Chapter 6
Antiviral Drugs Against Alphaherpesvirus

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Abstract The discovery of acyclovir and penciclovir has led to the development of a successful systemic therapy for treating herpes simplex virus infection and varicella-zoster virus infection, and the orally available prodrugs, valacyclovir and famciclovir, have improved antiviral treatment compliance. Acyclovir and penciclovir are phosphorylated by viral thymidine kinase and are incorporated into the DNA chain by viral DNA polymerase, resulting in chain termination. Helicase-primase plays an initial step in DNA synthesis to separate the double strand into two single strands (replication fork) and is a new target of antiviral therapy. The helicase-primase inhibitors (HPIs) pritelivir and amenamevir have novel mechanisms of action, drug resistance properties, pharmacokinetic characteristics, and clinical efficacy for treating genital herpes. The clinical study of amenamevir in herpes zoster has been completed, and amenamevir has been submitted for approval for treating herpes zoster in Japan. The clinical use of HPIs will be the beginning of a new era of anti-herpes therapy.

Keywords Acyclovir · Prodrug · Valacyclovir · Famciclovir · Antivirals · Helicase-primase · Amenamevir · Chain termination · Resistance

6.1 Introduction

Dr. Elion, a Nobel laureate, has pioneered an anti-herpetic drug, acyclovir (ACV), capable for systemic administration in herpes simplex virus (HSV) and varicella-zoster (VZV) infection with a wide safety margin and a very high therapeutic index (Elion 1989; Elion et al. 1977), leading to the development and situation of current anti-herpes medicine treatment. Various anti-influenza virus drugs have been developed such as neuraminidase inhibitors (Von Itzstein et al. 1993), RNA polymerase inhibitor (favipiravir) (Furuta et al. 2002), a proton pump inhibitor (amantadine)

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(Jackson et al. 1963), and cap-dependent endonuclease inhibitor (S-033188) (Koszalka et al. 2017). By contrast, for the last 40 years, antiherpetic drugs have been limited to inhibitors of viral DNA synthesis because ACV and penciclovir (PCV) and prodrugs, valacyclovir and famciclovir, are satisfactory for the treatment and prevention of apparent HSV and VZV diseases. In addition to current antiherpetic therapy inhibiting DNA synthesis through DNA polymerase (DNApol), it has taken time to develop new antiherpetic drugs with different mechanism of action, and novel helicase-primase (HP) inhibitors (HPIs) of HSV and VZV have been developed and will expand new anti-herpes drug therapy.

DNA synthesis inhibitors affecting DNApol are categorized into five groups by their mechanism of action as shown in Fig. 6.1. The first group inhibits DNA polymerization by blocking the incorporation of normal deoxyribonucleoside triphosphates (dNTPs), but the inhibitor itself is not incorporated into DNA. Foscarnet, vidarabine, and sorivudine belong to this group.

The second group functions through chain termination by inhibiting chain elongation that stops at the incorporated site due to the lack of the 3′OH group in the deoxyribose part for the binding to the next base. ACV is phosphorylated by viral thymidine kinase (TK), and its triphosphate form is incorporated into the DNA chain. Next, the chain can no longer incorporate dNTPs, resulting in the inhibition of DNA synthesis because ACV lacking 3′OH cannot form the phosphodiester bonding with the 5′OH- of the next dNTP. Valacyclovir is a prodrug of ACV with improved oral absorption and compliance.

In the third group, the incorporation of the agents into the DNA chain does not stop at the incorporated site but terminates several bases ahead of the newly incorporated DNA chain. PCV, its prodrug famciclovir, and ganciclovir (GCV) possess 3′OH- groups for the phosphodiester bonding with the 5′OH- of normal dNTPs and stop after chain elongation of several bases possibly due to the unstable structure.

Brivudin, idoxuridine, and ribavirin are incorporated into the RNA or DNA strand, respectively, and the complementary strand synthesis to this incorporated strand generates many mismatches, resulting in the production of nonfunctional proteins leading to replication-incompetent virus production (lethal mutagenesis).

Recent advancement in antiherpetic drugs has resulted in the development of novel viral HPIs, and clinical studies on genital herpes using two HPIs, ASP2151 (amenamevir) and BAY 57-1293 (pritelivir), have been successfully conducted (Tyring et al. 2012; Wald et al. 2014, 2016). HPIs inhibit the initial stage of DNA replication before DNApol functions and have no effect on viral DNApol activity. Although the modes of DNA synthesis inhibition are different and specific to each antiviral agent, they exhibit similar efficacy by inhibiting viral DNA synthesis in vitro and in vivo. One of helicase-primase inhibitors, amenamevir, has recently completed its clinical trial on herpes zoster using once-daily dose and been approved and used for the treatment of herpes zoster in Japan. Thus, the clinical application of HPIs suggests the possibility that they may assume an important position similar to ACV in HSV and VZV therapy.

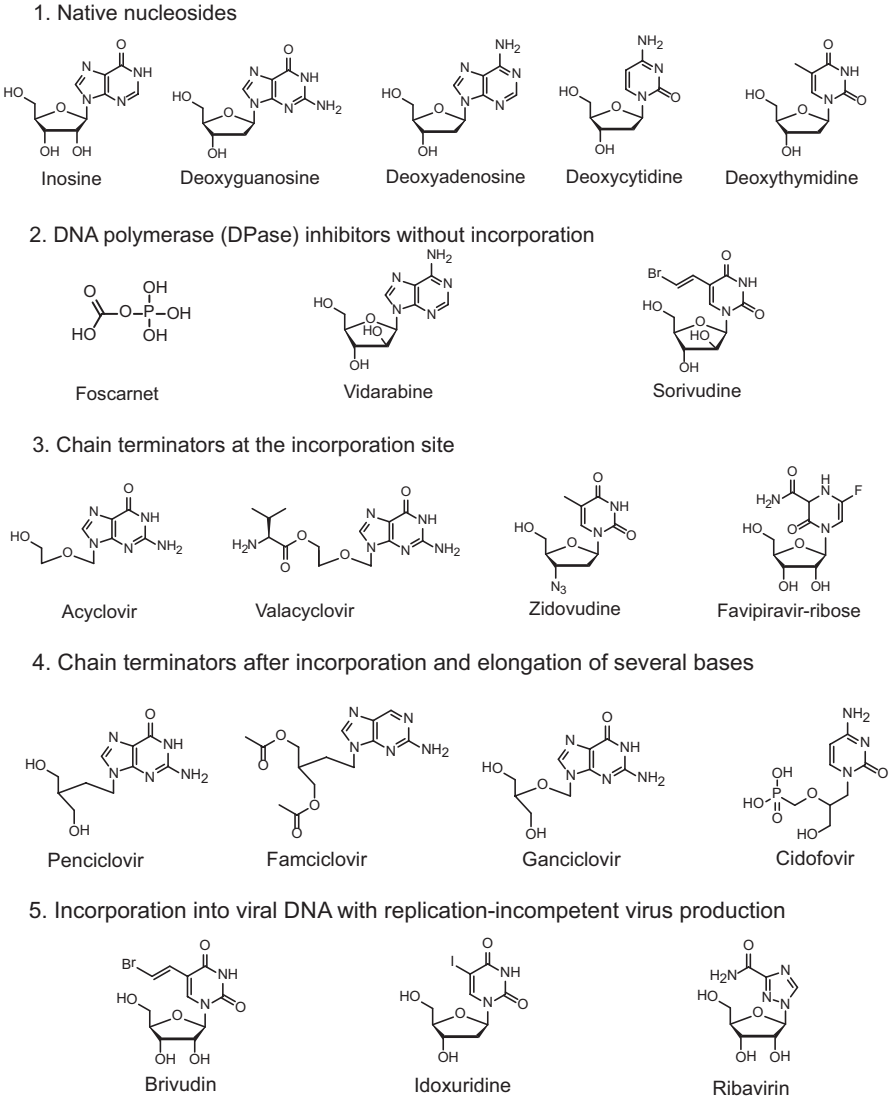


Fig. 6.1 Antiviral compounds acting on viral DNA polymerase and their categories. (*1*) Native nucleosides are inosine, deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxythymidine. (*2*) DNA polymerase (DNApol) inhibitors without incorporation are foscarnet, vidarabine, and sorivudine. (*3*) Chain terminators at the incorporation site are acyclovir and valacyclovir; an anti-human immunodeficiency virus drug, zidovudine; and an anti-influenza drug, favipiravir. (*4*) Chain terminators after incorporation and elongation of several bases are penciclovir, famciclovir, ganciclovir, and cidofovir. (*5*) Drugs that incorporate into viral DNA with replication-incompetent virus production are brivudin, idoxuridine, and ribavirin

6.2 Deoxyribonucleotide (dNTP) Synthesis in HSV- and VZV-Infected Cells

Purine is synthesized from amino acid to inosine monophosphate (IMP) and then to adenosine monophosphate (AMP) and guanosine monophosphate (GMP), and cytosine and uridine monophosphates are synthesized from amino acids as shown in Fig. 6.2. Ribonucleotide monophosphates (rNMPs) synthesized de novo are the ribose form (RNA type), and subsequently their triphosphate forms are the substrates for RNA. On the other hand, as the substrates for DNA, ribose forms of ribonucleotide diphosphate (rNDP) should be converted to the deoxyribose forms of dNDPs by ribonucleotide reductase (RR). The diphosphate form of dNTPs, except for that of uridine, is processed to the triphosphate form (dNTP) as the substrates of DNA. Uridine is not a substrate of DNA, and its diphosphate form, dUDP, becomes the monophosphate form dUMP. dUMP is a substrate for thymidylate synthase (TS) and is converted into thymidine monophosphate (dTMP) successively to thymidine triphosphate (dTTP) for DNA. TS is a key enzyme for TMP synthesis in a de novo pathway and requires folic acid as the coenzyme for C1 unit (methyl residue) transfer to produce TMP. Therefore, TS is a target anticancer drug of the chemotherapy agent 5-fluorouracil (5-FU); an immunosuppressant, methotrexate; and the anti-VZV agent sorivudine monophosphate. Thus, blocking the TS pathway results in a severe outcome of cancer cells or immunoregulatory cells depending on the importance of this pathway for DNA synthesis. TK is a key enzyme in the salvage pathway, which recycles nucleosides, to supply TMP, independent of the pathway from dUMP to TMP through TS. DNA polymerase (DNApol) is the final step for DNA synthesis using synthesized dNTPs through RR, TS, and TK.

Thus, RR, TS, and TK are important enzymes for dNTP synthesis and their supply to herpes virus DNA synthesis. HSV encodes RR and TK, while VZV encodes RR, TS, and TK. HSV and VZV can replicate in cells that do not synthesize DNA at the time of infection, but these cells synthesize proteins through mRNA synthesis. Thus, most of the cells synthesize RNA without DNA synthesis; therefore, rNTPs are abundant in the cells. HSV and VZV infection induce viral RR in these cells, and rNDPs are converted to dNDPs by viral RR. In addition to viral TK, dNMPs, including TMP, are supplied for viral DNA synthesis via the salvage pathway; thus, cells infected with HSV and VZV are supported for viral DNA synthesis by supplying dNMPs in infected cells through viral RR from rNDP and TK from thymidine. VZV encodes TS in addition to RR and TK, thus supporting viral DNA synthesis. Thus, viral RR, TK, and TS play important roles for the replication of HSV and VZV in infected cells. Cytomegalovirus (CMV) encodes UL97 that phosphorylates ganciclovir instead of TK (Littler et al. 1992; Sullivan et al. 1992), but RR (large subunit) lacks many catalytic residues (Patrone et al. 2003). Thus, CMV has a different dNTP supply pathway for DNA synthesis with HSV and VZV.

On the other hand, host cells possess RR, TK, and TS for their replication, and they are dependent on the cell cycle or cellular activity. Cellular TK phosphorylates thymidine, while cellular TK does not phosphorylate ACV and PCV; therefore,

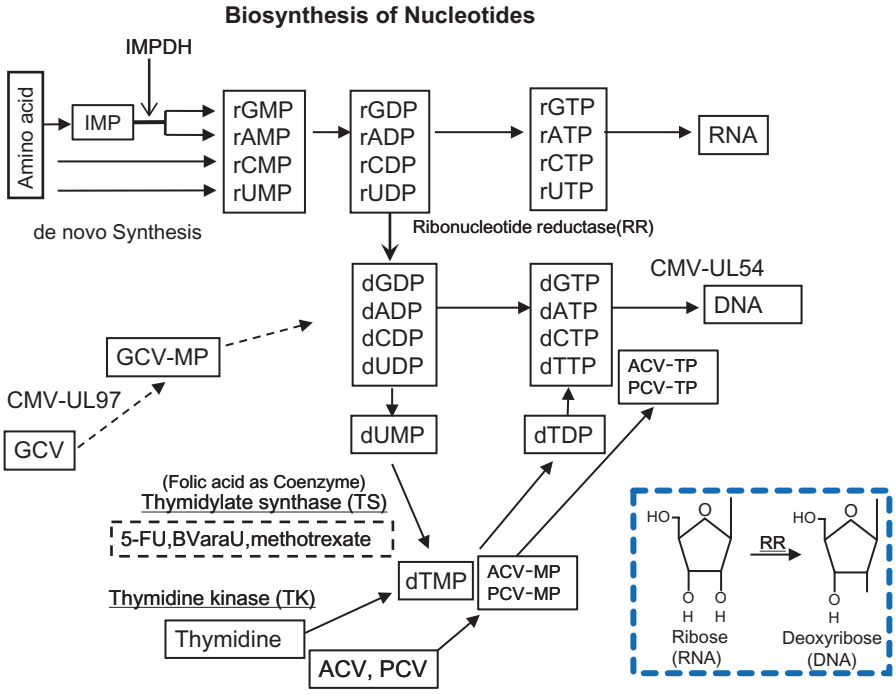


Fig. 6.2 Biosynthesis of nucleotides. Purine and pyrimidine are synthesized de novo from amino acids as ribose form nucleotides and inosine monophosphate (IMP) that are modified by IMP dehydrogenase to adenosine monophosphate (rAMP) and guanosine monophosphate (rGMP). Next, nucleotide monophosphate (rNMP) is phosphorylated to triphosphate forms (rNTP), and these become the substrate for RNA. The ribose form of nucleotide diphosphate (rNDP) is converted to the 2′-deoxyribose form (dNDP) by cellular or viral ribonucleotide reductase (RR) as shown in the lower box. When viral RR is induced by HSV and VZV infection, dNDPs are synthesized in the early phase of infection and are supplied for viral DNA synthesis to facilitate and activate viral DNA synthesis even in cells that do not actively synthesize cellular DNA. Thymidine is an important substrate of DNA and is supplied in two ways—from uridine monophosphate (UMP) to thymidine monophosphate (TMP) by thymidylate synthase (TS) (de novo pathway) and from the systemic circulation by thymidine kinase (TK) (salvage pathway). The important role of TS in thymidine biosynthesis can be easily understood by blocking this pathway with the anticancer drug 5-fluorouracil (5-FU) and immunosuppressant methotrexate. Sorivudine (BVaraU) is an anti-VZV agent, and its monophosphate form inhibits TS. Because sorivudine itself is phosphorylated and its monophosphate blocks TMP formation by the inhibition of TS activity, sorivudine shows potent anti-VZV action at the low concentration by the reduction of the competing TMP supply on viral DNA polymerase (DNApol). Acyclovir (ACV) and penciclovir (PCV) are phosphorylated by viral TK and are further phosphorylated to the triphosphate form by cellular enzymes. ACV-TP and PCV-TP are incorporated into viral DNA by viral DNApol, resulting in chain termination. Concerning the anti-CMV drug ganciclovir, it is phosphorylated by CMV-UL97 and incorporated into viral DNA by CMV-UL54 DNA polymerase

these antivirals have little effect on non-infected cells. Thus, cellular enzymes are present, but viral infection induces viral enzymes for viral replication only in infected cells. Therefore, uninfected cells do not have viral TK, and ACV or PCV is not phosphorylated for the inhibition of DNA synthesis. This specificity of ACV phosphorylation by viral TK only in infected cells is evaluated as a drug with a wide safety margin and a very high therapeutic index.

6.3 Pathway of Acyclovir (ACV) (Valaciclovir), Penciclovir (PCV) (Famciclovir), and Ganciclovir (Valganciclovir)

Viral RR and TS supply dNDPs from the ribonucleotides of RNA metabolism and facilitate viral DNA synthesis in the early phase of infection as shown in Fig. 6.2. Thus, HSV and VZV can promote viral DNA synthesis and complete replication even in infected cells that are not synthesizing DNA, such as neurons and keratinocytes. Viral TK phosphorylates ACV and PCV, which are further phosphorylated to ACV triphosphate (TP) and PCV-TP, respectively. ACV-TP and PCV-TP compete with dGTP for incorporation into viral DNA through viral DNApol. ACV-TP lacking 3′OH- is incorporated into the DNA chain and terminates the elongation at the incorporated site because the 5′-3′ phosphodiester bond cannot be formed between the 3′-end of the sugar part of ACV and 5′-OH- of the next dNTP that should be incorporated. By contrast, PCV possessing the 3′OH-sugar moiety is incorporated into the DNA chain, and subsequently, dNTP is incorporated into the elongated DNA chain. However, this chain elongation terminates after the addition of several dNTPs into DNA chain (Vere Hodge and Cheng 1993). Thus, ACV and PCV are phosphorylated by viral TK and terminate chain elongation in a different way.

ACV and PCV are phosphorylated to ACV-TP and PCV-TP, and both terminate viral DNA synthesis. dGTP competes ACV-TP or PCV-TP on viral DNApol, and an increased amount of dGTP reduces the antiviral action of ACV-TP or PCV-TP by suppressing the incorporation of ACV-TP or PCV-TP. Regarding the dNTP supply and antiviral activity of ACV and PCV, dGTP is supplied from rGDP by viral RR, and the supplied dGTP reduces the molar ratio of ACV-TP or PCV-TP versus dGTP as the substrate of viral DNApol. In the early phase of infection, the supply of dGTP through RR is limited compared with the amount of ACV-TP or PCV-TP, and the ratio of ACV-TP or PCV-TP per dGTP is large enough to show strong inhibition of viral DNA synthesis. However, when viral DNA synthesis becomes active by the abundant supply of dGTP through viral RR in the late phase, active viral DNA synthesis phase, of infected cells, ACV-TP, or PCV-TP per dGTP is reduced, and thus the antiviral activity of ACV or PCV was attenuated by a sufficient supply of dGTP competing with ACV-TP or PCV-TP (Shiraki et al. 1992; Yajima et al. 2017). Antiviral activity of ACV and PCV is influenced by the amount of competing dGTP in the infected cells, while antiviral activities of foscarnet and HPIs are not influenced by viral DNA synthesis (Yajima et al. 2017).

6.4 ACV-/PCV-Resistant Mutants

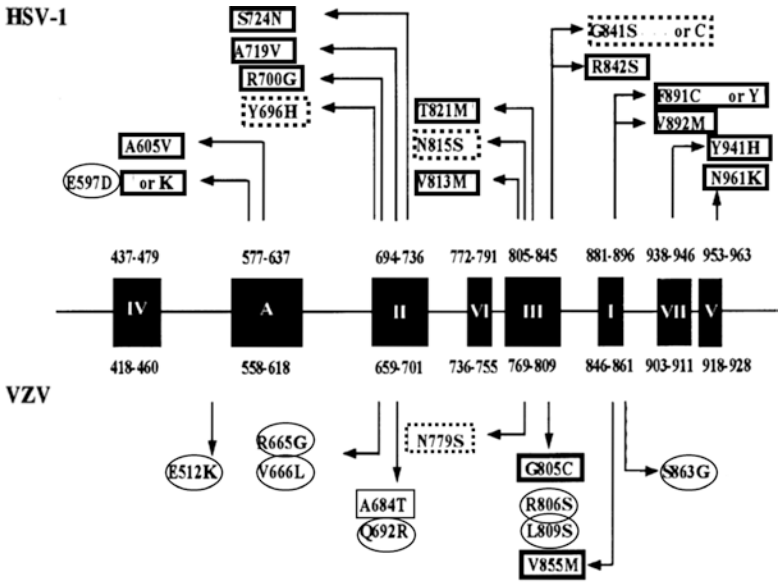
Most of the ACV- or PCV-resistant viruses are TK-deficient mutants, and mutants with altered TK and DNApol mutants are not common. When TK function is lost by mutation or decreased activity to phosphorylate ACV, they are called TK deficient or TK altered, and these viruses are called as ACV-resistant viruses (TK deficient or TK altered, respectively). The TK gene is nonessential for viral replication, and TK-deficient mutants can replicate in even with the loss of its enzymatic activity. This is a specific feature of drug-resistant virus related only to ACV and PCV. Altered TK mutants have altered substrate specificity to ACV with preservation of TK activity but fail to phosphorylate ACV, resulting in preserving the growth capability in the presence of ACV.

Viral DNApol is the essential gene product for replication, and DNApol function cannot be lost in viral replication. Therefore, DNApol mutants resistant to ACV have the amino acid change in the conserved domains that preserve the DNApol function but do not incorporate ACV-TP with the change in the substrate specificity of DNApol. These mutations do not occur randomly, but specific amino acid alterations occur in the conserved domains as shown in Fig. 6.3. Amino acid alterations are clustered, and each antiviral agent has its own specific locations in the DNApol. Interestingly, N779S of VZV confers ACV resistance but renders hypersensitivity to vidarabine and foscarnet, and G805C and V855M of ACV-resistant VZV confer vidarabine and foscarnet resistance but render hypersensitivity to aphidicolin. Alterations in the recognition sites of viral DNApol to ACV-TP influence those to vidarabine and foscarnet and the aphidicolin-acyclic ribose structure of ACV. The recognition sites of mutant DNApol to ACV are divided into the foscarnet-arabinose (vidarabine) moiety group and acyclic ribose structure of the ACV groups (Fig. 6.3). Mutations of DNApol at the foscarnet-arabinose (vidarabine) recognition site are more sensitive to aphidicolin, and mutations at the aphidicolin-acyclic ribose structure are resistant to vidarabine and foscarnet. ACV-resistant mutants with foscarnet-arabinose (vidarabine) hypersensitivity are more resistant to ACV than those with foscarnet-arabinose (vidarabine) resistance.

ACV-resistant mutants are mostly TK mutants; moreover, all ACV resistance are not associated with foscarnet resistance of the DNApol mutants. These observations indicate that foscarnet treatment is effective in most ACV-resistant mutant infections.

6.5 Subclinical Generation of ACV-Resistant Mutants During ACV Treatment

Susceptibility to ACV of viral isolates from patients treated with ACV is not influenced by ACV treatment, and ACV-resistant mutants do not appear during episodic therapy or suppressive therapy for genital herpes with long-term antiviral therapy in



Susceptibility of VZV mutants to antivirals (μg/ml) n= 3 and more

	Amino acid	ACV	Phosphonoacetic acid	Vidarabine	Aphidicolin
Kawaguchi	Original strain	1.83±0.11	5.24+0.19	4.56+0.65	0.37+0.08
A2	V855M	19.9±5.6	39.0+6.1	13.9+2.3	0.11+0.03
A3	G805C	19.2±1.9	31.1+8.2	9.36+1.42	0.15+0.06
A6	N779S	69.3±1.5	1.28+0.08	0.24+0.03	0.50+0.10

Fig. 6.3 Viral DNA polymerase mutations of ACV-resistant mutants in the HSV-1 and VZV DNA polymerase gene (Kamiyama et al. 2001) Filled boxes show the conserved regions I–VII of the HSV-1 DNA polymerase gene. The reported mutation sites of HSV and VZV DNA polymerase mutants are summarized, and these sites are substrate recognition sites for ACV, foscarnet, vidarabine, and aphidicolin

The lower table shows the susceptibility of VZV V855M, G805C, and N779S mutants to ACV, foscarnet/phosphonoacetic acid, vidarabine, and aphidicolin. These three mutants indicate the recognition sites of antiviral drugs between the foscarnet-arabinose moiety of the vidarabine group and aphidicolin group

indicates ACV and foscarnet/phosphonoacetic acid-resistant mutants

indicates ACV-resistant but foscarnet/phosphonoacetic acid-hypersensitive mutants

indicates foscarnet/phosphonoacetic acid-resistant mutants

immunocompetent patients (Daikoku et al. 2016; Englund et al. 1990; Honda et al. 2001; Okuda et al. 2004; Reyes et al. 2003; Stranska et al. 2005). ACV treatment of infected cells increased the frequency of guanosine homopolymeric (G-string)-string mutation in the TK gene of HSV and VZV (Daikoku et al. 2016; Ida et al. 1999; Sasadeusz et al. 1997), while PCV treatment induced TK mutation quite rarer in VZV-infected cells than ACV treatment (Ida et al. 1999). This contrasting action between ACV and PCV is due to the mode of chain termination and the proofreading activity of herpesvirus DNApol. Herpesvirus DNApol has alkaline deoxyribonuclease (DNase) activity, which functions as the proofreading activity of DNApol.

RNA-dependent RNA polymerase or Taq DNA polymerase does not demonstrate proofreading activity. The presence of proofreading activity results in higher fidelity (1 in 10^6) of DNA polymerase than the lower fidelity (1 in 10^4) of RNA polymerase of RNA viruses or Taq DNA polymerase (Drake 1993). Thus, RNA viruses generate mutations more frequently than herpesviruses, and the proofreading activity is important in maintaining the fidelity of the genome during herpesvirus replication. Incorporation of ACV and proofreading activity induce mutation in the G-string parts as follows. When ACV is incorporated into the DNA chain at the terminus, the misincorporated ACV is removed by proofreading DNase and replaced with dGTP. These frequent correction cycles of the incorporation and removal of ACV are repeated at the G-string parts, and these G-string parts become the hot spots of mutation by the misincorporation of ACV, resulting in the deletion, addition, or substitution of nucleotides in the G-string parts of the TK gene. This type of mutation may occur in the whole genome, but some in the essential genes become fatal to the virus by the loss of function. The TK gene is nonessential and a target of ACV resistance, and the TK mutants are selected in the presence of ACV and are visualized. Although subclinical, this process was detected in the clinical isolates from patients with genital herpes treated with ACV. ACV treatment induces G-string mutations in the virus population in the genital lesions, and these mutants become latent, reactivate, and appear in the genital lesions in the patients (Daikoku et al. 2016). There is no problem as current ACV therapy, but such a change is subclinically occurring.

While PCV is incorporated into DNA but allows to the elongation of several bases, proofreading does not occur, and, subsequently, the G-string parts are not the hot spots of mutation, resulting in a quite lower mutation rate than that of ACV. Thus, mutants isolated in PCV treatment are rare.

6.6 Sorivudine

Sorivudine is phosphorylated to the diphosphate form by TK of HSV-1 and VZV, and the inhibition of TS activity by sorivudine monophosphate caused VZV to be quite susceptible to sorivudine. The TMP supply from UMP is blocked through inhibiting TS, and this increases the ratio of antiviral sorivudine monophosphate per TMP in VZV-infected cells (Cohen and Seidel 1993; De Clercq 2005; Kawai et al. 1993; Machida et al. 1982; Yokota et al. 1989). The IC_{50} of sorivudine is extremely low at 0.0035 μM, and sorivudine showed potent anti-VZV activity and better efficacy than acyclovir. Sorivudine 40 mg/day showed significantly more efficacy than ACV 4 g/day in the treatment of herpes zoster in HIV-infected adults (Bodsworth et al. 1997). Sorivudine was licensed for herpes zoster in Japan in 1993.

Bromovinyluracil of the sorivudine metabolite irreversibly binds and inhibits dihydropyrimidine dehydrogenase activity, and this enzyme is important as the degrading enzyme of 5-fluorouracil (5FU), an anticancer drug. When sorivudine was used in patients with cancer treated with 5-FU, the 5-FU concentration in the

blood was increased by interfering with the 5-FU catabolism by inhibition of the 5-FU-degrading enzyme by bromovinyluracil. The increased 5-FU caused severe hematopoietic toxicity of 5-FU in the reduction of leukocytes and platelets causing 15 patient deaths with 5-FU and sorivudine treatment. If not combined with 5-FU, sorivudine is an excellent anti-VZV drug.

6.7 Brivudin

(E)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU/brivudin) is phosphorylated by viral TK, inhibits viral DNA synthesis, and shows strong activity toward VZV at lower concentrations than ACV (De Clercq 2004; De Clercq et al. 1982). A double-blind survey study was conducted on 608 herpes zoster patients treated with 1 × 125 mg oral brivudin (n = 309) or 5 × 800 mg ACV (n = 299), both for 7 days, during two prospective, randomized clinical herpes zoster trials. The survey was aimed to evaluate the outcome of the two treatment regimens in postherpetic neuralgia (PHN). The incidence of PHN, defined as zoster-associated pain occurring or persisting after rash healing, was significantly lower in brivudin recipients (32.7%) than in ACV recipients (43.5%, P = 0.006) (Wassilew et al. 2003). Brivudin is used for the treatment of herpes zoster in adult patients and may reduce the incidence of PHN. The use of brivudin with 5-FU requires caution, because like sorivudine, it can enhance the hematopoietic toxicity of 5-FU.

6.8 DNA Polymerase Inhibitors

The viral DNA polymerase inhibitors foscarnet and rarely vidarabine in intravenous preparations are used for drug-resistant HSV and VZV infection. Current treatment with ACV or PCV is satisfactory in HSV and VZV infection because immunocompromised patients, especially those with human immunodeficiency virus infection, are well controlled, and immunocompromised patients who need prolonged antiviral treatment are limited. Foscarnet and vidarabine do not require phosphorylation for their antiviral action and are used for TK-deficient HSV and VZV. Vidarabine inhibits viral DNA synthesis at concentrations below those required to inhibit host cell DNA synthesis (Shipman Jr et al. 1976) and may have multiple sites of action within an infected cell (Kamiyama et al. 2001; Suzuki et al. 2006). It is phosphorylated to its active triphosphate form by cellular kinases (Schwartz et al. 1984). Thus, vidarabine can inhibit TK-deficient mutants of HSV and VZV that are resistant to ACV, and the active site of vidarabine on DNApol is different from that of ACV but similar to that of foscarnet (Kamiyama et al. 2001; Larder and Darby 1986; Miwa et al. 2005; Shiraki et al. 1990). Vidarabine is less efficient with more adverse events than ACV (Whitley et al. 1986). Therefore, the clinical use of vidarabine is limited.

Foscarnet is a pyrophosphate analog that is released from dNTPs on DNApol; thus, foscarnet directly acts on DNApol (Kern et al. 1981; Ostrander and Cheng 1980). The inhibitory action of foscarnet depends on the ratio of the numbers of DNApol and foscarnet molecules; therefore, foscarnet is not influenced by the supply of deoxyribonucleotides, such as ACV or PCV (Yajima et al. 2017). Foscarnet is available as an intravenous preparation and requires attention regarding the electrolyte balance in the blood and renal function after its administration. Foscarnet is used for ACV- or ganciclovir-resistant virus.

The mutation sites of HSV and VZV DNApol are shown in Fig. 6.3. There are two recognition groups of ACV-resistant HSV and VZV in their DNApols: the foscarnet-arabinose moiety of the vidarabine group versus the aphidicolin group as described in the section of ACV-/PCV-resistant mutants. ACV-resistant mutants with foscarnet-vidarabine resistance (VZV G805C, V855M) are more sensitive to aphidicolin than the wild-type parent virus, and those with foscarnet-vidarabine hypersensitivity are more resistant to aphidicolin. ACV-resistant mutants with foscarnet-arabinose (vidarabine) hypersensitivity (VZV N779S) are more resistant to ACV than those with foscarnet-arabinose (vidarabine) resistance (Kamiyama et al. 2001).

Cidofovir has a phosphorylated form and does not require initial phosphorylation by TK or CMV-UL97 (Cundy 1999; Safrin et al. 1997). Cidofovir is an injectable antiviral medication used for the treatment of CMV retinitis in individuals with AIDS. Cidofovir-diphosphate inhibits viral DNApol of herpesviruses, orthopoxviruses, adenoviruses, polyomaviruses, and papillomaviruses (Beadle et al. 2002). A prodrug form of cidofovir, brincidofovir (CMX001), is orally available, and clinical studies are under way including Ebolavirus infection (Dunning et al. 2016).

6.9 Helicase-Primase in DNA Synthesis

Double-stranded DNA needs to become separated into two single strands (replication fork) before DNA synthesis, and their complementary strands are synthesized from each DNA strand to make two new double-stranded DNA molecules in the process of DNA replication (Fig. 6.4). Helicase-primase is responsible for both unwinding viral DNA at the replication fork, separating double-stranded DNA into two single strands, and synthesizing RNA primers (Okazaki fragment) in the lagging strand for DNA synthesis. DNApol starts complementary DNA synthesis from these two strands. This HP enzyme complex consists of three proteins—a helicase, a primase, and cofactor subunits—which are well conserved among *Herpesviridae* viruses and are called UL5 (helicase, VZVORF55), UL52 (primase, VZVORF6), and UL8 (cofactor, VZVORF52), respectively. UL5 unwinds duplex DNA ahead of the fork and separates the double strand into two single strands. UL52 lays down RNA primers that the two-subunit DNA polymerase (UL30/UL42) extends. The helicase-primase complex possesses multi-enzymatic activities, including DNA-dependent ATPase and helicase localized in the helicase subunit and primase in the

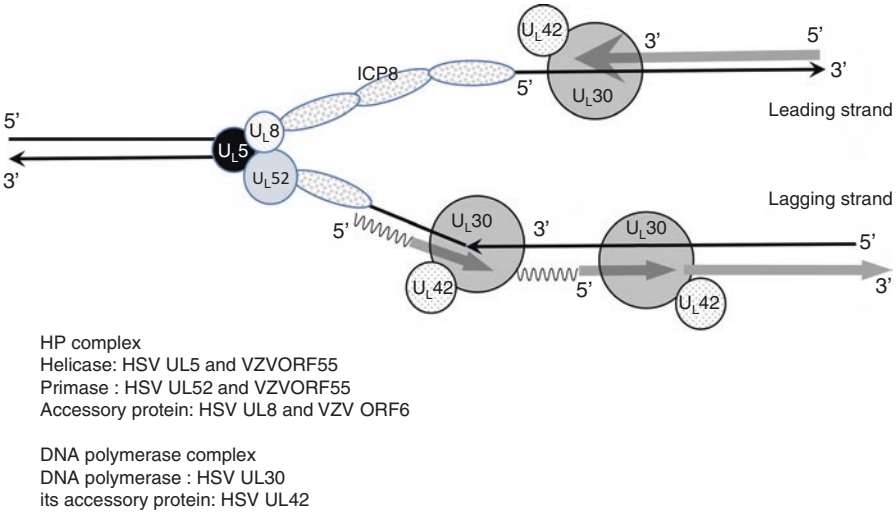


Fig. 6.4 Mechanism of DNA synthesis and viral helicase-primase (HP) complex (modified from Boehmer and Lehman 1997). Figure shows the role of the helicase-primase complex (UL5, UL8, UL52 of HSV and ORF55, ORF6, ORF52 of VZV), DNA polymerase complex (UL42, UL30 of HSVDNA polymerase), and ICP8 single-stranded DNA-binding protein of HSV. HSV UL5 and VZVORF55 (helicase) unwind double-stranded DNA and separate double strands into two single strands, making the replication fork. HSV UL52 and VZVORF55 (primase) synthesize RNA primers (Okazaki fragments) for DNA synthesis. DNA polymerase and its accessory protein (UL42) bind to each single strand and synthesize complementary DNA to each strand. The single-stranded DNA-binding protein, ICP8, binds to single-stranded template DNA. The arrows indicate the direction of movement of the DNA replication proteins (Shiraki 2017)

primase subunit; all of these enzymatic activities are needed for the helicase-primase complex to function in viral DNA replication. HP is quite different from topoisomerases to wind or relax double-stranded DNA. Therefore, HP is an important enzyme for DNA synthesis and a conserved enzyme from *E. coli* to *Homo sapiens*, and HSV, VZV, and CMV have their own HP as an essential gene product in their replication.

6.10 Helicase-Primase Inhibitor (HPI)

HPI inhibits the single-stranded, DNA-dependent ATPase, helicase, and primase activities by binding to the helicase-primase complex (Biswas et al. 2014; Chono et al. 2010, 2012; James et al. 2015). There are three classes of herpesvirus HPIs – thiazole urea, BAY 57-1293 (pritelivir) (Kleymann et al. 2002), 2-aminothiazolylphenyl derivatives, BILS 179 BS (Crute et al. 2002; Spector et al. 1998), and oxadiazolylphenyl type ASP2151 (amenamevir) (Fig. 6.5) (Chono et al. 2010). Interestingly, the former two classes of HPIs, BAY 57-1293 and BILS 179 BS,

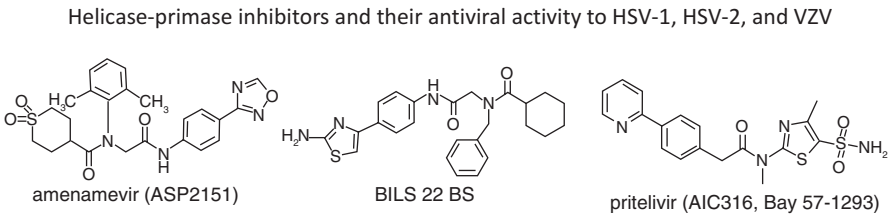


Fig. 6.5 Structure and antiviral activity of helicase-primase inhibitors. The structures of three helicase-primase inhibitors (HPI) and their spectrum of antiviral activity are shown in the figure and table, respectively. Three HPIs of amenamevir—oxadiazolylphenyl type (ASP2151) (Chono et al. 2010); BILS 179 BS, 2-amino-thiazolylphenyl derivatives (Crute et al. 2002; Spector et al. 1998); and pritelivir, thiazole urea (BAY 57-1293) (Kleymann et al. 2002)—were developed and exhibited lower EC_{50} concentrations than those of acyclovir, and amenamevir and pritelivir have been evaluated for their clinical efficacy. Interestingly, amenamevir possesses anti-VZV activity and is based on the anti-VZV activity. A clinical study on amenamevir for herpes zoster has been completed and submitted for approval of the licensure of herpes zoster in Japan

inhibit HSV-1 and HSV-2 but not VZV, and amenamevir possesses antiviral activity not only against HSV-1 and HSV-2 but also against VZV. HPIs require low concentrations to inhibit viral growth of HSV-1 and HSV-2 at the effective concentrations for 50% plaque reduction (EC_{50}s) of 0.014 to 0.060 μM and 0.023 to 0.046 μM, respectively, and the anti-VZV activity of amenamevir (0.038–0.10 μM) is more potent against all strains tested than against ACV (1.3-5.9 μM) (Chono et al. 2010).

These HPIs are virus specific with low cytotoxicity in vitro, are orally available and effective against HSV infection, and are well tolerated in mice. As the target molecules are different from ACV, PCV, foscarnet, and vidarabine, their mechanism of action and antiviral and pharmacokinetic profiles are unique to HPIs. Foscarnet inhibits viral DNA polymerase activity through direct binding to the pyrophosphate binding site (James and Prichard 2014). Both HPIs and foscarnet directly inhibit viral enzymes. On the other hand, ACV is phosphorylated by HSV TK and cellular kinase, which convert it to the active form, ACV-TP, and then incorporate it into the elongating viral DNA strand, resulting in chain termination (James and Prichard 2014). Because ACV-TP competes with dGTP to inhibit viral DNA synthesis, the ratio of ACV-TP to dGTP directly affects the effectiveness of ACV in virus replication. Thus, it is reasonable that amenamevir and foscarnet, which directly inhibit the viral enzyme, suppressed viral replication more effectively than ACV after viral DNA synthesis becomes active (Yajima et al. 2017). ACV-TP competes with dGTP on viral DNApol in infected cells, and anti-HSV activity is attenuated when dGTP supply becomes abundant after infection. The EC_{50}s of ACV to HSV-1 was approximately 1.5 μM up 5 h after infection and increased after 7.5 h to 12.4 μM at 12.5 h after infection as shown in Fig. 6.6a. In contrast, HPIs target the enzyme and not the nucleoside analog, and anti-HSV activity of amenamevir is not influenced regardless of the time course after infection. Thus, the susceptibility to ACV increased to 8 to 11 times that of initial EC_{50} at 12 h after infection when active DNA synthesis is progressing by the supply of dGTP, while the susceptibility of HSV-1 to foscarnet

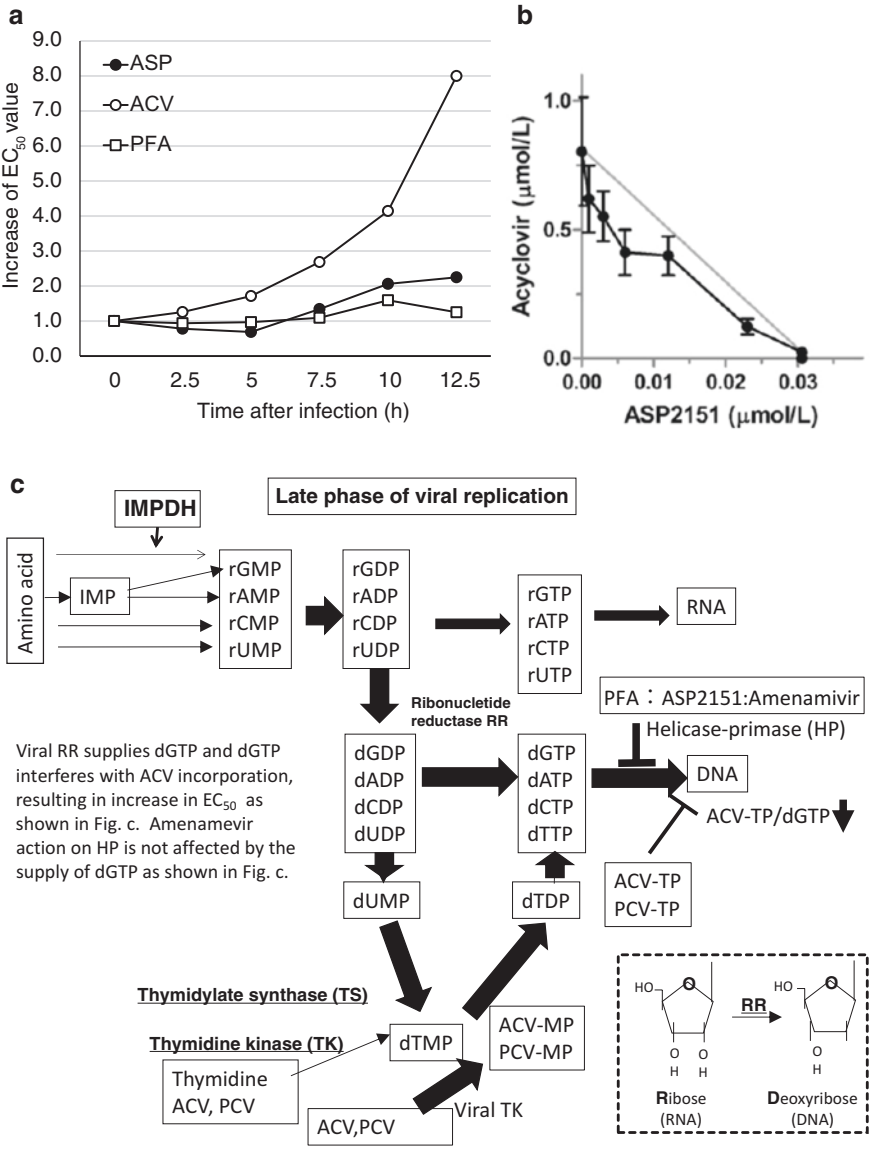


Fig. 6.6 Synergism of amenamevir (ASP2151) with ACV against the VZV Kawaguchi strain analyzed by isobologram (Chono et al. 2013). (**a**) Time course of the susceptibility (EC_{50}) changes in infected cells to ACV, ASP2151, and foscarnet (PFA) every 2.5 h after infection (Yajima et al. 2017). The increase in EC_{50} values is expressed as the ratio of those at 0 h. The susceptibility of HSV-1 to ACV increased more than 7.5 h after infection, but ASP2151 and PFA were not influenced. The effects of the increase of dGTP for viral DNA synthesis in the late phase of infected cells reduced the antiviral activity of ACV, but ASP2151 and PFA were not affected by viral DNA synthesis and its related cellular factors. (**b**) The solid straight line (gray) indicates the theoretical additive antiviral activity in combination with ASP2151 and ACV (Chono et al. 2013).

and amenamevir was not influenced by the replication cycle. The antiviral activity that is not affected by the replication cycle is a great advantage of HPIs over current anti-herpetic drugs and allows the once-daily dose for human use.

6.11 HPI-Resistant Viruses

HPI-resistant virus and ACV-resistant virus in the wild-type virus stock were compared in HSV-1 and HSV-2. The ACV-resistant virus was found in one in 10^3 to 10^4 plaque-forming units (PFU) (Chono et al. 2012), and this value was consistent with that in the other reports (Coen et al. 1982; Daikoku et al. 2016; Parris and Harrington 1982). HPI-resistant virus was found in one in 10^6 to 10^7 PFU (Chono et al. 2012); thus, the HPI-resistant virus is rare. Any mutation to lose function in the TK gene leads to the ACV-resistant virus; by contrast, HPI mutants should preserve the function of HP in the restricted amino acid change in UL5 or UL52 (Chono et al. 2010, 2012). Thus, HPI-resistant mutants are quite rarer than ACV-resistant mutants.

The HPI-resistant viruses have been isolated, and their mutation sites have been analyzed. HP is an essential gene product, and these mutations maintain their basic function for replication but avoid the interaction of HPIs by changing the structures of helicase and primase. Sequencing analyses revealed several single-base-pair substitutions resulting in amino acid changes in the helicase and primase of amenamevir-resistant HSV mutants (Chono et al. 2010). Amino acid alterations in the helicase subunit were commonly clustered near helicase motif IV in the UL5 helicase gene of both HSV-1 and HSV-2 among HPIs, while the primase subunit substitution associated with reduced susceptibility was found only in amenamevir-resistant HSV-1 mutants. Interestingly, we found that R367H with S364G substitution in the UL52 primase gene (double mutation) enhanced the resistance to amenamevir compared with S364G substitution alone (single mutation). HPI-resistant HSV mutants show susceptibility to ACV and attenuated growth capability in vitro and pathogenicity than the parent virus in HSV-infected mice (Chono et al. 2012). Mutations in either helicase or primase of HP complex against amenamevir might confer defects in viral replication and pathogenicity.

Fig. 6.6 (continued) Each point (EC_{50}) is shown as the mean ± standard error from four independent experiments. Significant synergism was observed by the combination of ASP2151 and ACV ($P = 0.0005$), and a low concentration of ASP2151 showed strong synergism with ACV (**c**) Metabolic pathway in the stage of viral DNA synthesis when ribonucleotides are efficiently converted to deoxyribonucleotides by viral RR for viral DNA synthesis (Shiraki 2017). dGTP is massively supplied for DNA synthesis at about 60,000 and 90,000 dGTPs per one DNA molecule of VZV and HSV, respectively, and this supply of dGTP reduces the incorporation of ACV-TP into viral DNA with the competition of ACV-TP with the massive supply of dGTP, resulting in attenuation of the inhibition of viral DNA synthesis by ACV. This results in the increased EC_{50} value of ACV as shown in Fig. (**a**). PFA and ASP2151 directly act on DNApol and HP, respectively, and inhibit viral DNA synthesis without any influence by the supply of dGTP. Amenamevir efficiently inhibits viral growth in the early and late phases of infection, in contrast to ACV, as indicated in Fig. (**c**)

Synergism of amenamevir was observed with ACV, PCV, and vidarabine in HSV-2 and VZV. Synergism of amenamevir with ACV was observed at all concentrations in vitro by isobologram analysis, and amenamevir achieved synergism at its low concentrations in HSV-1, HSV-2, and VZV (Fig. 6.6b) (Chono et al. 2013). The combination of amenamevir and valacyclovir in oral administration showed significant synergistic activity in HSV-infected mice. This synergistic activity of amenamevir and ACV or PCV indicates the maximization of anti-herpetic therapy, possible reduction in increased toxicity by increasing the dose of ACV or PCV, and possible reduction in the generation of resistant virus in the prolonged treatment of chronic infection in immunocompromised patients. Combination therapy may be a useful approach to treat herpes infections suspected to be caused by nucleoside analog drug-resistant virus variants and represents more effective therapeutic options than monotherapy, particularly for severe disease conditions, such as herpes encephalitis or patients with immunosuppression.

The pharmacokinetic profile of HPIs suggests the oral 1-day dose can attain the concentration exhibiting antiherpetic activity for the entire day, and this excellent property exceeds that of valacyclovir and famciclovir in maintaining the antiviral level, when they are used in the suppressive therapy of genital herpes. This long-lasting antiviral status indicates that reactivation from the ganglia would be completely inhibited with subsequent viral shedding, leading to the sexual transmission of HSV. Thus, HPIs might stop genital lesions and viral shedding in healthy persons with genital herpes and subsequent sexual transmission of HSV; additionally, HPIs would have favorable characteristics as antiherpetic drugs in suppressive therapy.

Due to promising preclinical profiles on antiviral activity, safety, tolerability, and pharmacokinetics, HPIs, pritelivir and amenamevir, were selected as development candidates, and their clinical efficacies have been evaluated in two phase-2 clinical studies for patients with genital herpes (Tyring et al. 2012; Wald et al. 2014, 2016). The clinical study of pritelivir on the viral shedding of genital herpes comparing daily oral doses of 100 mg of pritelivir with 500 mg of valacyclovir showed better efficacy on genital lesions and viral shedding than valacyclovir. Genital lesions were present on 1.9% of days in the pritelivir group vs 3.9% in the valacyclovir group (RR, 0.40; 95% CI, 0.17-0.96; $P = 0.04$). The frequency of shedding episodes did not differ by group, with 1.3 per person-month for pritelivir and 1.6 per person-month for valacyclovir (RR, 0.80; 95% CI, 0.52 to 1.22; $P = 0.29$) (Wald et al. 2016). HSV shedding among placebo recipients was detected on 16.6% of days; shedding among pritelivir recipients was detected on 18.2% of days among those receiving 5 mg daily, 9.3% of days among those receiving 25 mg daily, 2.1% of days among those receiving 75 mg daily, and 5.3% of days among those receiving 400 mg weekly. The percentage of days with genital lesions was also significantly reduced, from 9.0% in the placebo group to 1.2% in both the group receiving 75 mg of pritelivir daily (relative risk, 0.13; 95% CI, 0.02 to 0.70) and group receiving 400 mg weekly (relative risk, 0.13; 95% CI, 0.03 to 0.52). Pritelivir reduced the rates of genital HSV shedding and days with lesions in a dose-dependent manner in otherwise healthy persons with genital herpes (Wald et al. 2014). One dose of valacyclovir does not maintain antiviral activity for the entire day but suppresses apparent

reactivation with some breakthrough. By contrast, the excellent pharmacokinetic profile of HPIs with administration once a day can maintain the anti-HSV activity for the entire day, suggesting that HPIs would inhibit HSV reactivation, even viral shedding, in patients with genital herpes, as well as the transmission of HSV completely from a healthy person with genital herpes.

The clinical study of amenamevir on herpes zoster compares once-daily oral doses of amenamevir with three doses valacyclovir. Amenamevir has been approved as the first HPI drug in clinical use and successfully used for the treatment of herpes zoster in Japan.

6.12 Conclusion

This chapter has introduced the current anti-herpetic drugs and newly developed HPIs. HPI works at a low concentration in vitro, and the resistant virus is rarer than acyclovir and synergistic with ACV. HPIs have shown efficacy in genital herpes in a once-daily dose. The investigation of amenamevir has been completed a clinical study on herpes zoster, and the drug has been used for the treatment of herpes zoster in Japan. Thus, HPIs will be the next-generation drugs for HSV and VZV. Moreover, various anti-herpes virus drugs are under development (Disease NIOaaI Herpes Drugs in Development n.d.)

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