 Cartoon representation of B family DNA polymerases. (**a**) pUL30 DNA polymerase of HSV-1 in apo form (PDB 2GV9). Pre-NH_2-terminal domain (cyan), NH_2-terminal domain (light yellow), exonuclease domain (red), palm domain (magenta), fingers domain (blue), and thumb domain (green). (**b**) gp43 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

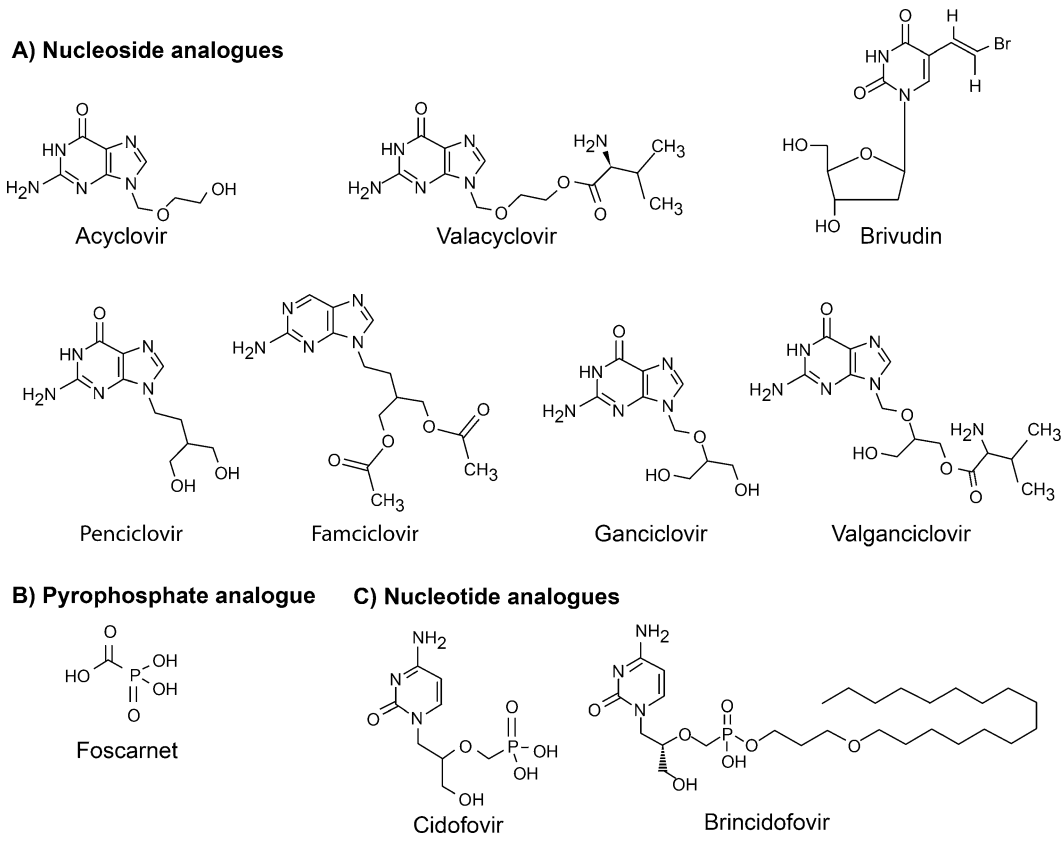

Fig. 1.3 Antiviral agents targeting the viral DNA polymerase

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

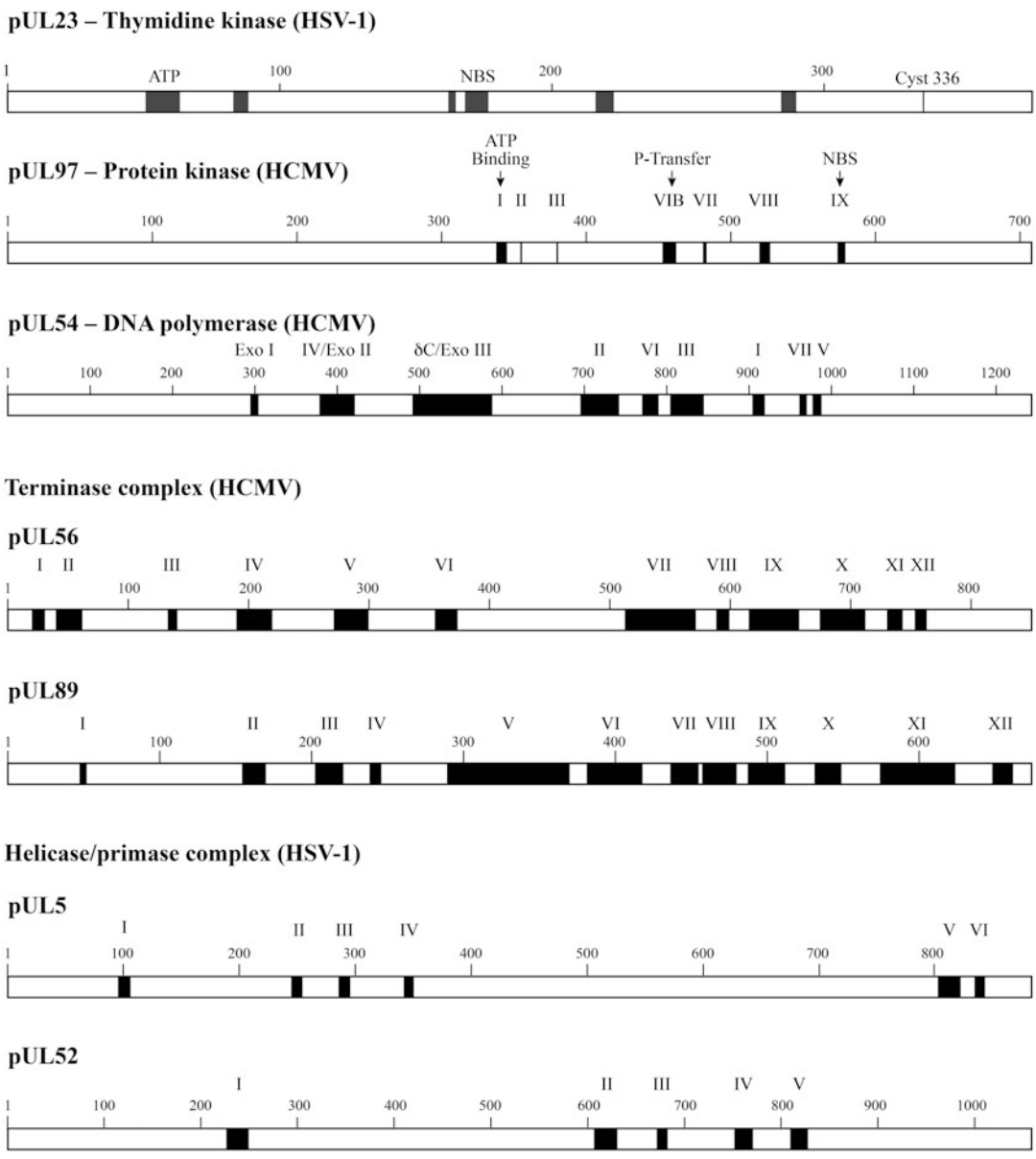

Fig. 1.4 Schematic representations of the viral proteins targeted by anti-herpetic agents. The pUL23 thymidine kinase of HSV-1 was taken as representative of pUL23 of HSV-2, ORF36 of VZV, and pU69 of HHV-6. pUL97 corresponds to the protein kinase of HCMV. The pUL54 DNA polymerase of HCMV was chosen as 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

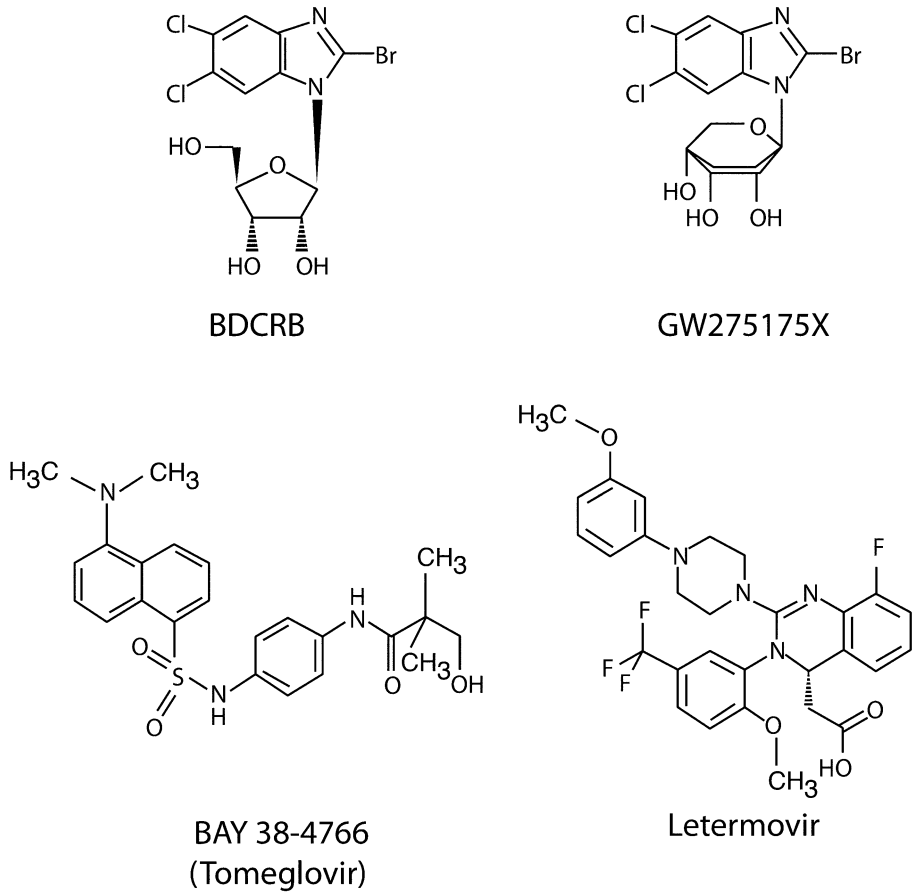

Fig. 1.5 Antiviral agents targeting the viral terminase complex

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

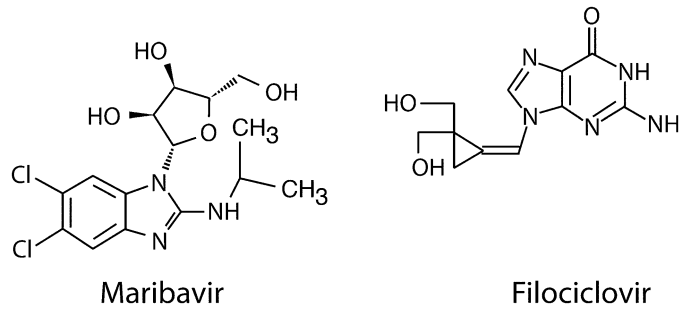

Fig. 1.6 Antiviral agents targeting the viral protein kinase and both the viral protein kinase and DNA polymerase

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

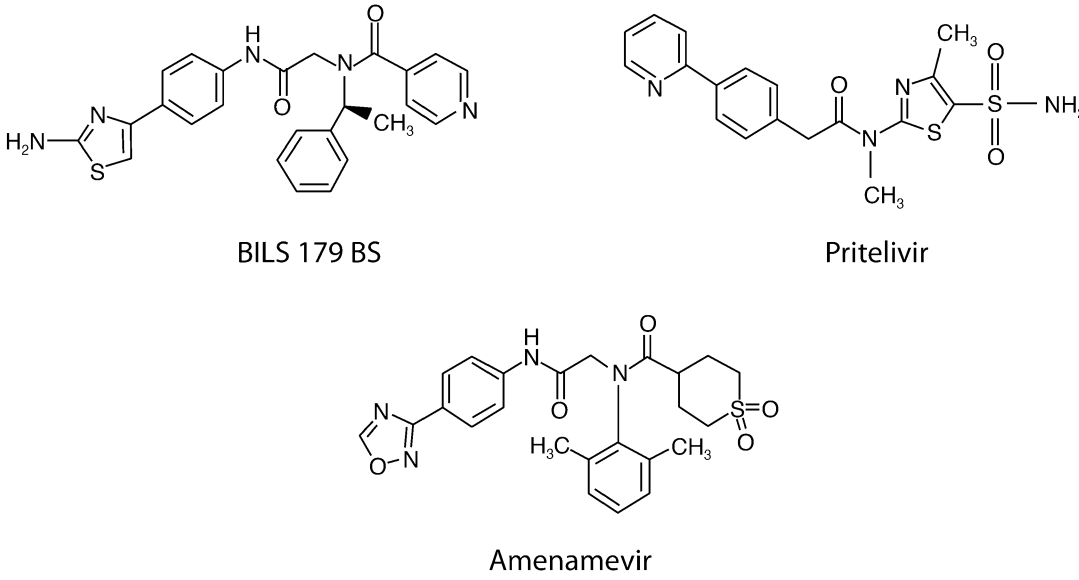

Fig. 1.7 Antiviral agents targeting the viral helicase-primase complex

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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