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CHEMICAL REVIEWS

Review

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# Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections

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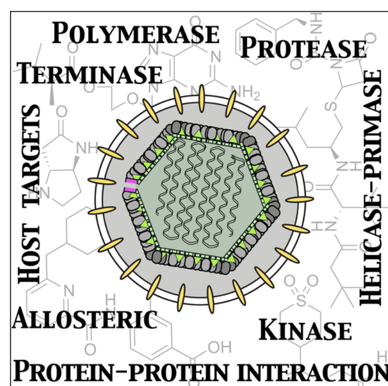


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## 1. INTRODUCTION

More than 90% of the world's population is infected with a herpesvirus.[1] Despite this staggering fact, only a handful of approved drugs exist for the general treatment of herpesvirus infections. To date, all of these drugs inhibit the same enzyme, the viral DNA polymerase. Nine human herpesviruses have been identified, and each has been associated with disease. In immune-competent individuals, herpesvirus infections are the causes of unpleasant but typically non-life-threatening diseases such as oral and genital herpes, chickenpox and shingles, skin rash in infants (roseola infantum), and infectious mononucleosis (also known simply as mono). In individuals with immature or compromised immune systems, herpesvirus infection can be devastating. Developmental disabilities, loss of sight and hearing, cancer, life-threatening pneumonia, encephalitis (inflammation of the brain), and death comprise only a partial list of the cost herpesviruses have on well being in this subset of the population.

The tremendous complexity of herpesvirus biology brings with it many potential avenues for therapeutic interventions that remain in their infancy. However, the past two decades have seen progress toward novel treatments for herpesviruses; this is the subject of the current review. Previous reviews of the subject are either more than 10 years old or cover a subsection of the field. Herein we provide a comprehensive review of herpesvirus drug discovery with an emphasis on the most recent advances in the field and their progression from early discovery to clinical development. The focus is on small-molecule inhibitor development so we do not cover biologics and vaccine development in as much detail. There is little work on antiherpes biologics outside the context of vaccine development, which is reviewed elsewhere.[2] We will, however, discuss some exciting biologics targeting viral polypeptides that appear to drive oncogenesis, though they are not required for the viral replication cycle. The necessary herpesvirus biology is introduced, and a more detailed review of that biology/virology can be found elsewhere.[3] By highlighting the exciting recent work in herpesvirus drug development, and the historical studies that enabled it, we hope to spur interest in the many potential therapeutic targets for this ubiquitous but neglected virus family.

## 2. HERPESVIRUS BIOLOGY

### 2.1. Viral Classification

All herpesviruses are large enveloped double-stranded DNA viruses. The viral genome is composed of a linear chain of 125−290 kbp and contains ∼70−200 protein coding genes, depending on the specific virus. Herpesvirus virions (the infectious particles) have three major components: the nucleocapsid, the tegument, and the envelope. Herpesviruses have an icosahedral nucleocapsid ($T$ = 16) composed of 162 capsomeres (150 hexons and 12 pentons) where the viral genome resides. A matrix of viral proteins called the tegument exists between the lipid bilayer envelope and the nucleocapsid. The envelope contains glycoproteins critical to cell attachment and entry. Virions are approximately 200 nm in diameter.[4]

### 2.2. Subfamilies

The taxonomic family *Herpesviridae* consists of herpesviruses that infect mammals, birds, and reptiles. This family does not include herpesviruses infecting fish and frogs (*Alloherpesviridae*) or bivalves (*Malacoherpesviridae*). Human herpesviruses are further broken down into three subfamilies, the $\alpha$-, $\beta$-, and $\gamma$-herpesviruses (Figure 1). Differences in biology and genetics

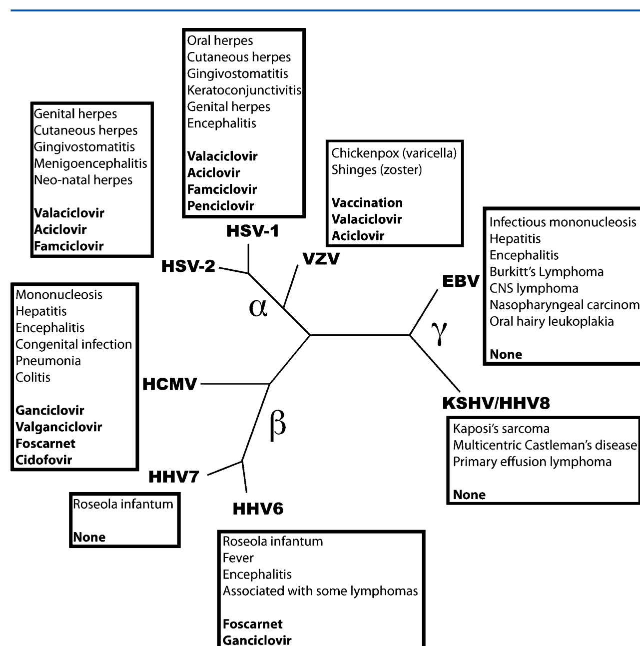


**Figure 1.** Human herpesviruses, diseases, and antiherpesvirus treatments organized by subfamilies ($\alpha$, $\beta$, and $\gamma$).

give rise to these classifications. To date nine human herpesviruses have been identified: herpes simplex virus 1 and 2 (HSV1 and 2, $\alpha$), Varicella zoster virus (VZV, $\alpha$), human cytomegalovirus (HCMV, $\beta$), human herpesvirus 6a, 6b, and 7 (HHV6a, 6b, 7, $\beta$), Epstein−Barr virus (EBV, $\gamma$) and Kaposi's sarcoma-associated herpesvirus (KSHV AKA human herpesvirus 8 or HHV8, $\gamma$). All of these viruses establish life-long latent infections with potential for periodic lytic reactivation—and all can cause disease (Figures 1 and 2).

### 2.3. Cell Tropism

The $\alpha$-human herpesviruses (HSV1, HSV2, and VZV; Figure 1) establish latent infection in cells of the peripheral nervous system. HSV1, the primary cause of oral herpes, resides primarily in the trigeminal ganglia while HSV2, the primary cause of genital herpes, tends to reside in sacral ganglia. VZV, the etiologic agent of chickenpox and shingles, latently infects both the trigeminal ganglia and the dorsal basal ganglia. Primary infection with HSV1 and HSV2 occurs in mucoepithelial cells where lytic replication also takes place. Unlike the herpes simplex viruses, VZV requires transport from mucoepithelial cells in the upper respiratory tract where infection is established to skin cells where disease most often manifests. Infection of T-cells is thought to enable this transport.

The $\beta$-herpesviruses (HCMV, HHV7, and HHV6; Figure 1) have a complex cell tropism infecting an array of immune cells as well as endothelial cells and fibroblasts. Polymorphonuclear leukocytes (PMNLs), peripheral blood mononucleated cells (PBMCs) including macrophages and monocytes, endothelial cells, and fibroblasts can all support both lytic and latent infection *in vivo*. Hematopoietic stem cells and CD14+ monocytes are thought to be the main source of reactivation.[5] The trafficking of human $\beta$-herpesviruses from among different cell types is an active area of research, particularly for human cytomegalovirus.[6]

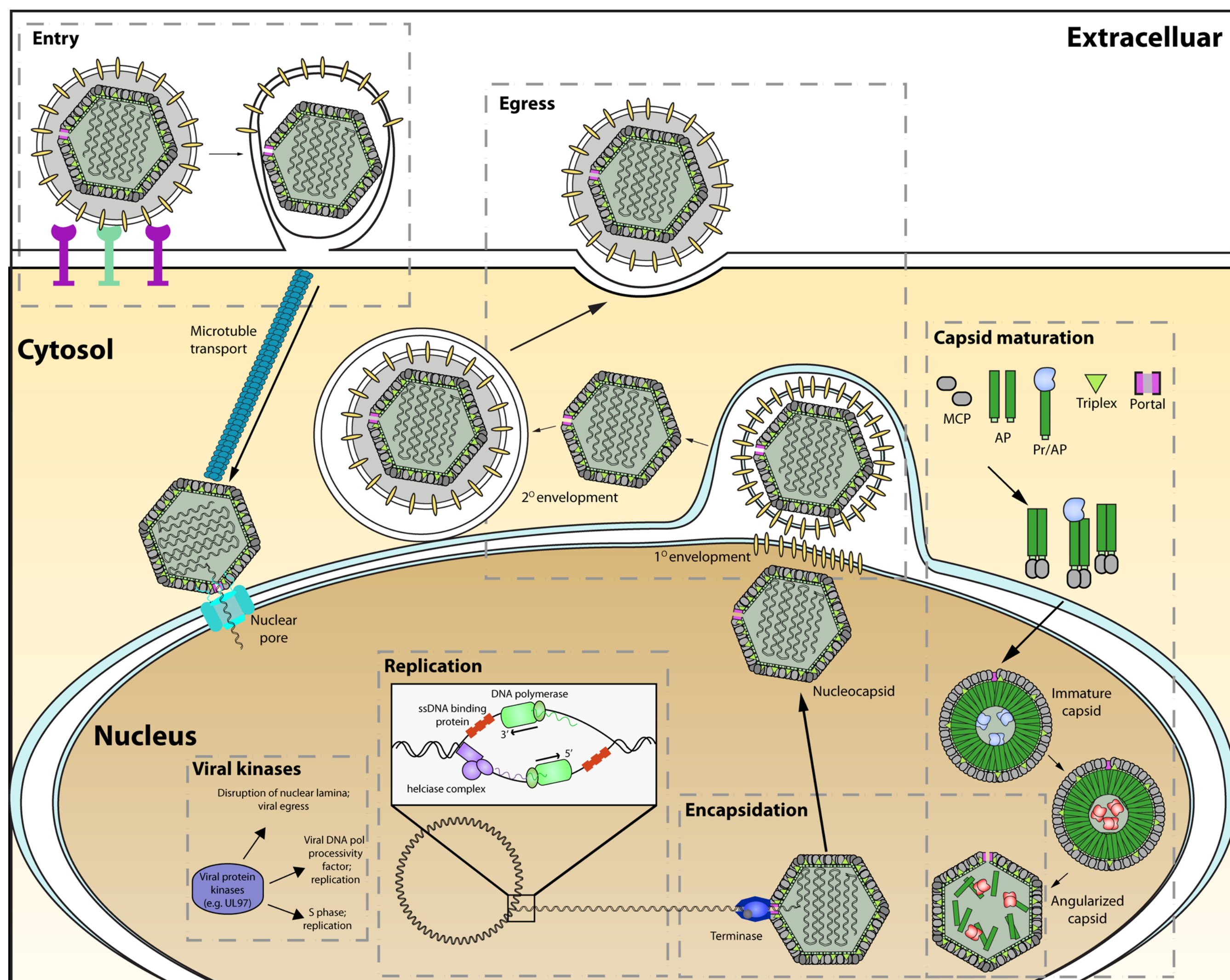


**Figure 2.** "Druggable" viral replication cycle. The processes of entry, viral DNA replication, encapsidation, capsid maturation, egress, and the role of viral kinases are depicted diagrammatically. Each process offers opportunities for inhibition of the viral replication cycle.

The $\gamma$-herpesviruses infect epithelial, endothelial, and B cells. For EBV both lytic and latent infection can take place in B cells. EBV specifically infects B cells through interaction between EBV envelope glycoproteins and complement receptor 2 (CD21) on host cells. The exact site of primary infection and the mechanism by which EBV is transferred from B cells to epithelial cells is still an area of active research. Primary EBV infection may be mediated by epithelial cells; however, some have postulated tonsillar B cells are the site of primary infection as B cells are much more efficiently infected than are epithelial cells.[7] It is also possible that both are implicated in primary infection. Numerous methods have been proposed for cell-to-cell transfer of EBV, and this process is thought to be critical for infection of epithelial cells.[7] Nasopharyngeal carcinoma and oral hairy leukoplakia both provide direct evidence for the importance of EBV infection in epithelial cells. KSHV establishes and maintains latent infection in CD19+ B cells. Multicentric Castleman's disease and primary effusion lymphoma are examples where disease manifests in B cells. However Kaposi's sarcoma arises from endothelial infection, again highlighting the importance of the complex cell tropism of $\gamma$-herpesviruses. These KSHV-associated diseases are further discussed in section 3.2.

## 2.4. Viral Replication Cycle

The viral replication cycle is depicted in Figure 2. During entry, the nucleocapsid is released from the envelope upon fusion with the host cell membrane and is internalized. Dynein/dynactin motor protein complexes then transport the nucleocapsid to the nucleus along tubulin microtubules. Capsids bind nuclear pores, and their DNA genome is released into the nucleus (Figure 2).

In productive infection, the viral genome must be replicated. A viral replisome composed of a DNA polymerase, the helicase-primase complex, and an ssDNA-binding protein perform this task (Figure 2, replication). Cellular RNA Polymerase II generates viral transcripts. A viral transactivator (i.e., HSV1 VP16), introduced into the cell as part of the tegument, kick-starts a cascade of viral gene expression controlled by the intermediate-early promoters. A variety of viral proteins, such as endoribonucleases, rapidly affect host mRNA stability, resulting in decreased expression of host proteins and a competitive advantage for the translation of viral transcripts.[8] The major capsid protein (MCP), assembly protein (AP), and maturational protease (Pr) are expressed in the cytoplasm and translocate to the nucleus where they assemble to form the immature capsid. Processing by the viral protease allows for

maturation of the capsid, formation of the portal vertex, and ultimately encapsidation of the viral genome by the terminase complex (Figure 2, capsid maturation).[9] Once the mature nucleocapsid has formed, it must associate with tegument proteins, become enveloped, and egress from the cell (Figure 2, egress). Acquisition of the tegument was recently reviewed.[10] The viral capsid is too large to be transported through the nuclear pore. Instead, herpesviruses remodel host membranes, a process mediated in part by viral kinases (i.e., HCMV pUL97, Figure 2). Primary envelopment occurs with transport into the inner nuclear membrane, de-envelopment with transport out of the perinuclear space and into the cytoplasm, and finally secondary envelopment moving from the cytoplasm to the extracellular space (Figure 2, egress). This process requires a range of viral proteins and was recently reviewed.[11] The role of host and viral proteins in HCMV maturation, and its comparison to that of other herpesviruses, was also recently reviewed.[12]

During latency, maintenance of the viral episome varies between herpesviruses, in large part based on whether the cells they latently infect replicate. The $\alpha$-herpesviruses infect cells of the peripheral nervous system that do not normally replicate, so no DNA replication or segregation is required. $\beta$- and $\gamma$-Herpesviruses establish latency in replicative cells and thus require mechanisms to ensure the viral genome is properly replicated and segregated into daughter cells. Proteins such as latency-associated nuclear antigen (LANA) in KSHV have evolved to do this, for instance, by tethering the viral episome to host chromosomes during segregation and enabling replication of the viral episome by host replisome machinery.[13] EBV protein Epstein-Barr virus nuclear antigen 1 (EBNA-1) plays a similar role.[14] The $\beta$-herpesviruses (Figure 1), such as HCMV, have no known analog of LANA/EBNA-1. The mechanism by which the $\beta$-herpesviruses maintain life-long latent infection is still relatively poorly understood and an area of active research. Recently Mücke et al. determined that HCMV major immediate early 1 protein (ME1P) binds host nucleosomes in a way analogous to that of LANA and postulated ME1P may play the role of LANA/EBNA-1 for HCMV.[15]

## 3. UNMET CLINICAL NEED: KSHV- AND HCMV-ASSOCIATED DISEASE

Although all of the human herpesviruses cause disease and would benefit from novel treatments, KSHV and HCMV cause especially devastating disease in immunocompromised individuals. These patients may be living in developing nations with limited resources, thus diminishing the incentive for drug discovery targeting the diseases that affect them. In other cases, such as congenital cytomegalovirus, regulatory concerns and the difficulty associated with clinical trial recruitment can disincentivize research in the field. For these reasons, and given the theme of this journal issue, we focus our description of disease on those associated with KSHV and HCMV infection. We also highlight drug discovery efforts in this area, many of which are directly applied or highly relevant to the other seven human herpesviruses.

### 3.1. Kaposi's Sarcoma-Associated Herpesvirus (KSHV)/HHV8

KSHV was first discovered in 1994 during the height of the AIDS pandemic in the United States. On the basis of epidemiological data the scientific and medical communities suspected Kaposi's sarcoma (KS) had an infectious etiology. At that time, human cytomegalovirus (HCMV), human herpesvirus 6 (HHV6), hepatitis B virus, HIV, and *Mycoplasma penetrans* were all considered possible causes; however, etiologic association between KS and these infections was not established.[16] Researchers at Columbia University, New York, used representational difference analysis to identify novel sequences found in KS lesions but not in normal-tissue samples from the same patient.[16h] Two novel sequences were identified and characterized. The first had a 51% sequence identity to herpesvirus saimiri capsid protein, a $\gamma$-herpesvirus that infects New World Monkeys and causes lymphoma. This sequence also had moderate sequence identity (39%) to Epstein−Barr Virus (EBV) capsid protein, a known human herpesvirus-associated with lymphoproliferative disorders.[17] The second sequence was homologous to protein in the tegument between the nucleocapsid and the virus envelope of both herpesvirus saimiri and EBV. Later, the full genome of KSHV was sequenced.[18]

The presence of KSHV DNA in KS lesions described in 1994 was not, however, sufficient to provide a causal link, and this point remained contentious as of 1995.[19] Ganem and colleagues provided critical evidence in support of the etiologic connection between KSHV and KS. They first showed that most KS cells exhibited latent infection. This, in combination with establishing a cell line for the study of KSHV, enabled the development of a serologic test against the latency-associated nuclear antigen (LANA), mirroring an assay previously developed for the EBV homologue EBNA-1.[20] Use of this assay determined whether the presence of KSHV mirrored KS risk in different populations. For example, KS risk was known to be higher for HIV-positive homosexual men than HIV-positive patients who contracted HIV through exposure to blood products. Indeed, this study established that KSHV infection tracks with KS risk and suggested that KSHV can be sexually transmitted and does not always accompany HIV infection (i.e., HIV-positive patients who contracted infection from blood products had a rate of KSHV infection similar to the HIV-negative population).[20a] By 1997, a strong case for a causal link between KSHV and KS had been made. KSHV DNA was present in KS lesions, KSHV infection preceded development of KS, KSHV infection tracked with risk of developing KS, and KSHV was shown to infect the tissue implicated in disease not only for KS but also for multicentric Castleman's disease and primary effusion lymphoma.[21]

KSHV, like all other herpesviruses, is a large enveloped double-stranded DNA virus. It has a ∼165 kb genome and encodes 86 proteins. The lytic stage of KSHV has a complex gene expression pattern and includes formerly unappreciated small ORFs of unknown function.[22] KSHV establishes latency in B cells but can infect a variety of endothelial, epithelial, and hematopoietic cells. The viral life cycle for KSHV mirrors that of other herpesviruses. Integrins and heparan sulfate have been implicated in KSHV cell entry via interaction with KSHV glycoproteins. Since integrins and heparan sulfate are present on most cell types, it was not immediately apparent why and how KSHV selectively infects endothelial cells and B cells. Recently, it was shown that the ephrin receptor tyrosine kinase A2 is critical for viral cell entry and fusion with endothelial cells, however not for cell attachment.[23]

After fusion and release of the nucleocapsid into the cytoplasm, the viral genome is transported to the nucleus (Figure 2, entry). Once in the nucleus, viral DNA is transcribed.

KSHV has distinct genetic programs corresponding to latency and the lytic cycle. Within the lytic cycle KSHV—like all other herpesviruses—has immediate-early, early, and late genes.[22] Latently expressed genes are involved in immune evasion and maintenance of the latent infection. Lytic genes are involved in large part with generation of new capsids and the productive spread of those capsids to neighboring cells. Proteins involved in capsid assembly are expressed in the cytoplasm, but viral capsid assembly occurs in the nucleus of the cell. In a process mediated by the assembly protein-protease fusion (AP/Pr, KSHV ORF17), a procapsid forms which then angularizes with the removal of the assembly protein by cleavage from the major capsid protein (Figure 2, capsid maturation). Liberation of the assembly protein allows for conformational changes and packaging of viral DNA into the nucleocapsid. The nucleocapsid then exits from the nucleus, joins tegument proteins, acquires glycoproteins and enveloping lipids, and ruptures from the cell (Figure 2, egress).

## 3.2. HHV8-Associated Disease

**3.2.1. Kapsosi's Sarcoma (KS).** Four primary types of KS are observed: AIDS-KS, classic KS, iatrogenic KS, and endemic KS. Endemic KS exists primarily in Central and Eastern Africa and was observed prior to the HIV pandemic. This form of KS often affects children with disseminated lymphadenopathy. Classic KS typically affects elderly men of Mediterranean or Ashkenazi Jewish origin. Iatrogenic KS occurs in patients that are immunosuppressed for medical reasons, such as organ transplant. AIDS-KS is by far the most prevalent form of KS, though its frequency in western nations has diminished drastically with the availability of highly active antiretroviral therapy (HAART). AIDS-KS remains an enormous health burden in much of Sub-Saharan Africa where effective HIV/AIDS treatments are not available and HIV/AIDS incidence is high.

**3.2.2. B Cell Malignancies: Primary Effusion Lymphoma and Multicentric Castleman's Disease.** Both primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) arise from KSHV-infection of B cells. Their diagnosis, clinical manifestations, and viral etiology have been recently reviewed.[24] Most instances of PEL occur in HIV/AIDS patients. These cases make up 4% of all HIV-related non-Hodgkin's lymphomas. Interestingly, PEL cancer cells have a B-cell genotype but do not express B-cell antigens. This affects PEL treatment strategies (see section 3.2.3 below). Extensive mutations are found in immunoglobulin genes of PEL B-cells. KSHV is found in all PEL samples, but many HIV-positive KSHV-positive patients never develop PEL. This has led to the conclusion that KSHV is necessary but not sufficient for PEL. The high frequency of coinfection with EBV in PEL cancer cells has led some to suggest EBV may be a cofactor that acts in concert with KSHV to give rise to this lymphoma. This is highlighted by an abundance of EBV latency protein EBNA1 and EBV microRNA expression, both thought to play a role in tumorogenesis.[25]

KSHV is not necessary for MCD; however, KSHV infection is found in roughly half of the cases in immunocompetent patients and nearly all cases in the immunocompromised population. There are two types of MCD, hyaline vascular and plasma cell types. The plasma cell type is more common, especially in the disseminated multicentric Castleman's disease (as opposed to unicentric CD).[24d] A hallmark of MCD is cytokine disregulation, especially interleukin 6 (IL-6). This is due both to upregulation of human IL-6 as well as expression of viral IL-6, an IL-6 mimic encoded by the KSHV genome.[24b] Recent success in treating MCD with anti-IL-6 or anti-IL-6 receptor antibodies strongly suggests a role for IL-6 in disease.[26] A host of viral proteins that suppress apoptosis are also implicated in tumorogenesis. Interestingly, many of these are lytic genes suggesting that treatment with antiherpesvirus drugs that target replicating (lytic) virus could be efficacious.[24a]

**3.2.3. Current Treatments.** Current treatments for KS depend on epidemiological classification of the disease. AIDS-KS treatment focuses primarily on anti-HIV/AIDS therapeutics—though even with HAART only ~50% of patients experience complete remission. For this reason, chemotherapies and radiation are often also used to treat KS. Liposomal anthracyclines such as Doxil and DaunoXome are the most commonly used chemotherapy for KS. The anthracyclines function through DNA intercalation. Paclitaxel (Taxol), gemcitabine (Gemzar), and vinorelbine (Navelbine) can also be used. Paclitaxel and vinorelbine are mitotic inhibitors, while gemcitabine is a fluorinated nucleoside analog. Due to the side effects of these more traditional chemotherapies an interest in immunotherapies for KS treatment has arisen. For some time interferon $\alpha$ was used; however, this too suffers from severe side effects. Clinical trials are ongoing for drugs such as bevacizumab, interleukin-12, lenalidomide, pomalidomide, bortezomib, and sorafenib though these are not yet approved for treatment. Work on HIV vaccines and decreasing cost of antiretrovirals may still be the best hope for much of the population suffering from AIDS-KS. KSHV *in vitro* susceptibility to antiherpesvirus treatments (Figure 3) has been extensively tested with the general conclusion that ganciclovir (**1**) was the most potent guanosine nucleoside analog drug (discussed in section 4.1).[27] Only recently, it was shown in a randomized double-blind clinical trial that valganciclovir (**2**), the prodrug of ganciclovir (**1**), reduces viral load *in vivo*.[28] This is consistent with previous reports that note a reduction in KS frequency in HIV/AIDS patients being treated with ganciclovir

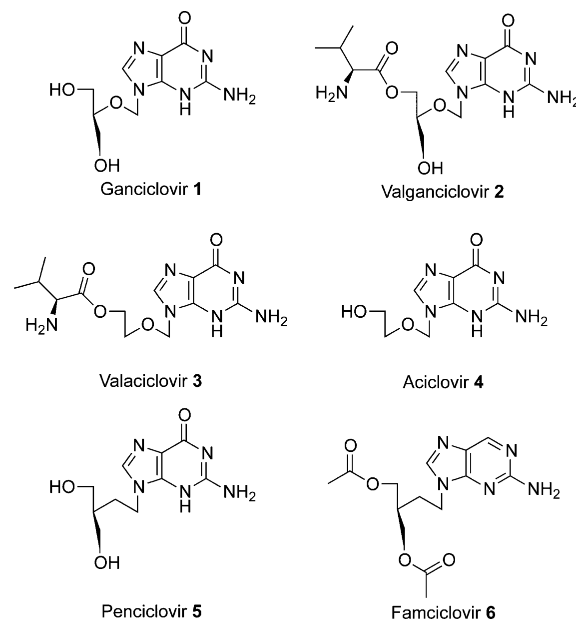


**Figure 3.** Approved guanosine analog inhibitors of herpesvirus replication (Figure 2, replication).

(**1**) for HCMV infection, though no measurements of KSHV were made.[29] Antiherpesvirus treatments have also shown utility in the iatrogenic/organ transplant setting.[30] In combination, these studies suggest antiherpesvirus treatments such as ganciclovir (**1**) and valganciclovir (**2**) aid in the treatment of KS but are not efficacious enough to be used as monotherapies.

In an HIV/AIDS setting, HAART is recommended for treatment of KS, PEL, and MCD. PEL and MCD are both lymphoproliferative disorders; however, their treatment varies due to expression of B-cell antigens in MCD and the lack thereof in PEL. For MCD (but not PEL), rituximab, a chimeric monoclonal antibody that targets CD20 on B cells, is often used in treatment.[31] Valganciclovir (**2**) as a component of combination therapy has also shown some promise in clinical trials for KSHV-positive MCD treatment, consistent with a strong correlation between viral replication and disease progression and severity.[32] Due to involvement of IL-6 and viral IL-6 (a KSHV-encoded IL-6 mimic), anti-IL-6 and anti-IL 6 receptor antibodies are also being investigated as possible treatments.[26] This year an anti-IL-6 antibody, siltuximab, was approved for the treatment of HIV-negative KSHV-negative MCD. Unfortunately, siltuximab does not bind tightly to viral IL-6, and so approval did not include KSHV-positive MCD that makes up about half of cases in the immunocompetent population and nearly all cases in immunocompromised individuals.[24a,31]

PEL is not treated with rituximab because PEL tumors do not express most B-cell antigens, including CD20. Instead, first-line PEL treatment consists of standard chemotherapy combination treatments. Autologous stem cell transplant is an additional option. Some temporary remission has been seen with administration of antiherpesvirus agents directly into the pleural cavity. However, this approach is thought to be hampered by the fact that only a small population of infected cells in PEL are undergoing lytic replication and are thus sensitive to current antiherpesvirus agents.[33]

### 3.3. Cytomegalovirus Disease

HCMV infects a large segment of the population with overall age-adjusted seroprevalence in the United States estimated at 50.4%.[34] In countries such as Brazil, Chile, South Africa, Turkey, and India, seroprevalence is estimated to exceed 90%.[35] Importantly, seroprevalence increases substantially with age. During their reproductive years many women and their partners are seronegative. This leaves many pregnant women at risk of primary HCMV infection, a high risk factor for congenital HCMV. This is especially the case in countries with relatively low overall seroprevalence, such as the United States, where the percent of seropositive women can nearly double between women ages 12−19 and women ages 30−39.[34] Transmission of infectious HCMV is through body fluids into which the virus is shed such as saliva, tears, breast milk, urine, genital secretions, semen, and blood. In the case of congenital HCMV, transfer can be intrauterine. HCMV infection is largely asymptomatic for individuals with a healthy immune system; however, in neonates, organ transplant patients, and individuals with HIV/AIDS this infection has devastating consequences as discussed below.

**3.3.1. Congenital Setting.** The United States Centers for Disease Control and Prevention (CDC) estimates that 8000 children born in the United States each year suffer from permanent health problems such as hearing and/or vision loss, mental disability, seizures, or in rare cases death due to congenital HCMV infection.[36] This outpaces the incidence of Down syndrome (4000/yr), fetal alcohol syndrome (5000/yr), or spina bifida (3500/yr) while receiving considerably less attention and awareness from the public. In a recent review, Manicklal et al. describe how scientists, doctors, and the public alike neglect congenital HCMV infection not just in the United States, but also globally.[37] The biology that gives rise to HCMV neuropathies and their resultant developmental disabilities remains elusive and is reviewed elsewhere.[38] Mounting evidence from clinical trials suggests treating neonates confirmed to have HCMV disease with antivirals such as ganciclovir (**1**) and valganciclovir (**2**) (discussed in section 4.1) limits disease manifestation, though it cannot reverse damage caused before birth or prior to treatment.[39] Pregnant mothers cannot be treated with ganciclovir (**1**) due to documented mutagenic character of this drug in animal studies, though valaciclovir (**3**) may provide some benefit to these patients and their children.[40] These limited treatment options have no approved guidelines in the United States. Given the potential for severe toxicity in neonates and pregnant mothers, parents are left with the challenge of weighing the risks of potential clinical manifestation of HCMV in their children and the potential harm of current treatments.

**3.3.2. Organ Transplant Setting.** HCMV infection causes significant morbidity and mortality in organ transplant patients, operating primarily through symptomatic viremia (CMV syndrome), invasion and damage of specific tissues, HCMV pneumonia, as well as an increased chance of graft rejection. Improved diagnostics, prophylaxis, and treatment of HCMV have significantly reduced mortality for organ transplant patients infected with HCMV. As recently as the 1980s and early 1990s, mortality due to HCMV pneumonia in bone marrow recipients was 70−95%.[41] Today the incidence of HCMV pneumonia has been much reduced, and mortality in that patient population is 15−50%, still extremely significant but much improved.[42] Overall mortality stands at 1−2%.[43] The guanosine nucleotide analog ganciclovir (**1**) and its prodrug valganciclovir (**2**) have been critical to this achievement and can be part of the standard of care for transplant patients. Strategies and guidelines both in the United States and internationally for managing HCMV infection in the solid organ transplantation setting were recently reviewed.[44] Despite the relative success of these drugs, toxicity and emerging resistance are substantial limitations of the current treatment paradigms.[45]

**3.3.3. HIV/AIDS Setting.** End-organ disease in the gastrointestinal track and eye are common manifestations of HCMV infection in HIV/AIDS patients and uncommon in other settings. It is unclear what gives rise to gastrointestinal symptoms in HIV/AIDS patients and not in organ transplant or congenital settings. Current studies cannot rule out contributions from pathogens other than HCMV. HCMV retinitis however is a well-known symptom of both congenital HCMV infection and infection in HIV/AIDS patients, though not in organ transplant recipients. In severe cases of HCMV retinitis, loss of vision can occur. Prior to the development of highly active antiretroviral therapy (HAART), both systemic and topical antivirals were used to treat HCMV infection in HIV/AIDS patients. One particularly interesting treatment, fomivirsen, was the first antisense drug to be approved by the FDA in August 1998. This antisense oligonucleotide prevents HCMV replication by binding mRNA encoding the major

immediate-early transcription factor, a critical regulator of the viral lifecycle.[46] Unfortunately this treatment is limited to HCMV retinitis due to poor pharmacokinetics that require intraocular injection as the route of administration.

### 3.4. Assays for Drug Discovery

Three broad types of assays are available for herpesvirus drug discovery: *in vitro* assays with recombinant protein, cell culture viral assays, and animal models of viral infection and disease. The first type is highly varied, reflecting the multitude of targets available in herpesvirus drug discovery. They are discussed throughout and include measurements of kinase activity by Western blot, protease activity through fluorogenic substrates, and fluorescence polarization to monitor protein−protein interaction, to name a few.

Yield reduction assays (YRA), plaque reduction assays (PRA), and cytopathic effect assays (CPE) are common cell culture viral assays.[47] CPE can be measured by visual microscopic inspection or by any of a number of dyes: Crystal Violet dye stains the remaining attached cells in a plate, neutral red dye stains the lysosomes of live cells, and MTT/XTT/MTS dyes measure mitochondrial activity. If a test compound prevents viral infection or the lytic cycle, then fewer cells will die. CPE assays use a higher multiplicity of infection than PRA assays. Plaque reduction assays also measure cell death. Inoculums contain fewer virions such that infection of a monolayer of cells gives rise to individual plaques that can be enumerated. Each plaque is indicative of a viral infection. The plaque forms as neighboring cells are infected and die.[48] The yield reduction assay relies on extent of reinfection as a measure of virion yield. Serial dilution of spent media containing virions is used to reinfect fresh monolayers of cells, and the extent of reinfection correlates with how many infectious virions were produced in the presence of potential inhibitor. Extent of reinfection can be measured in numerous ways including number of plaques, presence of a virally encoded fluorophore such as GFP, or cell death.[47a] Alternatively, an YRA can be performed via ELISA to directly quantify the amount of virus in the media when appropriate antibodies are available.

Animal models for herpesviruses rely on the conservation of this family of viruses throughout evolutionary history.[49] For example in CMV, murine CMV (MCMV), rat CMV (RCMV), guinea pig CMV (gpCMV), and rhesus CMV (RhCMV) are critical models in drug and vaccine development. As in most fields, different animal models afford different benefits. Mice and rat models of CMV have a long history with well-understood immunology and great availability of reagents.[49] However, MCMV and RCMV cannot cross the placental barrier and thus are not useful as models of congenital CMV. In contrast, the ability of gpCMV to infect the fetus provides a congenital model, although fewer reagents are available and gestation periods are considerably longer.[49] Animal models for HCMV are reviewed in detail elsewhere.[49,50] Throughout this review we reference studies employing many of these assays in the process of herpesvirus drug discovery.

## 4. NONPROTEASE DRUG TARGETS

### 4.1. Current Treatments and Their Molecular Mechanisms of Action

All currently approved treatments in the United States target the viral DNA polymerase (with the exception of the antisense oligonucleotide fomivirsen which is limited to intraocular injections for treatment of HCMV retinitis in HIV/AIDS patients). Ganciclovir (**1**), valganciclovir (**2**), valaciclovir (**3**), acyclovir (**4**), penciclovir (**5**), and famciclovir (**6**) are all guanosine analogs (Figure 3). For the prodrug forms, valaciclovir (**3**), valganciclovir (**2**), and famciclovir (**6**), a valyl ester or an ester acetate group is cleaved to release the parent compound. A viral kinase phosphorylates the drug to a monophosphate form. Host kinases then convert the monophosphate to the active triphosphate. The identity of the viral kinase responsible for that initial phosphorylation event varies between herpesviruses and can be a source of resistance mutations.[51] The active triphosphate preferentially inhibits the viral DNA polymerase and incorporates into the viral DNA, preventing viral DNA replication. Acyclovir (**4**) and ganciclovir (**1**) (as well as their respective prodrugs) have different affinities for herpesvirus kinases. For instance, ganciclovir (**1**) is a good substrate for HCMV UL97, but acyclovir (**4**), while still phosphorylated by UL97, is a worse substrate. Acyclovir (**4**) is a better substrate for the herpes simplex virus thymidine kinases than it is for HCMV UL97.[51a−c,52] It is this variability that makes ganciclovir (**1**) a more selective treatment for HCMV, while acyclovir is commonly used to treat the $\alpha$-herpesviruses. Penciclovir (**5**) is a topical agent for the treatment of oral herpes caused by HSV-1. Famciclovir (**6**), a prodrug of penciclovir (**5**) with improved oral availability, is used primarily to treat shingles (VZV, herpes zoster) and to a lesser extent recurrent HSV-1 and HSV-2 infections.

Brivudine (**7**), foscarnet (**8**), and cidofovir (**9**) are also approved herpesvirus treatments targeting the viral DNA polymerase; however, they are not guanosine analogs (Figure 4). Brivudine [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine, **7**] is a thymidine analog used primarily for the treatment of shingles (VZV, herpes zoster). It functions similarly to acyclovir and is active as a triphosphate, monophosphorylated by the VZV thymidine kinase and subsequently acted on by cellular kinases. Brivudine (**7**) is approved in some European and Central American countries.[53] Cidofovir (**9**) is a cytidine analog. In contrast to the guanosine analogs, it does not require the viral kinase for phosphorylation to the active form. Host kinases

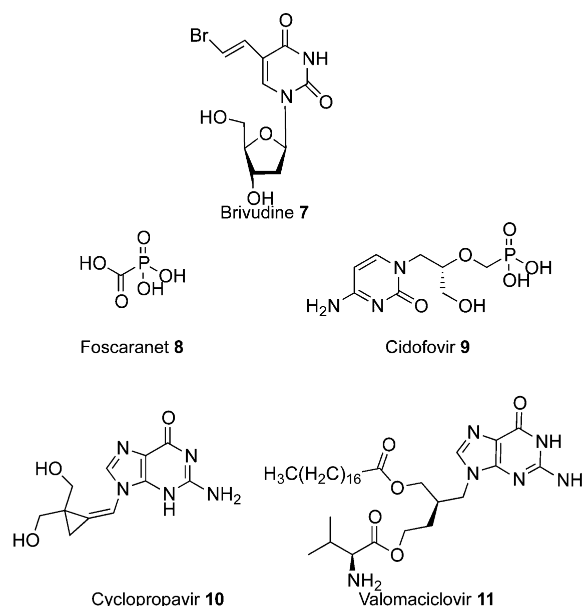


**Figure 4.** Additional inhibitors of the viral DNA polymerase (Figure 2, replication).

phosphorylate cidofovir (**9**) to the active diphosphate form where it binds to and competitively inhibits the viral DNA polymerase, and like the guanosine analogs, it is also incorporated into the viral DNA, although two consecutive cidofovir incorporations are required to fully inhibit HCMV DNA elongation.[54] These two actions prevent viral DNA replication.[55] Foscarnet (**8**) is phosphonoformic acid, a phosphonic acid derivative. It acts at the pyrophosphate binding site of the viral DNA polymerase, preventing chain elongation. It too does not require phosphorylation by a viral kinase.[56] Both cidofovir (**9**) and foscarnet (**8**) are second-line treatments for resistant herpesvirus infection. Because they rely solely on host kinases for conversion to the active form, they can readily be used to treat patients with viral infections where resistance mutations have arisen in a viral kinase, blocking guanosine analog treatments. Unfortunately, foscarnet (**8**) and cidofovir (**9**) also both require intravenous administration and exhibit severe dose-limiting toxicities. Both can cause severe nephrotoxicity, and cidofovir (**9**) can also cause myelosuppression.[55,56]

## 4.2. Improving Existing Treatments

Viral DNA polymerase inhibitors are enormously successful drugs. While there is a need for drugs with novel mechanisms of action, the track record of success, well-understood ADMET characteristics, and clear opportunity for improvement have driven continued development of molecules based on these existing approved treatments.

**4.2.1. Nucleoside Analogs.** Efforts to improve the original nucleoside analog, acyclovir, gave rise to compounds such as valaciclovir (**3**), ganciclovir (**1**), and valganciclovir (**2**) that are now standard of care treatments for herpesviral infection. Much of this history has been previously reviewed in the literature.[57] In the current review, we focus on guanosine nucleoside analogs that have recently entered clinical trials for the first time. Cyclopropavir (**10**) and valomaciclovir (**11**) are both novel guanosine nucleoside analogs (Figure 4).[58] Cyclopropavir (**10**) is a dihydroxymethyl methylenecyclopropane nucleoside analog that binds tightly to and is phosphorylated by the HCMV protein kinase UL97. The monophosphate form is then converted to the active triphosphate by cellular guanosine monophosphate kinase. The triphosphate form inhibits the viral DNA polymerase and prevents viral replication. As with other guanosine analogs used to treat HCMV, resistance mutations for cyclopropavir (**10**) map to UL97 kinase. Cyclopropavir's potent binding to UL97 makes it not only an inhibitor of viral DNA synthesis, but also a competitive inhibitor of UL97. Cyclopropavir (**10**) is active against HCMV in cell culture and murine CMV in mice. A phase 1 study to determine safety and pharmacokinetics of cyclopropavir (**10**) in healthy volunteers was completed in August 2013. Preclinical development on this chemical scaffold is ongoing. Monohydroxymethyl (rather than dihydroxymethyl) methylenecyclopropane nucleoside analogs with a broader antiherpetic activity and improved resistance profiles were recently reported and appear to function similarly through a UL97-mediated process.[59]

Valomaciclovir (**11**), unlike cyclopropavir, is active against the $\alpha$-herpesviruses (HSV-1, HSV-2, and VZV) and EBV, but not the $\beta$-herpesviruses or KSHV. Valomaciclovir (**11**) is a prodrug form of ([(*R*)-9[4-hydroxy-2-(hydroxymethyl)butyl]-guanine] (H2G), an antiherpesviral compound identified in the early 1990s. H2G, like valomaciclovir (**11**), is most potent against the $\alpha$-herpesviruses and EBV, although some weak inhibition of HHV-6 and KSHV was observed.[60] H2G had poor bioavailability in animal models, and thus, analogs with short-chain alkyl esters, valine and divaline esters, mono- or difatty acid esters, and diesters with an amino acid and a long-chain fatty acid were generated to improve aqueous solubility and cell permeability. Ultimately the stearyl/valyl diester found in valomaciclovir (**11**) was found to be optimal.[60c,61] Valomaciclovir phase 2 clinical trials for the treatment of shingles (herpes zoster, VZV) and infectious mononucleosis (EBV) were completed in 2009 and 2010, respectively. In August 2012 the results of a phase 2b randomized, double-blind, active-controlled trial versus valaciclovir (**3**) for the treatment of shingles were reported.[62] Noninferiority was achieved with the two highest doses of once-daily valomaciclovir (**11**) when compared to the approved active competitor, 3-times-daily valaciclovir (**3**). Small sample sizes (given the large coefficient of variation in some measures) and a lack of placebo control were noted as concerns for the study and reason to pursue additional trials with this compound. No study results for the infectious mononucleosis trial have yet been published, and the sponsor, Epiphany Biosciences, had not initiated additional trials in the United States or European Union at the time this review was written.[63]

Bicyclic nucleoside analogs (BCNAs, Figure 5) have recently been developed with high specificity for VZV, selectively

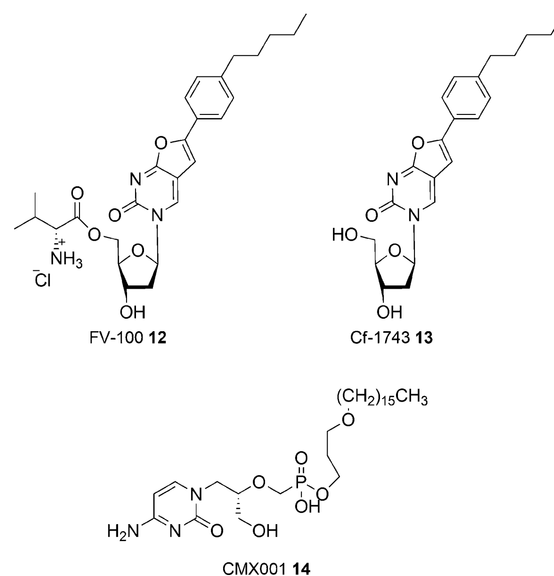


**Figure 5.** Bicyclic nucleoside analogs and a lipid ester analog of cidofovir. These inhibitors target viral replication (Figure 2, replication).

targeting the VZV thymidine kinase over even closely related HSV-1 and HSV-2 thymidine kinases. BCNAs are not broken down by catabolic enzymes, a problem that was observed with VZV inhibitor brivudine [7, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, BVDU]. That BCNAs are not broken down by catabolic enzymes was a welcome result, providing additional stability and avoiding significant increases in fluorouracil observed with BVDU (**7**) due to inhibition of dihydropyrimidine dehydrogenase by the free base of BVDU (**7**), (*E*)-5-(2-bromovinyl)uracil. Unexpectedly, the triphosphate forms of BCNAs are not detected in cells, and thus, it is unclear whether BCNAs act via direct inhibition of DNA polymerase. A valyl-ester prodrug, FV-100 (**12**), was developed from the highly potent BCNA cf-1743

**Table 1. Herpesvirus Genes and Their Associated Inhibitors[a]**

| common name | HSV ORF | VZV ORF | HCMV ORF | HHV6/7 ORF | EBV ORF | KSHV ORF | inhibitor/compd no. | function |
|---|---|---|---|---|---|---|---|---|
| DNA polymerase | UL30 | 28 | UL54 | 38 | BALF5 | 9 | nucleoside analogs and related (compds **1–14**) | viral DNA replication |
| processivity factor | UL54 | 16 | UL44 | 27 | BMRF1 | 59 | | viral DNA replication |
| helicase-primase: helicase | UL5 | 55 | UL105 | 77 | BBLF2 | 40 | pritelivir (**15**), BILS 179 BS (**16**) | helicase activity, viral DNA replication |
| helicase-primase: ATPase | UL8 | 52 | UL102 | 74 | BBLF3 | 41 | | ATPase activity, viral DNA replication |
| helicase-primase: RNA polymerase (primase) | UL52 | 6 | UL70 | 43 | BSLF1 | 56 | | primase activity, viral DNA replication |
| ssDNA binding protein | UL29 | 29 | UL57 | U41 | BALF2 | 6 | | viral DNA replication |
| ribonucleotide reductase large subunit | UL39 | 19 | UL45 | U28 | BORF2 | 61 | BILD 1263 and BILD 1351 (**34**, **36**) | metabolism |
| ribonucleotide reductase small subunit | UL40 | 18 | N/A | N/A | BaRF1 | 60 | | metabolism |
| Major capsid protein | UL19 | 40 | UL86 | U57 | BcLF1 | 25 | | capsid structure |
| capsid triplex protein 1 | UL38 | 20 | UL46 | U29 | BORF1 | 62 | | capsid structure |
| capsid triplex protein 2 | UL18 | 41 | UL85 | U56 | BDLF1 | 26 | | capsid structure |
| assembly protein (AP) | UL26.5 | 33.5 | UL80.5 | | | 17.5 | | capsid assembly |
| maturational protease (PR) | UL26 | 33 | UL80 | U53 | BVRF2 | 17 | see Figures 15–17, 19 | capsid assembly and DNA encapsidation |
| portal vertex | UL6 | 54 | UL104 | U76 | BBRF1 | 43 | WAY-150138 and related (**23–27**) | capsid structure and DNA encapsidation |
| terminase ATPase subunit 1 | UL15 | 42, 45 | UL89 | U66 | BGRF1 BDRF1 | 29 | TCRB (**17**), BDCRB (**18**), BAY 38–4766 (**20**) | viral DNA replication and encapsidation, putative role in DNA translocation |
| terminase ATPase subunit 2 | UL28 | 30 | UL56 | U40 | BALF3 | 7 | TCRB (**17**), BDCRB (**18**), BAY 38-4766 (**20**), GW275175X (**21**), AIC246 (**22**) | viral DNA replication and encapsidation, DNA binding and cleavage |
| terminase binding protein | UL33 | 25 | UL51 | UL35 | BFRF1A | 67 | | viral DNA replication and encapsidation |
| glycoprotein B | UL27 | 31 | UL55 | U39 | BALF4 | 8 | compds **28–33** | fusion and entry, heparin-binding |
| CDK-like protein kinases | N/A | N/A | UL97 | U69 | BGLF4 | 36 | cyclopropavir (**10**), maribivir (**19**) | multifunctional, prodrug activation |
| thymidine kinases | UL23 | 36 | N/A | N/A | BXLF1 | 21 | | metabolism, prodrug activation |

[a]Adapted with permission from ref 76b. Copyright 2007 Cambridge University Press.

(3-(2-deoxy-$\beta$-D-ribofuanosyl)-6-(p-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one) (**13**). A phase 2 active comparator clinical trial against valaciclovir (**3**) in patients with shingles (VZV, herpes zoster) was completed in 2010. While FV-100 (**12**) appeared to show some improvement over valaciclovir (**3**) in reduction of pain related to shingles, this result was not statistically significant and did not meet the primary end point. After changing hands a number of times, FV-100 (**12**) is now being pursued by ContraVir. Careful clinical trial design and outcome measurements could enable FV-100 (**12**) to progress into the clinic. It will be exciting to see if these relative newcomers in the long history of antiherpesvirus nucleoside analogs make it to the clinic.

FV-100 (**12**), valaciclovir (**3**), and valganciclovir (**2**) are all peptidyl prodrugs of their parent compounds (cf-1743 (**13**), acyclovir (**4**), and ganciclovir (**1**), respectively). Taking this a step further, Velázquez and colleagues have developed "double-prodrugs" of cf-1743 (**13**) and acyclovir (**4**). In these prodrugs two enzymatic events must take place, cleavage of a peptide by proteases and cleavage of a peptidyl-ester by any of a number of hydrolases.[64] Previously this group established that peptides cleaved preferentially by dipeptidyl peptidase could serve as the "first" prodrug component. A variety of peptidyl esters, including the valyl-ester used in valaciclovir, served as the "second" prodrug component. These compounds exhibited better solubility, serum stability, cell permeability, and oral bioavailability in mice. The novel prodrugs were converted to their active compounds and had substantial antiviral activity in cell culture.[64,65]

**4.2.2. Nucleoside Phosphonates.** CMX001 (Brincidofovir) (**14**), a lipid ester analog of cidofovir (**9**), is an additional example of leveraging a tried-and-true mechanism of action and a previously approved drug to produce a new chemical entity with improved pharmacological qualities and efficacy. Cidofovir (**9**) is a nucleoside phosphonate that targets viral DNA replication both by inhibition of the viral DNA polymerase as well as incorporation into viral DNA, preventing replication.[66] Lipid ester analogs of cidofovir not only improve bioavailability, but also improve antiviral activity by 3−4 orders of magnitude against HSV-1 and 2, VZV, HCMV, murine CMV, HHV-6, EBV, and KSHV.[67] Hexadecyloxypropyl cidofovir (CMX001) (**14**) also showed activity against HCMV strains resistant to standard of care, including unmodified cidofovir (**9**). Importantly, the incorporation of lipid esters enabled oral bioavailability for a drug that previously could only be administered intravenously. On top of that, it had significantly reduced accumulation in the kidney, reducing the likelihood of the dose limiting nephrotoxicity characteristic of cidofovir (**9**).[68] Excitingly, CMX001 (**14**) has progressed through phase 1 and 2 trials and is now being assessed in phase 3 trials for the treatment of adenovirus and HCMV infection (ClinicalTrials.gov identifiers NCT02087306 and NCT01769170).

## 4.3. Non-Nucleoside DNA Replication Inhibitors: Targeting Helicase-Primase

The helicase-primase complex performs three critical functions for herpesviruses during viral DNA replication; it (1) unwinds the viral DNA, (2) forms the replication fork, and (3) primes the leading and lagging strands (Figure 2, replication). This complex has been best studied in HSV-1 where it was first identified and determined to consist of gene products UL5, UL8, and UL52 (Table 1).[69] Biochemical studies revealed that UL5 has DNA helicase activity, UL52 has RNA polymerase (primase) activity, and UL8 has ATPase activity that is stimulated by ssDNA.[70] The first example of a HSV helicase inhibitor, a 2-aminothiazole, was published in 1998.[71] Kleymann et al. from Bayer AG and Crute et al. from Boehringer Ingelheim Pharmaceuticals, Inc., independently published two structurally similar thiazole-based inhibitors of the helicase-primase complex in 2002 (Figure 6).[72] The optimal

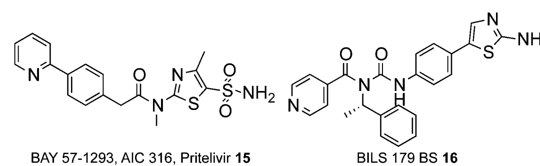


**Figure 6.** Helicase-primase inhibitors (Figure 2, replication).

Bayer compound *N*-[5-(aminosulfonyl)-4-methyl-1,3-thiazol-2-yl]-*N*-methyl-2-[4-(2-pyridinyl)phenyl]acetamide, BAY 57-1293 (also known as AIC316 or Pritelivir, **15**), exhibited $ED_{50}$ values of 0.5 mg/kg in a mouse lethal challenge model for both HSV-1 and HSV-2. Generation of escape mutants and biochemical analysis with recombinant helicase-primase suggest BAY 57−1293 (**15**) simultaneously binds UL5 and UL52 and stabilizes the helicase-primase complex at the replication fork, stalling DNA replication.[72b] The Boehringer Ingelheim compound 1-benzyl-1-cyclohexanecarbonyl-3-[4-(2-methyl-1,3-thiazol-5-yl)phenyl]urea, BILS 179 BS (**16**), was also shown to be a potent inhibitor of HSV, acts on the helicase-primase complex, and was effective in animal models.[72a] Replacing the methyl group in **16** with an amino group brought the potency in cell culture from an $IC_{50}$ of about 30 nM to 6 nM.[73] Studies on the potential for escape mutants for helicase-primase inhibitors were recently summarized and are ongoing.[74]

In terms of clinical development, BAY 57-1293 (**15**) has shown the most promise and is now being developed by AiCuris as AIC316 (**15**) or Pritelivir. In January of 2014 results from a double-blind randomized, placebo controlled, phase 2 study in otherwise healthy individuals with HSV-2 positive genital herpes showed significant reduction in viral shedding and hinted at reduced occurrence of genital lesion outbreaks. In May of 2013 the Food and Drug Administration placed a hold on further clinical development of AIC316 due to dermal and hematologic toxicities in monkeys that could not be readily explained. These monkeys received roughly 15−250 times the highest dose administered in the phase 2 trial (and 70−900 times more than the most effective dose determined by this trial, which was not the highest). No such toxicities were observed in the phase 2 trial; however, research into the reasons for these toxicities in monkeys is reportedly underway.[75]

## 4.4. Targeting Encapsidation and Packaging

**4.4.1. Terminase Inhibitors.** Targeting the terminase complex has great potential for the inhibition of HCMV replication. The terminase, composed of UL89 and UL56 (Table 1) in HCMV, is functionally conserved across herpesviruses.[76] It cuts the replicating viral genome into genome-length segments and packages them into the capsid. Human cells do not share any analogous complex, nor do they process their DNA in this way. This makes the terminase an exciting target in terms of selectively inhibiting viral replication, while limiting host off-target effects.

Inhibitors of the terminase (Figure 7) began to surface in 1995 with the identification of 2,5,6-trichloro-1-ß-D-ribofuranosyl benzimidazole (TCRB, **17**), initially intended as an anticancer agent, and its 2-bromo analog 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB **18**). As nucleoside analogs, these inhibitors were expected to undergo phosphorylation and inhibit viral DNA synthesis. Surprisingly, neither of these expectations held true. TCRB (**17**) and BDCRB (**18**) act without chemical modification, and resistance mutations map to UL56 and UL89 (Table 1) of the HCMV viral terminase.[77] TCRB (**17**) and BDCRB (**18**) are potent inhibitors of the β-herpesvirus HCMV, but not of α-herpesviruses (HSV-1, 2, and VZV), β-herpesvirus HHV-6, or the γ-herpesviruses (EBV and KSHV/HHV8).[78] At a concentration of 125 μM, BDCRB (**18**) inhibits the nuclease activity of UL89, though only slightly. This modest effect observed with recombinant UL89 is insufficient to explain BDCRB (**18**) activity in cell culture.[79] This may reflect proposed binding of both UL89 and UL56 (Table 1) by this class of molecules.[80] The development and improvement of these benzimidazole terminase inhibitors and how they ironically gave rise to an inhibitor of kinase UL97, maribavir (**19**), were recently reviewed by Biron et al.[81] Further discussion of maribavir (**19**) can be found below (section 4.5.2).

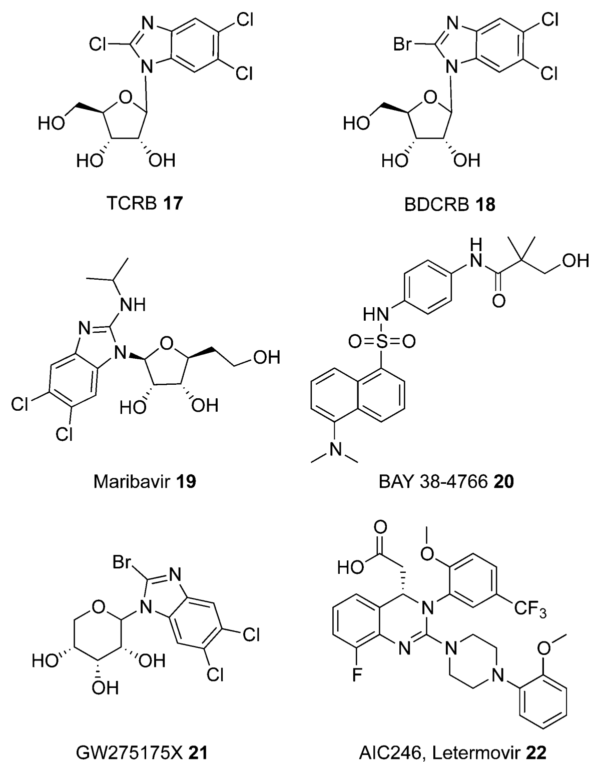


**Figure 7.** Terminase inhibitors (Figure 2, encapsidation).

In 2001 Bayer reported a significantly different chemical scaffold linked to terminase inhibition. The sulfonamide 3-hydroxy-2,2-dimethyl-N-[4([[5-(dimethylamino)-1-naphthyl]-sulfonyl]amino)-phenyl]propanamide, BAY 38-4766 (**20**), inhibits HCMV, and like the benzimidazoles TCRB (**17**) and BDCRB (**18**), resistance mutations map to UL56 and UL89.[80] BAY 38-4766 (**20**) is well-tolerated and effective in murine and guinea pig CMV infection models, including the prevention of guinea pig CMV in immunocompromised animals. It is also effective against some monkey CMV strains, though to a lesser extent than the rodent models.[82] BAY 38-4766 (**20**) was taken into phase 1 trials and showed safety in healthy volunteers at doses up to 2 g. However, there is no evidence that BAY 38-4766 (**20**) progressed further in clinical development.[45]

The benzimidazole terminase inhibitors suffer from metabolic instability; the glycosidic bond is cleaved *in vivo*.[83] Substitution of the furan sugar found in BDCRB for β-D-ribose gives GW275175X (**21**, 2-bromo-5,6-dichloro-1-β-D-ribopyranosyl-1*H*-benzimidazole) and eliminates the major metabolic liability of BDCRB and TCRB. Developed and sponsored by GlaxoSmithKline, this compound advanced through a phase 1 trial but was dropped to pursue maribavir (**19**).[45,84]

The most advanced terminase inhibitor to date is AIC246 (**22**) (Letermovir), a 3,4-dihydro-quinazoline-4-yl-acetic acid derivative that was developed by AiCuris as a potent inhibitor of HCMV through extensive hit-to-lead optimization.[85] In cell culture across three different HCMV strains, AIC246 (**22**) shows low (4−5 nM) $EC_{50}$ as measured by cytopathic effect reduction and GFP reduction in fibroblasts. Ganciclovir (**1**), the standard of care for HCMV, is more than 400-fold less potent in these same assays. AIC246 (**22**) is effective against HCMV strains resistant to ganciclovir. This is consistent with the fact that AIC246 (**22**) activity is independent of the viral kinases and the viral DNA polymerase, the two primary sources of resistance mutations for nucleoside analog inhibitors. AIC246 (**22**) also shows potent antiviral activity in a mouse xenograft model of HCMV. In this assay AIC246 (**22**) is about 5-fold more potent than valganciclovir (**2**), an orally bioavailable form of ganciclovir (**1**). Murine CMV and guinea pig CMV models could not be used since AIC246 (**22**) is inactive against these viruses in cell culture.[85c] AIC246 (**22**) is thought to act on UL56 of the HCMV terminase. This is based on generation and genotyping of mutant viruses that escape AIC246 (**22**). These data leave open the possibility that AIC246 (**22**) binds UL89 and mutations in UL56 (Table 1) perturb this binding indirectly, though the simplest explanation is direct interaction with UL56. Interestingly, AIC246-resistant viruses were not resistant to putative sulfonamide and benzimidazole terminase inhibitors, suggesting distinct structural determinants of binding.[85a,b] In December 2011 AIC246 (**22**) completed a phase 2 study, and in April 2012 AiCuris announced that it had passed all primary efficacy end points. The drug has received Orphan Drug status in the European Union and Fast Track Designation in the United States. Late in 2012 AiCuris announced they have signed on to an exclusive worldwide licensing agreement with Merck for AiCuris' portfolio of anti-HCMV leads, including AIC246 (**22**).

**4.4.2. Portal Vertex Inhibitors.** Researchers at Wyeth (now part of Pfizer) identified and developed thiourea derivatives that inhibit correct formation of the portal vertex (Figure 8). One series of compounds was relatively selective for HSV-1 while exhibiting some inhibition of HSV-2 and HCMV.[86] Another related series selectively targeted VZV.[87] The initial thiourea compound identified, *N*-(3-chloro-4-(3-(5-chloro-2,4-dimethoxyphenyl)thioureido)phenyl)formamide (**23**), inhibits HSV-1 virus production with an $IC_{50}$ of 7.9 μM for the Patton strain, and 24.5 μM in the E377 strain. Substitution of the formamide for the 2-fluoro-phenyl ring resulted in WAY-150138 (**24**) and improved potency ∼20-fold for both viral strains. Mechanism of action was determined by interrogating five critical aspects of inhibitor effect: time-

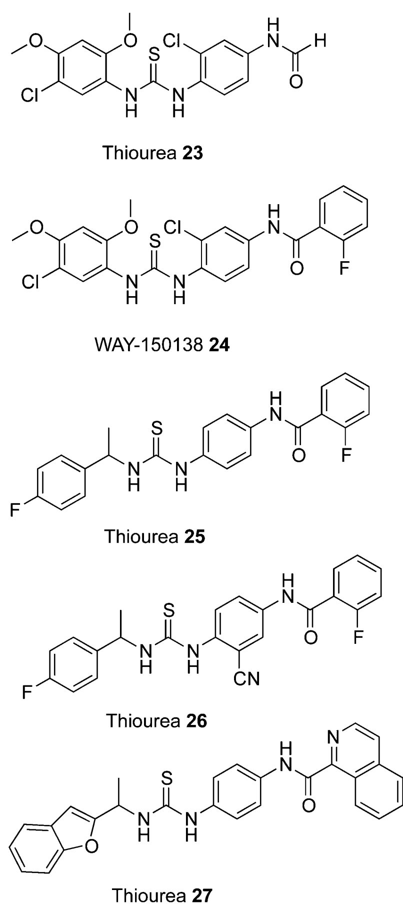


**Figure 8.** Portal vertex inhibitors (Figure 2, encapsidation).

dependence (relative to time of infection), viral DNA replication, viral DNA cleavage, capsid morphology, and resistance mutations. WAY-150138 (**24**) did not prevent viral DNA replication since it inhibits viral production after DNA replication is expected to be complete, suggesting it acts later in the viral replication cycle. Southern blot analysis revealed viral DNA cleavage was not taking place in the presence of inhibitor. Consistent with those data, no C-capsids were observed in the presence of inhibitor. Finally three single point mutations that conferred resistance were identified, all mapped to the portal protein (HSV-1 UL6).[86b] A follow-up study further investigated the mechanism of action of WAY-150138 (**24**). It ruled out selective inhibition of UL6 (Table 1) protein synthesis, degradation of UL6 after translation, and extraction of UL6 from already formed B-capsids. Western blots performed on capsids from compound-treated cells revealed depletion of UL6 and UL15. The authors propose that WAY-150138 (**24**) prevents incorporation of UL6 into capsids. Two key arguments are made in favor of this over the alternative conclusion of interaction with UL15. First, resistance mutations mapped only to UL6 and not UL15 (Table 1). Second, it has been shown that UL15 associates with capsids only in the presence of UL6. Thus, if only UL15 incorporation was prevented, one would expect to still observe the presence of UL6. In summary, these thiourea derivatives appear to prevent incorporation of the portal vertex (UL6) into capsids, thus preventing DNA encapsidation.[86a] Closely related compounds (**25**, **26**, and **27**) are reported to function in a similar way against VZV, though mechanism of action was not as thoroughly pursued, so additional studies are warranted for further development of this series.[87] On the basis of reports on related thiourea inhibitors of HCMV (section 4.5.1) these compounds were likely dropped due to a lack of stability from hydrolysis of the thiourea as well as poor bioavailability.[88]

### 4.5. Other Viral Targets and Inhibitors with Unknown Mechanism of Action

Inhibitors of entry and attachment, of viral kinases, and vaccine development are additional potential approaches to herpesvirus treatment. Progress has been made in each case and is discussed below.

**4.5.1. Targeting Viral Entry.** Structural and biochemical studies of entry and attachment have paved the way for inhibitor development, though much remains to be done in this field, and no currently approved drugs act via this mechanism. The majority of molecules that inhibit entry or attachment are peptides or large charged molecules such as heparin. While such compounds benefit from having extracellular targets such as herpesvirus glycoproteins, they suffer from poor oral bioavailability and metabolic stability. Arguably the most advanced-stage example of a compound thought to function by blocking viral entry is SP-303 (also known as Virend), a proanthocyandin oligomer tested in phase 2 clinical trials as a topical treatment for anal and genital herpes. This compound showed some promise; however, there is no evidence that it was further pursued for FDA approval. Connolly et al. further review advances in our structural and functional understanding of herpesvirus entry and the potential for targeting the process therapeutically.[89]

Another interesting example of entry inhibitors is a thiourea derivative that is reported to block HCMV glycoprotein B-mediated fusion with host cells (Figure 9). This small molecule came out of SAR studies from the hit compounds against HSV-1 that gave rise to the portal vertex inhibitors described in section 4.4.2. Substitutions at the acyl group in **28** demonstrate how SAR for HSV-1 and HCMV rapidly diverged. Introduction of a phenyl group improved potency against both viruses by 10-fold; however, a 2-furoyl (**29**), and other heteroaromatic groups, increased potency against HCMV but lost activity against HSV-1. The 2-furoyl compound (**29**) was selective and potent with an $IC_{50}$ against HCMV of 0.2 $\mu$g/mL and an $IC_{50}$ against HSV-1 of >10 $\mu$g/mL. This became the starting point for additional SAR around ring 1. In a series of alkyl and electron withdrawing groups that had improved potency, introduction of trifluoromethyl and chloro groups meta and para, respectively, to the thiourea nitrogen was optimal (**30**, $IC_{50}$ 0.03 $\mu$g/mL). No substitutions on ring 2 were tolerated, though changes in selectivity were observed. For instance, the 3,6-dimethoxy analog (**31**) had modest activity against VZV and no activity against HCMV. Introduction of a thiazole (**32**) in place of the furoyl resulted in further improvement in potency, with an $IC_{50}$ of 0.008 $\mu$g/mL. Unfortunately, the thiourea group in this series was readily hydrolyzed under acidic conditions at elevated temperatures.[90]

A follow-up study sought to overcome this stability problem. The authors presumed that thiourea hydrolysis took place via protonation of the thiourea nitrogen bound directly to ring 1. They further hypothesized that the electronegative character of the ring 1 substituents (i.e., trifluoromethyl) made for a better leaving group and that insertion of a linker that is not electron withdrawing between the substituted phenyl and the thiourea would improve stability. A simple methylene spacer was sufficient to improve stability. Nearly all of compound **33**

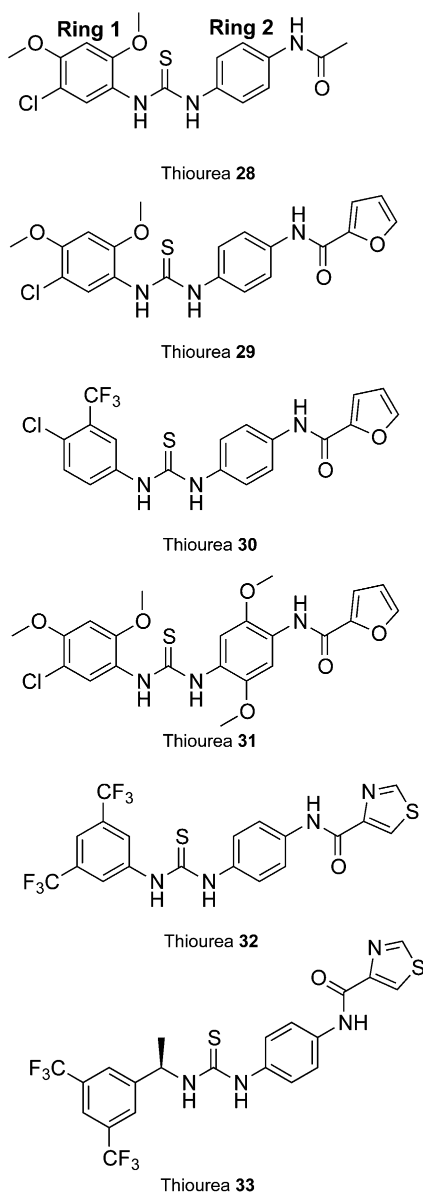


**Figure 9.** Putative HCMV fusion inhibitors (Figure 2, entry).

remained intact after 7 days at 37 °C, while 66% of compound **32** decomposed under those same conditions. Likewise, compound **33** had improved stability under both acidic conditions (0.1 or 1 N HCl) and basic conditions (0.1 N NaOH) at 37 °C. Fortuitously, this spacer also improved potency 10- to 20-fold across all seven reported linkers.[88]

Somewhat surprisingly, mechanism of action studies were carried out with the less stable thiourea series represented by **32**. As described by Jones et al., there is significant evidence that this series inhibits glycoprotein-B mediated fusion with the host cell membrane.[91] Resistance mutations were generated with compound **32** that could be confirmed via marker transfer experiments to map to the viral glycoprotein B (UL55, Table 1). As the authors acknowledge, however, they did not look for mutations in other glycoproteins involved in fusion and thus cannot rule out additional targets beyond glycoprotein B. To further strengthen their case that these compounds specifically block fusion, they assessed the effect of time-of-addition of inhibitor, initial events in the replication cycle such as degumentation and cytoplasm-to-nucleus transfer, interaction of inhibitor with host cell membrane proteins, and direct measurement of fusion in lipid mixing assays. Inhibitor reduced viral yield when administered at the time of infection; however, no effect was observed if treatment occurred just 2 h post infection. This strongly supports activity very early in the replication cycle. Detegumentation and cytoplasm-to-nucleus transport, which normally occur rapidly after fusion, did not occur in the presence of compound as measured by the lack of abundant tegument protein pp65 in the nucleus. Pretreatment of host cells with inhibitor prior to infection had no effect, suggesting no highly stable interaction with the host is made, though weaker interactions are still quite possible. Finally, the use of virions labeled with fluorescent lipids enabled direct measurement of fusion inhibition. In the presence of compound, in contrast to vehicle alone, fluorescent lipids were retained on the virion and not transferred to the host cell.[91] In summary, there is strong evidence that these inhibitors prevent HCMV fusion and interact with viral envelope glycoprotein B; however, additional targets for this compound series cannot be ruled out. Many other targets in viral entry exist, and Connolly et al. further review advances in our structural and functional understanding of herpesvirus entry and the potential for targeting the process therapeutically.[89]

For KSHV specifically, the ephrin receptor tyrosine kinase A2 (EphA2) recently emerged as a potential therapeutic target. KSHV requires binding to EphA2 for entry into endothelial cells.[23] This could be relevant to the treatment of Kaposi's sarcoma, though likely not B cell malignancies PEL and MCD (section 3.2.2). Both EphA2 binding alone and downstream signaling by EphA2 contribute to KSHV cell entry. The former greatly enhances entry, while the latter appears necessary in at least a subset of endothelial cells.[23a] This makes EphA2 an attractive target both for kinase inhibitors targeting the intracellular tyrosine kinase domain and for small molecules, peptides, or antibodies that directly block KSHV glycoprotein–EphA2 interaction.[23] Other proteins involved in EphA2 signaling may also prove to be novel therapeutic targets.[92] The discovery that KSHV requires binding to EphA2 for entry in some cell types and recent discoveries about herpesvirus entry were recently described.[23,89,92] Targets for the viral entry pathway of HSV-1 and HSV-2 are discussed elsewhere.[93]

#### 4.5.2. Targeting Viral Kinases.

Maribavir (**19**) [2-isopropylamino-5,6-dichloro-1($\beta$-D-ribofuranosyl)-benzimidazole] was identified during the development of benzimidazole inhibitors of the herpesvirus terminase (section 4.4.1). Maribavir (**19**) inhibits viral kinases, not the terminase that its chemical predecessors targeted. The full history of maribarvir's discovery and development has been extensively reviewed elsewhere.[81,94] In the current review, we focus on some highlights of early development, maribavir's (**19**) unique mechanism of action, specificity among the herpesviruses, and recent progress in its clinical development.

As with many drug development campaigns, resistance mutations were generated to guide identification of the drug target. Biron et al. identified mutations mapping to UL97 HCMV protein kinase (Table 1) that conferred resistance to maribavir (**19**). Inhibition of recombinant UL97 (Table 1) confirmed that it is maribavir's target. Maribavir (**19**) inhibits wild type UL97 (Table 1) potently, with an $IC_{50}$ of 3 nM in a histone phosphorylation assay. Somewhat alarmingly, the recombinant UL97 (Table 1) with the resistance mutation identified in the study, Leu397Arg, resulted in a 20 000-fold decrease in potency ($IC_{50}$ of 60 $\mu$M).[95] Maribavir (**19**) is

reported to competitively inhibit ATP binding to recombinant UL97 (Table 1) with a nanomolar $K_i$ (though the Lineweaver–Burk presented shows an inversion of the typical trend between 15 and 20 nM maribavir and thus warrants careful replication).[96] UL97 (Table 1) is also the kinase responsible for phosphorylating many nucleoside analog drugs, such as ganciclovir (**1**). Ganciclovir-resistant HCMV mutants are susceptible to maribavir (**19**). This supports structural models based on resistance mutations that suggest maribavir (**19**) interacts directly with the ATP-binding pocket. Likewise, maribavir-resistant mutants are susceptible to ganciclovir (**1**).[94,97] Interestingly, however, cyclopropavir (**9**) is reported to compete with maribavir (**19**) for binding to UL97 (Table 1), conflicting with the notion that maribavir does not interfere with guanosine nucleotide analogs binding as substrates. Gentry et al. note this unexpected result and hypothesize it could be indicative of conformational differences between ganciclovir (**1**) and cyclopropavir (**9**) binding, owing to the methylenecyclopropane of cyclopropavir.[98] Indeed, cyclopropavir (**9**) was recently used to generate HCMV strains resistant to ganciclovir (**1**) and maribavir (**19**), in addition to cyclopropavir (**9**).[99] These data hint at sites of potential overlap on UL97 between all three drugs or conformational changes to UL97 (Table 1) that preclude their binding.

Later studies found mutations conferring resistance to maribavir (**19**) were more commonly found in the viral gene pUL27, not the kinase, though resistance was mild compared to UL97 mutations. Study of the biological function of UL27 and its relation to UL97 and maribavir (**19**) resistance is in its infancy. Reitsma et al. provide the most compelling evidence of the relationship between UL97 and UL27 to date.[100] UL27 was shown to enhance proteasomal degradation of the acetyltransferase Tip60. Of note, the HIV virulence factor Tat also targets Tip60 for degradation, and Tat expression in cells infected with UL27-driven maribavir-resistant HCMV restores susceptibility to maribavir. Degradation of Tip60 results in increased levels of cyclin-dependent kinase (CDK) inhibitor $p21^{waf/CIP1}$, leading to a halt of cell cycle progression. Normally, protein kinase UL97 functions to drive cells into the S phase so that viral DNA replication can take place.[100] When maribavir (**19**) inhibits UL97 (Table 1), progression to the S phase is hindered leading to reduced viral replication (though this is only one of numerous effects of UL97 inhibition, Figure 2, viral kinases). If UL27 no longer drives the degradation of Tip60, then p21 expression is blocked and activity of CDKs allows progression to the S phase, overcoming that aspect of UL97 (Table 1) inhibition.[100] These findings are in agreement with previous work that showed differences in maribavir (**19**) susceptibility based on cell state and cellular kinase modulation.[101] This intriguing and complex mechanism of drug action and resistance still leaves numerous unanswered questions that will no doubt be the subject of future work.[102]

Maribavir (**19**) inhibits EBV as well as HCMV; however, the target for EBV is less clear. Multiple reports indicate that Maribavir (**19**) inhibits EBV protein kinase (also known as BGLF4 or EBV-PK, Table 1) in cell culture; however, it showed no inhibition of recombinant EBV-PK *in vitro*.[103] It is possible that the known EBV-PK substrates that are no longer phosphorylated in cells in the presence of maribavir can be acted on by other kinases, which are the true target of maribavir. This may also be an example of indirect (e.g., through blocking a cofactor) or substrate-dependent inhibition of EBV-PK. It is clear, however, that maribavir (**19**) prevents phosphorylation of the EBV DNA polymerase processivity factor (EA-D), a substrate for EBV-PK, and alters transcription of key viral genes leading to a reduction in viral fitness.[103,104]

Maribavir (**19**) exhibits acceptable ADMET characteristics that enabled progression into clinical trials. Phase 1 and phase 2 clinical trials were successful and established maribavir as having fewer deleterious side effects than current HCMV treatment options. Phase 3 clinical trials for prophylaxis in HCMV solid organ transplant and bone marrow transplant settings were established. Unfortunately, neither of these trials was successful in meeting their primary or secondary outcomes. No data were published for these trials, so it is unclear if study design, lack of full biological understanding of mechanism of action, or something inherent to the use of maribavir (**19**) in the selected patient populations is the most likely cause for these clinical failures.[94]

#### 4.5.3. Targeting the Viral Ribonucleotide Reductase.

Human herpesviruses encode a viral ribonucleotide reductase for the conversion of adequate amounts of deoxyribonucleoside diphosphates from the corresponding ribonucleoside diphosphates during active viral DNA synthesis.[105] The importance of the viral reductase in producing infectious particles has been the subject of conflicting studies. The reductase is composed of two subunits, R1 and R2, and their association is required for catalytic activity.[106] Temperature sensitive mutants with a lesion in the R1 subunit of the virally encoded reductase established 100-fold decrease in viral production in a tissue culture setting.[106] However, there were several reports that showed drastic differences in the importance of the viral reductase, and these differences were species specific in animal models.[107] Nevertheless, the viral reductase was seen as a tractable therapeutic target, and it was pursued for many years. Unfortunately, those efforts did not culminate in the approval of novel therapeutics and have waned in recent years. Below, we discuss examples of inhibiting the protein–protein interaction between the R1 and R2 subunits as this was the first example of an effective protein–protein interaction antagonist *in vivo*.

Two studies published simultaneously first described the inhibition of the viral reductase protein–protein interaction (PPI) with slightly different synthetic peptides incorporating similar sequences from the C-terminus of subunit 2.[108] Follow-up studies by researchers at Boehringer Ingelheim developed peptidomimetic compounds, such as BILD 1263 (**34**, Figure 10), that improved potency over shorter synthetic peptides by over 200 000-fold and that displayed *in vivo* efficacy in a mouse ocular model of infection: the first such example for a PPI antagonist.[109] The evolution of viral resistance to related compounds and identification of the sites on the R1 subunit of the HSV-1 reductase sufficient for resistance (A1091S and P1090L) were also described.[109b]

The SAR of the compounds was also reported and described in detail.[109c] The authors of the study noted an important observation regarding $IC_{50}$ values and cellular efficacy, namely that the radioligand binding assay used to establish $IC_{50}$ was only accurate to 1 nM and that the compounds showing cellular efficacy all displayed $IC_{50}$s lower than the sensitivity of their assay, making meaningful SAR interpretation difficult within the most potent series.[109c] Beginning at the N-terminal ($R_1$) position of compounds related to **35**, it was found that increasing lipophilicity improved potency and that the stereochemistry around methyl-substituted cyclohexyl moieties was important (**36**). Ultimately, a (dimethylcyclohexyl) amino

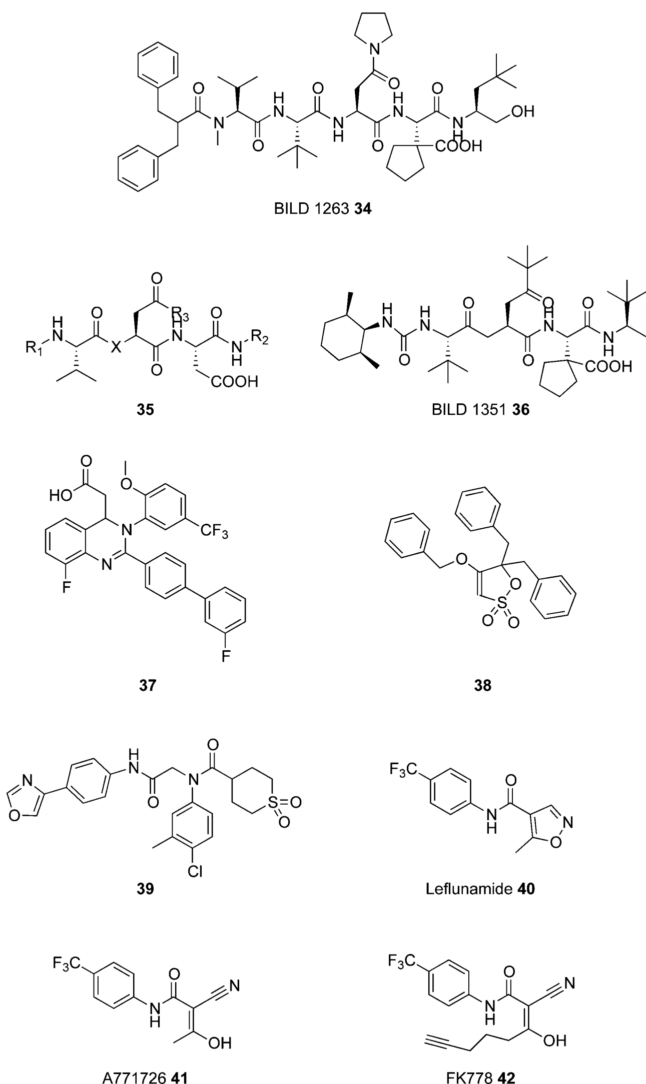


**Figure 10.** Ribonucleotide reductase protein−protein interaction inhibitors and inhibitors of unknown mechanism.

group improved potency over 7-fold (2 nM). This improved compound was used to probe the C-terminal ($R_2$, **35**) position and was shown to improve *in vivo* potency in an HSV-1 induced keratitis mouse model. Compounds containing the hydroxymethyl moiety at $R_2$ such as is found in BILD 1263 (**34**) were significantly less potent in the radioligand binding assay, but, for seemingly obvious permeability reasons, were more potent in the cell culture assays. The authors also describe a series of substitutions related to neopentylamine and discovered that the addition of a stereochemically defined methyl- or ethyl-substituted neopentylamine was optimal for activity in the cellular-based assay. Next, the authors explored the N-substituted pyrrolidine of BILD 1263 (**34**) and the amide linkage in the N-terminal direction of this side chain (**X** in **35**). The amide was replaced with a methylene linkage in order to lower desolvation, and the potency in the cellular assays was also found to improve by replacing the pyrrolidine with a *tert*-butyl group (i.e., **36**). These studies led to the discovery of BILD 1351 (**36**), which is significantly smaller than the previously described BILD 1263 (**34**) and equipotent in the cellular-based assays. BILD 1351 (**36**) and another congener more similar to BILD 1263 (**34**) both showed improved *in vivo* efficacy, but unfortunately, no public record of these compounds advancing further toward clinical trials has been published.[109c]

### 4.5.4. Inhibitors with Unknown Mechanism of Action.

A number of recent publications and patents report herpesvirus inhibitors with as of yet unknown mechanisms of action. To provide a comprehensive review and encourage further investigation of these compounds, they are discussed in brief below.

Patents disclosed by Wunberg et al. describe a series of dihydroquinazolines that exhibit anti-HCMV activity in cell culture.[110] The most potent inhibitors, similar to compound **37**, had $EC_{50}$ values of 0.01 $\mu$M with a 50% cytotoxic concentration ($CC_{50}$) of 15 $\mu$M, 1500 times that of the $EC_{50}$ value. No statement regarding the mechanism of action for these compounds is provided in the patent, though detailed SAR is described. The approved cancer treatment and kinase inhibitor gefitinib shares the quinazoline scaffold and was previously reported to have anti-HCMV activity in cell culture, though no activity in an animal model.[111] The quinazolines described by Wunberg et al. may also be acting on host or viral kinases, though testing of this hypothesis has not been reported in the literature.

Another example of antiviral compounds with unknown mechanism of action comes from a recent publication by De Castro et al. describing a series of 4-benzyloxy-$\gamma$-sultone (**38**) derivatives.[112] These compounds inhibit VZV and HCMV in cell culture with $EC_{50}$ values for the most potent compounds of approximately 10 $\mu$M. They are inactive against HSV-1 and HSV-2 as well as a number of other viruses. Activity against HHV6 and 7, EBV, and KSHV was not assayed. Extensive SAR revealed that the 4 and 5 positions of the $\gamma$-sultone are critical to antiviral activity. Specifically, a benzyloxyl group at the 4 position, as shown, and one benzyl group at the 5 position were required for both HCMV and VZV inhibition. The authors do not explore the stereochemical preferences for the antiviral activity. These compounds were equally active against HCMV strains resistant to ganciclovir, foscarnet, cidofovir, and acyclovir as well as mutant strains lacking HCMV protein kinase UL97 and VZV thymidine kinase (VZV-TK).[112] On the basis of these data, it is likely this series of inhibitors does not act through traditional DNA polymerase inhibition or inhibition of UL97 or VZV-TK. As we hope this review has made clear, this leaves many possible mechanisms by which inhibition could be taking place and additional research is warranted.

Yamanouchi Pharmaceutical Co., Ltd. (now a part of Astellas Pharma Inc.), disclosed tetrahydro-2*H*-thiopyran-4-carboxamide analogs (**39**) with potent antiviral activity against the $\alpha$-herpesviruses (HSV1 and 2, and VZV). Their most potent inhibitor (**39**) has an $EC_{50}$ of 0.033 $\mu$M in a VZV plaque reduction assay and 0.006 $\mu$M in an HSV-1 cytopathic effect inhibition assay. These compounds also show activity in an HSV-1 mouse model with 70−100% inhibition of lesions with a 10 mg/kg twice-daily dosing regimen over 5 days.[113] A more recent patent application from the same group also claims a reduction in pain caused by herpesvirus lesions.[114] To our knowledge, no information on the mechanism of action has been published.

Our final example of a herpesvirus inhibitor with an unknown mechanism is also an example of repurposing. Leflunomide (**40**), an immunosuppressive and inhibitor of mitochondrial dihydroorotate dehydrogenase in the pyrimidine synthesis pathway, is approved for the treatment of arthritis but

was found to have antiviral activity against resistant strains of HCMV and HSV-1. Its use in the treatment of HCMV in the organ transplant setting was recently reviewed, including *in vitro* studies on its potential mechanisms of action.[115] In a study by Waldman et al., electron microscopy revealed treatment with leflunomide in HCMV-infected cells results in formation of a mature nucleocapsid, but the tegument and envelope are not acquired.[116] The same was found to be true in HSV-1-infected cells treated with leflunomide.[117] Studies of the active metabolite of leflunomide (A771726, **41**) and an analog thereof (FK778, **42**), both of which also inhibit HCMV replication, propose a different mechanism of action. These molecules were found to interfere with HCMV signaling events and thus prevent some aspects of viral DNA replication and protein production, as well as apoptosis of HCMV-infected cells.[118] Differences in viral strain and cell type have been proposed as a potential explanation for some of the discrepancies between the studies.[118a] It is possible that treatments that so drastically affect cell metabolism could impact viral replication in multiple ways. While it is clear both biochemically and clinically that leflunomide can be useful in preventing HCMV replication, many questions about its mechanism of action remain unanswered.

**4.5.5. Vaccine Development.** Tremendous effort continues toward the goal of developing herpesvirus vaccines. To date, only the development of VZV vaccines (chickenpox and shingles) has been successful. Vaccine development has focused primarily on HSV-1 and 2, HCMV, and EBV. Both modified viruses as well as specific antigens are being used in this work.[2a] Development of HSV vaccines continues to be hampered by a poor understanding of why previous attempts at vaccination have failed, and, equally, if not more importantly, why some seropositive individuals are largely or completely asymptomatic while others have frequent clinical episodes. Across all herpesviruses it appears that while natural immunity is protective against reinfection and in some cases clinical manifestation of disease, it often does not prevent reactivation and shedding of the virus and thus transmission. In fact, concerns have been raised that vaccination could reduce symptoms but not shedding, leading to increased transmission. Past and current development and research to overcome these challenges have been reviewed elsewhere.[2a−c,119]

**4.5.6. Targeting Latency and Immune Evasion.** All current herpesvirus treatments target only replicating virus, while an ideal treatment also would target latency. To accomplish this one would need to block the action of those proteins responsible for maintaining latency. These can be broken into two categories: direct maintenance of latency (e.g., enabling segregation of DNA into daughter cells) and immune evasion. Both present interesting and challenging drug discovery opportunities. The macromolecules required for maintenance of latency vary substantially among the herpesviruses as one might expect given the diverse cell tropisms. Direct maintenance has been best studied in EBV and KSHV, where one can readily establish latent infection in cell culture. In recent years many papers have been published on herpesvirus latency, laying the groundwork for future therapeutic development. From a small-molecule discovery standpoint, the herpesvirus-encoded GPCRs offer exciting potential targets given the vast knowledge for targeting GPCRs in other indications.[120] Directly targeting mediators of latency such as LANA could prove more challenging from a druggability standpoint due to the protein−protein and protein−DNA interactions involved; however, promising research in this area is ongoing.[121] Finally, as we learn more about differences between uninfected and latently infected cells we are able to take advantage not only of viral proteins expressed during latency, but also differences in host protein expression that enable specific targeting of latently infected cells.[122] It will be exciting to watch as these strategies—alone or in combination—are brought to bear on the challenging goal of targeting herpesvirus latency.

## 4.6. Host Targets for Herpesvirus Inhibition

As with other viruses, such as HIV, some attempts to target host proteins for the inhibition of herpesviruses have emerged. In theory this approach could substantially reduce, if not eliminate, development of resistance to treatment. However, targeting host proteins raises serious concerns about toxicities, both on- and off-target. The three major host targets being currently explored are the protein kinases cyclin-dependent kinase (CDK) and mammalian target of rapamycin (mTOR), and cyclooxygenase 2 (COX2). Many other kinases are upregulated during HCMV infection and are potential therapeutic targets, some with known small-molecule inhibitors.[123] Another recent potential target is the ephrin receptor tyrosine kinase A2 (EphA2). KSHV cell entry requires binding to EphA2, and its down-regulation or addition of soluble ephrin ligand prevented viral entry.[23,92] This leaves open the possibility of targeting EphA2 both at the extracellular receptor as well as the intracellular kinase domain.

The use of CDK inhibitors to prevent viral replication in cell culture provided the first example of a host-targeted herpesvirus inhibitor, showing efficacy against at least HSV 1 and 2, VZV, and HCMV.[124] The exact mode of inhibition appears to differ between herpesviruses, stalling their replication at different stages of the viral lifecycle.[124a,125] Broadly, CDK inhibitors such as roscovitine (**43**, Figure 11) appear to inhibit viruses that require a nuclear phase of replication.

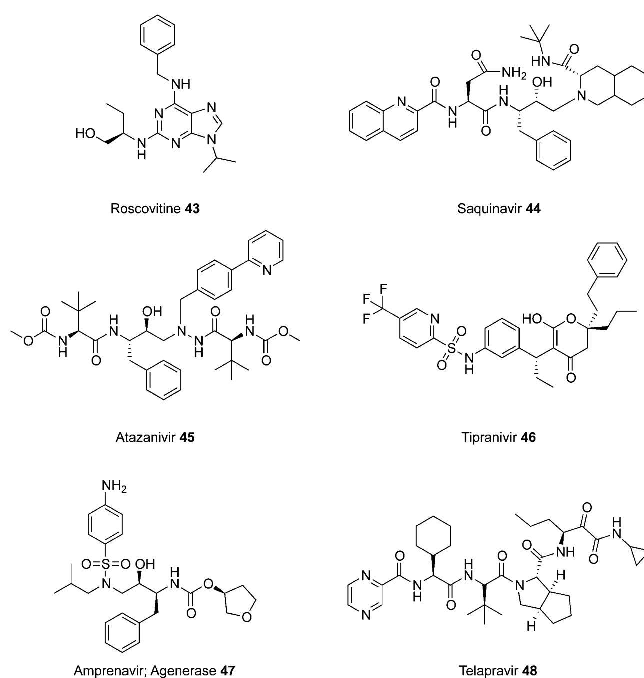


**Figure 11.** Roscovitine, a cyclin dependent kinase (CDK) inhibitor, and HIV and HCV protease inhibitors.

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After anecdotal evidence emerged that organ transplant patients receiving immunosuppressive agents targeting mTOR had fewer and less severe complications from HCMV, more extensive molecular and clinical studies were established to interrogate this observation. The underlying mechanism of action is complex and likely varies between herpesviruses and disease manifestations. Mechanistic studies using rapamycin recently revealed that the mTOR pathway is critical for modulation of HCMV late genes in infected macrophages.[126] Everolimus, an approved immunosuppressant and rapamycin analog, is now in clinical trials for treatment of HCMV disease in renal transplant patients (ClinicalTrials.gov identifier NCT00828503). Other approved mTOR inhibitors are in clinical trials for EBV- and KSHV-caused cancers, where molecular mechanism of action is likely much more complex (ClinicalTrials.gov identifiers NCT00918333 and NCT02110069). Li et al. recently reviewed the use of kinase inhibitors for the treatment of herpesvirus-associated disease, including but not limited to CDK, mTOR, and EphA2.[127] Brennan et al. summarize our understanding of the effect of immunosuppressive drugs on virus pathobiology, including the effect of mTOR inhibitors on herpesviruses.[128]

COX2 is one of the many genes that are upregulated during HCMV infection. This upregulation of COX2 directly results in a >50-fold increase in prostaglandin $E_2$. Treatment with noncytotoxic COX2 inhibitors decreases the accumulation of prostaglandin $E_2$ and reduces the production of HCMV virions from infected fibroblasts by more than 100-fold. Treatment with both a COX2 inhibitor and exogenous prostaglandin $E_2$ rescues viral production, highlighting an important role for prostaglandin $E_2$ in HCMV replication. More recently, Schröer et al. demonstrated that COX2 inhibition by approved anti-inflammatory drugs tolfenamic acid and indomethacin diminished direct cell-to-cell spread of HCMV in fibroblast cell culture and that this effect was reversed with the addition of prostaglandin $E_2$. These studies highlight a potential host target in the control of herpesvirus infection and build on a long history of research showing prostaglandins play a critical role in herpesvirus biology.[129]

## 5. SUCCESSES IN TARGETING VIRAL PROTEASES

To our knowledge, industry largely (if not entirely) halted efforts to develop HHV protease inhibitors in the late 1990s and early 2000s. Since then, myriad antiprotease therapies have entered the clinic, particularly HCV protease inhibitors and a variety of improved HIV protease inhibitors. Many lessons can be learned from these discovery efforts and applied to HHV protease inhibitor development. In combination with increasing structural, biochemical, and biological understanding of the HHV proteases, the success of HIV and HCV protease inhibitor development revives a long-standing interest in targeting the highly functionally conserved HHV protease family.

### 5.1. HIV Protease Drugs

The development of HIV protease inhibitors is one of the most successful examples of structure-based drug design to date and is expertly reviewed in the literature.[130] In 1981 reports first surfaced of what we now know as symptoms of HIV/AIDS, namely the severe opportunistic infections Kaposi's sarcoma and pneumocystis pneumonia.[131] Two years later HIV was isolated and deemed the likely cause of AIDS, and by 1985 full genome sequences for the virus had been published.[132] Amazingly, just 10 years after the HIV genome was sequenced, the US FDA approved the first HIV protease inhibitor, saquinavir (**44**).[133] Part of this rapid success came from synthetic compounds developed and lessons learned during failed attempts to develop inhibitors of renin, an aspartyl protease involved in control of blood pressure.[134]

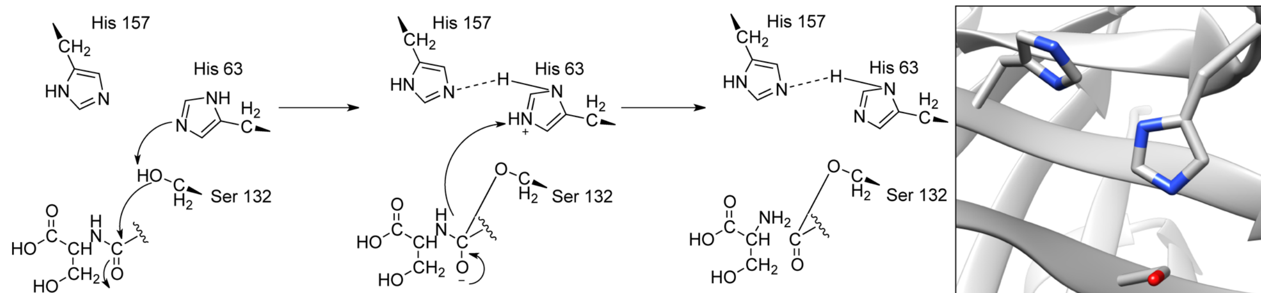
There are 10 HIV protease inhibitors currently marketed in the United States, and at least one new therapeutic, a deuterated form of the peptidomimetic inhibitor atazanavir (**45**), is currently in clinical development. All but one of these are peptidomimetic competitive active-site inhibitors with a hydroxyethylene core that acts as a transition state mimetic, displacing a critical water molecule from the active site and preventing proteolysis.[135] Tipranavir (**46**) is the exception, a nonpeptidomimetic inhibitor that uses a dihydropyrone ring in place of the hydroxyethylene core.[136] As described below, a similar focus on peptidomimetic inhibitors has dominated drug discovery efforts for herpesvirus protease inhibitors.

In retrospect, HIV protease is an excellent drug target. However, HIV protease inhibitors faced skepticism in early development, in part due to inherent challenges in targeting proteases and in part due to the success of reverse transcriptase inhibitors that entered the market prior to saquinavir's (**44**) approval. As resistance mutations developed against the reverse transcriptase inhibitors, another line of attack against these highly mutagenic viruses was necessary; protease inhibitors continue to play that central role in combination treatments for HIV/AIDS. The herpesvirus market mirrors, to some extent, the early HIV market where nucleoside analogs dominate and pharmaceutical companies, after significant effort, have largely abandoned the viral protease as a drug target. Ultimately, the success of HIV protease inhibitors, and later HCV protease inhibitors, continues to drive interest in and provide critical lessons for the development of small molecules targeting the highly conserved HHV proteases.

### 5.2. HCV Protease Drugs

In the late 1990s Vertex pharmaceuticals, the developer of HIV protease inhibitor amprenavir (Agenerase, **47**), initiated preclinical efforts to develop a clinical hepatitis C virus protease inhibitor. The combined efforts of academia and industry were able to overcome numerous substantial challenges: the target was not yet validated *in vivo*, the viral lifecycle was poorly understood, no robust preclinical models in cell culture or animals were available, and no structure of the viral protease had been solved. In a remarkable story that is expertly reviewed in the literature, scientists were able to achieve approval of telaprevir (**48**), the first HCV protease inhibitor, just 22 years after the virus was discovered in 1989.[137] Since then many other companies have clinical trials for or have approved HCV protease inhibitors.[138] Parallels can be drawn between the herpesvirus proteases and HCV protease. When first screened *in vitro*, no known serine protease inhibitors were effective against HCV protease. This was attributed to the active site being flat and solvent exposed, lacking a well-defined pocket into which an inhibitor could bind.[137d,139] In addition, it was shown that the protease was dynamic—binding to different inhibitors yielding different conformations of the enzyme. The same has been said of the human herpesvirus proteases, as described below (section 6). Initial inhibitor development against HCV protease focused on decamer peptidomimetics derived from the natural substrate. To simultaneously obtain low molecular weight and "drug-like" properties while

Scheme 1. Herpesvirus Protease Catalytic Mechanism and Ser-His-His Catalytic Triad




maintaining potency, the peptidomimetics were truncated to 5-mers spanning the P4−P1′ sites and a reversible covalent tetra-aldehyde moiety was introduced.[137f,140] Use of an $\alpha$-ketoamide warhead was determined to be optimal, and eventually exploration of that scaffold arrived at Telaprevir (**48**). Detailed discussion of medicinal chemistry for HCV protease inhibitors can be found elsewhere.[137f,140,141] A similar approach to HCMV protease inhibitors was taken, starting with peptidomimetics and utilizing covalent inhibitors to overcome lack of potency. An additional parallel can be drawn in the recent reports of allosteric regulation of HCV protease, specifically compounds that trap a closed and autoinhibited conformation of the helicase-protease fusion NS3-4A.[142] A similar allosteric inhibition of herpesviruses proteases involving the trapping of an inactive monomeric conformation of the enzyme is reviewed in detail below (sections 6.2−6.3 and 6.5−6.6).

## 6. HUMAN HERPESVIRUS PROTEASE

### 6.1. Biological Role in Viral Replication

Herpesvirus proteases have long been known to be essential for viral replication.[143] The protease (also known as assemblin and Pr) and the assembly protein are expressed from two overlapping open reading frames (i.e., KSHV ORF17 and ORF17.5, Table 1). The assembly protein alone (i.e., KSHV ORF17.5, Table 1) is expressed approximately 10-fold more than the protease−assembly protein fusion. The viral protease is expressed in the cytoplasm of virally infected cells as a monomeric fusion to the assembly protein (AP). The Pr-AP fusion binds the major capsid protein (MCP) and is translocated to the nucleus where the procapsid forms. Concentration-dependent dimerization in the nucleus allosterically activates protease, leading to protease-mediated cleavage of the release site (R-site) that frees the protease from AP. AP is proteolyzed from the MCP by hydrolysis at the maturation site (M-site). Cleavage at the M-site releases the internal assembly protein from the outer protein layer, enabling a conformational change in the outer shell as well as the portal vertex. These conformational changes allow packaging of viral DNA, and the mature capsid is formed (Figure 2, capsid maturation).[9a,144] In typical infections three forms of the viral capsid are produced. A-capsids are empty, lacking both the assembly protein and viral DNA. B-capsids contain the inner lining of assembly protein ORF17.5 but no viral DNA. C-capsids have expelled the scaffolding protein and contain viral DNA. Only C-capsids progress directly to mature virions. This is true across multiple herpesviruses.[145] When the viral protease is not present to perform its function, only B-capsids are formed and DNA packaging fails.[143b,146] The lack of A-capsid formation suggests A-capsids may be the result of premature release/cleavage of the assembly protein from B-capsids. This suggests that activators of the protease could block viral maturation, though further research is needed to confirm this hypothesis. Lacking genetic material and being morphologically malformed, these B-capsids are incapable of going on to become infectious virions. The results of herpesvirus protease knockout, as well as knockdown of the protease in murine cytomegalovirus that results in reduced viral load, provide genetic validation of HHV proteases as potential therapeutic targets.[147]

### 6.2. HHV Protease Structure

Many years of work have contributed to our current understanding of the HHV proteases. Cytomegalovirus protease was the first herpesvirus protease for which a crystal structure was determined, with four groups independently publishing HCMV protease structures in 1996.[148] These structures revealed a novel serine protease fold and a Ser-His-His catalytic triad providing a structural framework for a unique enzyme with low proteolytic activity due in part to the noncanonical catalytic triad (Scheme 1, Figure 12).[149] Consistent with biochemical studies suggesting numerous herpesvirus proteases form dimers, the HCMV protease crystal structure revealed a dimeric enzyme where each monomer contains an independent active site (Figure 12). The monomeric unit consists of a core $\beta$-barrel of mostly antiparallel $\beta$-sheets partially surrounded by seven $\alpha$-helices. Subsequently, structures of VZV, HSV1 and 2, KSHV, and EBV proteases were solved.[150] All six known HHV protease structures share the same fold, dimeric state, and Ser-His-His catalytic triad (Figure 13). These are the only proteins for which structures are known that have this fold, comprising their own superfamily in the SCOP database (MEROPS Clan SH, Family S21). Despite their structural and functional homology, the HHV proteases vary widely in sequence identity from 29% to 97% identical. Despite this unique fold, the Ser-His-His catalytic triad adopts a conformation very similar to that of the chymotrypsin-fold serine protease catalytic triad (MEROPS Clan PA, Family S1). The substrate binding pocket is shallow, dynamic, and solvent-exposed, as was discovered with HCV protease (Figure 12). The core $\beta$-barrel is conserved while helices are more divergent, and loops even more so. The structural configuration of the protease suggests autoproteolytic processing to liberate protease from assembly protein happens in trans, not in cis. Namely, the distance between the active site and the release site as well as structural constraints required for enzyme activity likely preclude intramolecular proteolysis.

### 6.3. Substrate Binding and Specificity

All herpesvirus proteases share a P1−P1′ specificity for Ala-Ser. Alignment of all M and R sites across the human herpesviruses

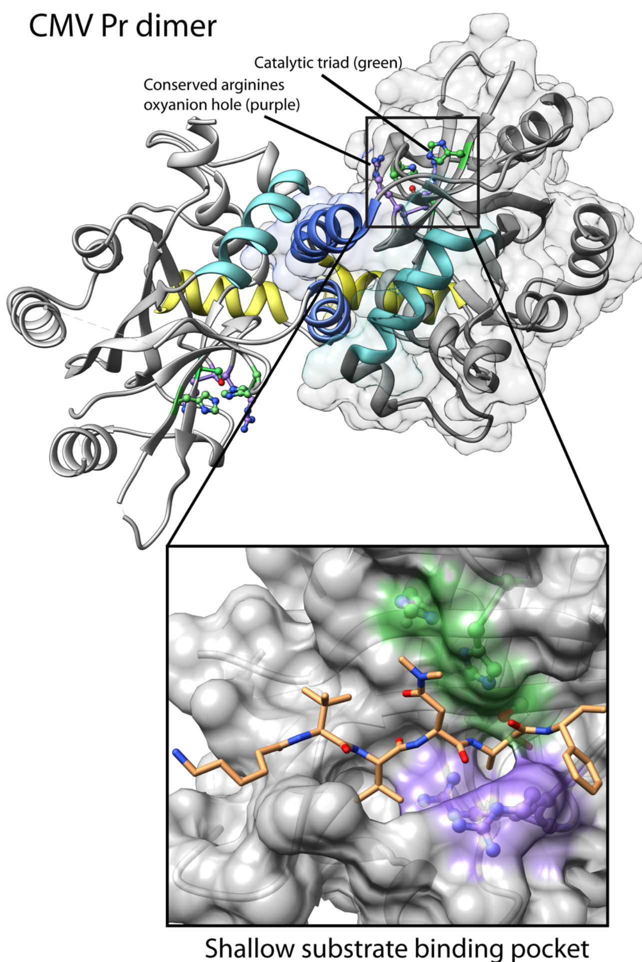



**Figure 12.** Dimeric herpesvirus protease and its shallow binding pocket. HCMV protease (PDB: 1NJU) is depicted. Helices 2 (cyan), 5 (blue), and 6 (yellow) are critical to dimerization and allosteric activation of the enzyme. The shallow binding pocket (inset) is shown with a peptidomimetic inhibitor bound. The catalytic triad (Ser-His-His) is shown in green and the conserved arginines of the oxyanion whole in purple.

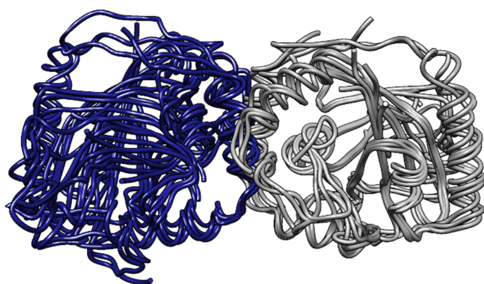


**Figure 13.** Overlay of known herpesvirus protease structures (HCMV, HSV2, EBV, VZV, and KSHV proteases).

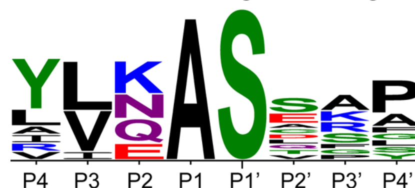



**Figure 14.** M- and R-site alignments for herpesvirus protease substrate specificity. WebLogo 3.3 was used to generate a specificity profile based on the M- and R-cleavage sites of KSHV, EBV, HHV-6, HCMV, HSV-1, and VZV assembly protein−protease fusion sequences.

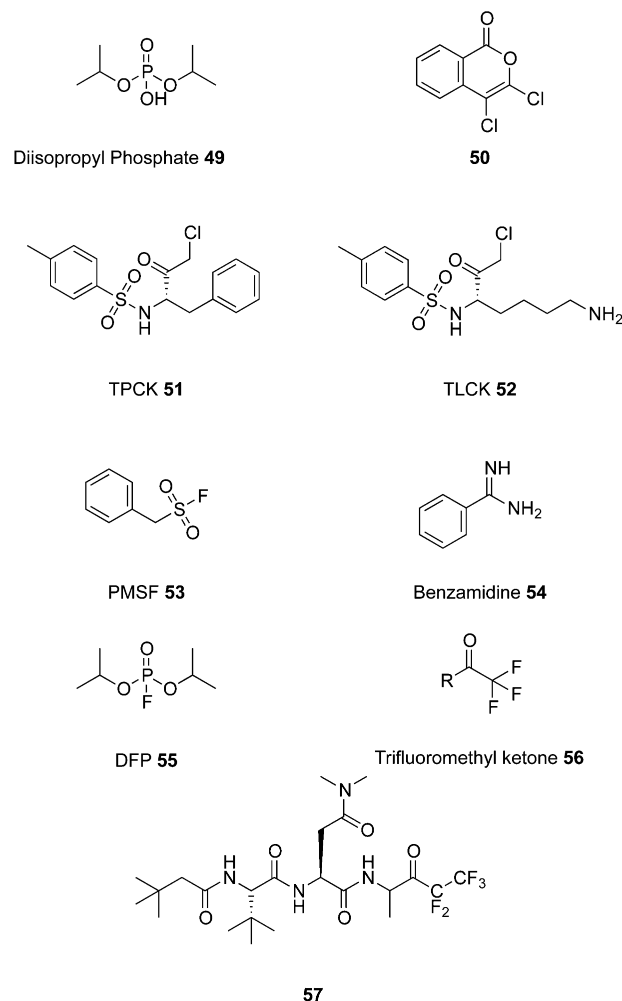



**Figure 15.** General protease inhibitors and an HCMV pentafluoroethyl ketone inhibitor (Figure 2, capsid maturation).

reveals a profile sequence showing the strict requirement for alanine in the P1 pocket and serine in the P1′ pocket. There is also a strong preference for tyrosine in the P4 pocket (Figure 14). While much of the specificity between these enzymes is shared, there are distinct differences that produce functional biochemical consequences. For example, HCMV protease is able to cleave its own endogenous substrate, HCMV assembly protein, as well as HSV2 assembly protein. Conversely, HSV2 protease is unable to efficiently cleave HCMV assembly protein. KSHV protease can cleave itself at the dimer interface leading to loss of activity resulting in additional regulation of proteolysis. The herpesviruses are recalcitrant to most standard serine protease inhibitors as described in section 6.4.1 and as was observed for HCV protease. This is likely due to their poor catalytic activity.[149] Antichaotropic agents such as high concentrations of sulfate or citrate, glycerol, and others can improve herpesvirus protease activity 10−100-fold.[151] At their most active, these highly selective processing enzymes are significantly less active than many other proteases with catalytic efficiencies 2−3 orders of magnitude less than typical digestive enzymes depending on substrates and conditions.

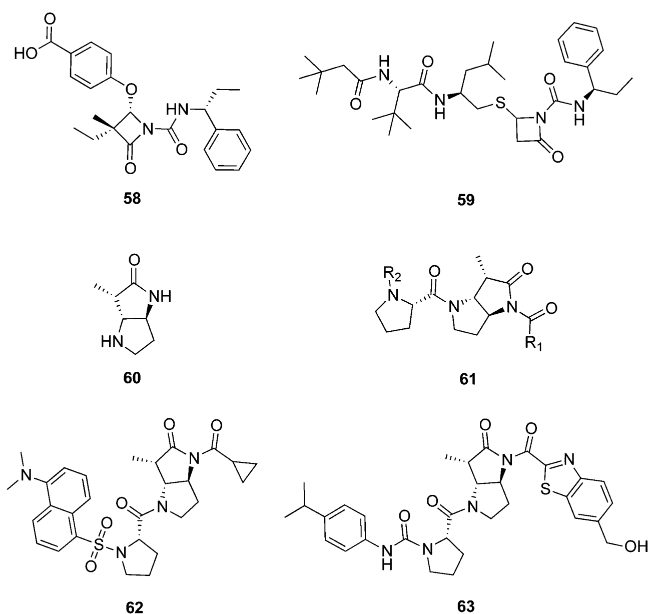



**Figure 16.** *β*- and *trans*-lacatam herpesvirus protease inhibitors (Figure 2, capsid maturation).

HSV2 protease was the first protease to be cocrystallized with an active-site inhibitor, diisopropyl phosphate (DIP, **49**). This allowed for direct identification of the oxyanion hole, involving two arginines and two leucines that are absolutely conserved across all known human herpesvirus proteases. Surface loops that are the most varied in sequence and structure are proposed to play a role in substrate recognition as is true for chymotrypsin-fold enzymes.[150a] Loops and helices, which are markedly less structurally conserved than the core *β*-barrel, contribute to defining the extended binding pocket.

For KSHV and HCMV proteases, substrate binding has been shown to occur through an induced-fit model.[152] Two distinct but related features have been reported: the effect of substrate binding on the active site and the effect of substrate binding on dimerization. Comparison of crystallographic structures between HCMV protease in its apoenzyme state and protease bound to a transition state-analog inhibitor reveals rearrangements of the conserved oxyanion hole arginine residues upon substrate binding. This result is supported by changes in tryptophan fluorescence of reporter Trp42, which upon substrate binding is packed against part of loop 9 that contributes a conserved arginine to stabilize the oxyanion hole (Figure 18). In KSHV protease, crystallographic studies reveal a large pocket in the S4 position to accommodate a P4 tyrosine when present; however, this pocket is not apparent from the apoenzyme state.[152d] In addition, substrate binding promotes dimerization. The literature to date does not make it clear whether substrate binding to a binding-competent monomer promotes a conformation that more readily dimerizes or whether substrate only binds the dimer but in doing so stabilizes the interface. In either case, the observation that addition of increasing concentration of substrate or peptidyl-inhibitor results in an increasing dimer population holds. Addition of a phosphonate inhibitor to KSHV protease results in a "super dimer" that no longer undergoes measurable monomer−dimer equilibrium. While this does not address whether substrate can bind to monomer and promote dimerization, it does confirm that substrate binding stabilizes the dimeric state of the enzyme.[152d]

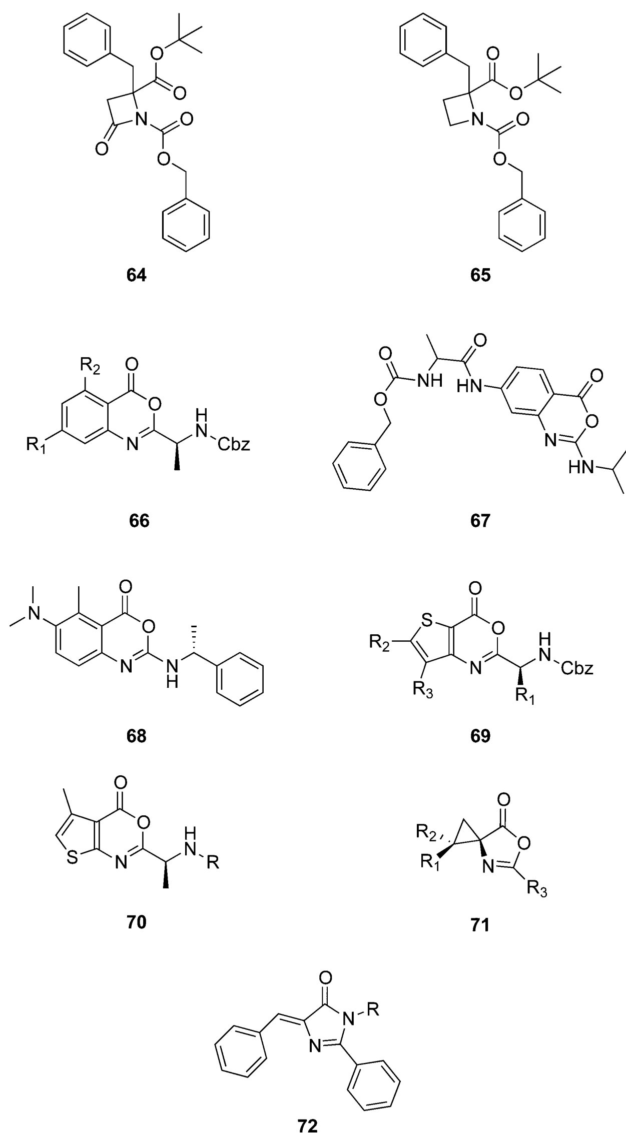



**Figure 17.** Azetidine, benzoxazinone, thieno[2,3-*d*]oxazinone, spirocyclopropyl oxazolones, and benzylidine *N*-sulphonyloxyimidazolone mechanism-based herpesvirus protease inhibitors (Figure 2, capsid maturation).

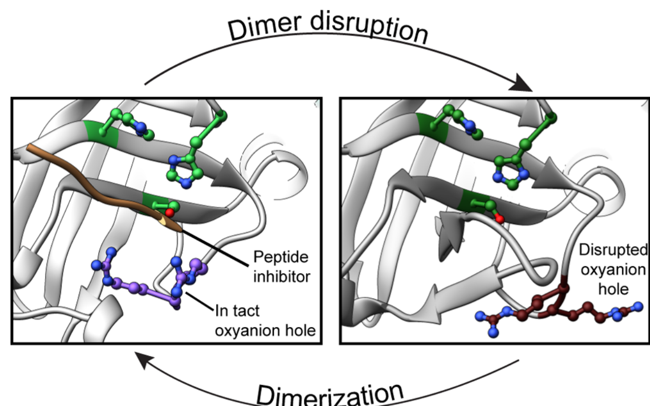



**Figure 18.** Order-to-disorder transition of conserved oxyanion hole arginines upon dimer disruption.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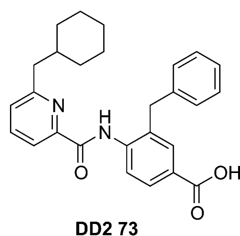



Figure 19. Dimer disruptor 2 (DD2), an allosteric herpesvirus protease inhibitor (Figure 2, capsid maturation).

## 6.4. Active-Site Inhibitors of HHV Proteases

**6.4.1. Generic Serine Protease Inhibitors.** HCMV protease has been the most actively pursued target of the HHV proteases both in academia and industry. As was the case for HCV protease, HCMV protease (and indeed all HHV proteases) is poorly inhibited by traditional serine protease inhibitors. A typical mechanism-based serine protease inhibitor, 3,4-dichloroisocoumarin (**50**), required high concentrations (0.1−1 mM) to achieve inhibition and was ultimately found to react with thiol groups of HCMV's five cysteine residues, not the active-site serine.[153] Widely used chloromethyl-ketones such as the chymotrypsin inhibitor tosylamido-2-phenylethyl-chloromethyl ketone (TPCK **51**) achieves only 33% inhibition of HCMV protease at 250 $\mu$M. Tosyl-L-lysine chloromethyl ketone (TLCK **52**), phenylmethylsulfonyl fluoride (PMSF **53**), and benzamidine (**54**) show no inhibition of HCMV protease at 250 $\mu$M. Diisopropylfluorophosphate (DFP **55**) inhibits both HCMV and HSV-1, albeit weakly, providing some of the early evidence that HHV proteases are serine proteases.[154]

Macromolecular protease inhibitors such as lima bean and soybean trypsin inhibitors, ovomucoid, Bowman−Birk inhibitor, and bovine pancreatic trypsin inhibitor likewise do not inhibit HCMV protease as expected due to the unique structural fold of the enzyme. Peptide aldehydes chymostatin, elastatinal, leupeptin, and antipain also fail to inhibit HCMV protease presumably due to the weak nucleophilicity of the active-site serine as a result of the noncanonical catalytic triad.[153] HCMV protease—HHV proteases in general—are clearly nontrivial protease targets.

**6.4.2. Activated Carbonyl Warheads.** Similar to the early development of HCV protease inhibitors, initial attempts to achieve specific inhibitors of HCMV protease relied on peptides and peptidomimetics, taking advantage of the substantial work describing substrate specificity for this enzyme. An 11-mer peptide spanning P6 to P5′ (GVVVNA/SCRLA) of the native M-site was both a substrate and competitive inhibitor with $K_i$ of 0.225 mM and $K_M$ of 0.515 mM. A similar peptide where the P1′ serine is replaced by alanine (GVVNA/ACRLA) showed substantial improvement in both $K_i$ (72 $\mu$M) and $K_M$ (0.112 mM).[155] Use of a reduced peptide bond at the cleavage site did relatively little to improve the native M-site peptide as an inhibitor.[156] The limited success of competitive noncovalent peptidic inhibitors led to the pursuit of mechanism-based inhibitors of HCMV protease. These utilized covalent warheads, such as trifluoromethyl ketone (**56**). In 1997 a group from Boehringer Ingelheim utilized a trifluoromethyl ketone warhead linked to M-site-derived peptides to interrogate the influence of peptide length and side-chain on inhibition.[157] They found that a tetramer was the minimal peptide that would achieve low micromolar $IC_{50}$ values. Lengthening the P1 side chain by a single carbon from methyl to ethyl was tolerated and somewhat improved inhibition; however adding two carbons to yield a propyl were not tolerated and decreased activity. Extensive SAR around the P2 side chain suggested that the S2 site was permissive while the S3 pocket preferred a bulkier *tert*-butyl glycine (Tbg, also known as tert-leucine) over the native valine residue. With the unnatural amino acid Tbg in place, the P4 capping group was explored and tolerant of a variety of substitutions. Several warheads were tolerated in place of the trifluoro methyl ketone. Interestingly, $\alpha$-ketobenzoxazoles were not specific to HCMV protease, while the other warheads maintained selectivity against a panel of serine proteases. The pentafluoroehtyl ketone inhibitor was the most potent with an $IC_{50}$ of 110 nM (**57**). Unfortunately, none of these compounds were efficacious in cell culture. The authors suspect this is due to cell permeability and/or metabolic stability.[157]

**6.4.3. $\beta$-Lactam Inhibitors.** In light of still limited success with activated carbonyls, an alternative strategy was pursued. As Waxman and Darke describe and as was noted by the group out of Boehinger Ingelheim that published extensively on protease active-site inhibitors, monocyclic $\beta$-lactam inhibitors of human leukocyte elastase were co-opted as a starting point for HCMV protease inhibitor development.[153,158] Human leukocyte elastase, like the HHV proteases, has a substrate preference dominated by small alkyl side chains.[159] Specifically, researchers at Merck had shown that N-carbonylamino derived $\beta$-lactams (**58**) inhibit leukocyte elastase.[160] Déziel and Malenfant reported peptidic substrate-based $\beta$-lactams built both from their previous work on substrate-based inhibitors described above and on the $\beta$-lactam scaffold.[157,158] These inhibitors incorporated the optimized peptidyl portion from the previous work onto the C4 position of the $\beta$-lactam through either an ether or thioether bond and probed substitutions at the $\beta$-lactam nitrogen leading to a 70 nM inhibitor (**59**). Replacing the peptidyl portion of the compounds with a variety of substituents failed to improve potency and/or selectivity across other proteases such as leukocyte elastase. However, some less potent analogs showed limited activity in cell culture ($EC_{50}$ of 60 $\mu$M in a plaque reduction assay).[158a] Further elaboration of this scaffold by the same group provides interesting SAR but ultimately failed to increase potency in enzyme assays or cell culture.[158b] Bonneau et al. elegantly show that these $\beta$-lactams are indeed acting through an acyl enzyme intermediate in the case of HCMV protease.[161] Using kinetic analyses with a fluorogenic $\beta$-lactam they were able to measure the rate of acylation and deacylation, and relate that to $IC_{50}$. These studies highlight the importance of whether the C4 position is occupied by a leaving group as well as the overall electrophilicity of the carbonyl carbon as it is influenced by the substitutions on the $\beta$-lactam ring.[161]

**6.4.4. *trans*-Lactam Inhibitors.** Derived from euphane triterpene natural product inhibitors of serine proteases, the 5,5-translactam scaffold similar to **60** has been optimized to achieve potent inhibition of HCMV protease. The work of Borthwick et al. skillfully explored and optimized this novel class of inhibitors and is expertly reviewed elsewhere.[162] In the present review we address the key findings of these studies and relevant details of the approach that aided in the inhibitor development against this challenging target.

A rational design was used beginning from the highly conserved cleavage sequence for HCMV protease, Val-Xxx-Ala/Ser where the slash denotes the site of cleavage. A methyl group was introduced $\alpha$ to the lactam carbonyl in order to mimic the alanine methyl that would hypothetically occupy the S1 pocket.

Given the apparent importance of this alanine, Borthwick et al. maintained this substituent and synthesized stereospecific analogs to determine which stereochemistry would best position the methyl group in the S1 pocket, ultimately favoring the *cis*-substitution as depicted in **60**.[163] The subsequent SAR focused first on the absolute stereochemistry for the active diastereomer of the translactam, which is also as depicted in compound **60**. Further functionalization of the lactam nitrogen from which the S1′ site is accessed showed a preference for trigonal substituents (not tetrahedral), with potency tracking with electron withdrawing ability, but also showing some preferences for the sterics of the various substituents that were analyzed.[164] In the same work, the authors utilize array chemistry to probe tolerable substitutions at the pyrrole nitrogen of the *trans*-lactam system and identify the monocyclic pyrrole derived from *S*-proline as an ideal substituent (**61**). With absolute stereochemistry assigned and evaluated, the authors modified the $R_1$ and $R_2$ positions of compound **61** and discovered the dansyl-cyclopropyl substituents as depicted in compound **62** to be optimal. The authors report a $K_i$ of 20 nM and an $IC_{50}$ of 340 nM for **62** and provide a thorough mechanistic study as well as docking results.[164] Substantial effort was then dedicated to establishing the fine balance of potency, pharmacokinetic properties, and selectivity necessary to advance a small molecule as a drug candidate.[165] The authors moved to a 4-isopropylphenylurea in place of the dansyl group and replaced the cyclopropyl with a benzothiazole derivative to obtain **63**, which showed a favorable PK and activity profile including a half-life greater than 24 h, 2 orders of magnitude selectivity for HCMV protease over acetylcholine esterase, elastase, and thrombin, and an excellent $IC_{50}$.[166] Importantly, this compound had high nanomolar to low micromolar antiviral activity in cell culture against lab strain AD169, equivalent to ganciclovir (**1**), and low cytotoxicity, creating an excellent therapeutic index. Finally, an unsubstituted benzothiazole derivative showed acceptable bioavailability in dogs and good brain and ocular penetration in guinea pigs.[165b] This exciting work is a quintessential example of hit-to-lead drug discovery utilizing a combination of rational SAR, combinatorial chemistry, and structure-based drug design to address not only *in vitro* potency, but also pharmacological considerations critical to drug discovery. Unfortunately, to our knowledge, there is no literature describing the progression of the *trans*-lactams into animal disease models or clinical trials.

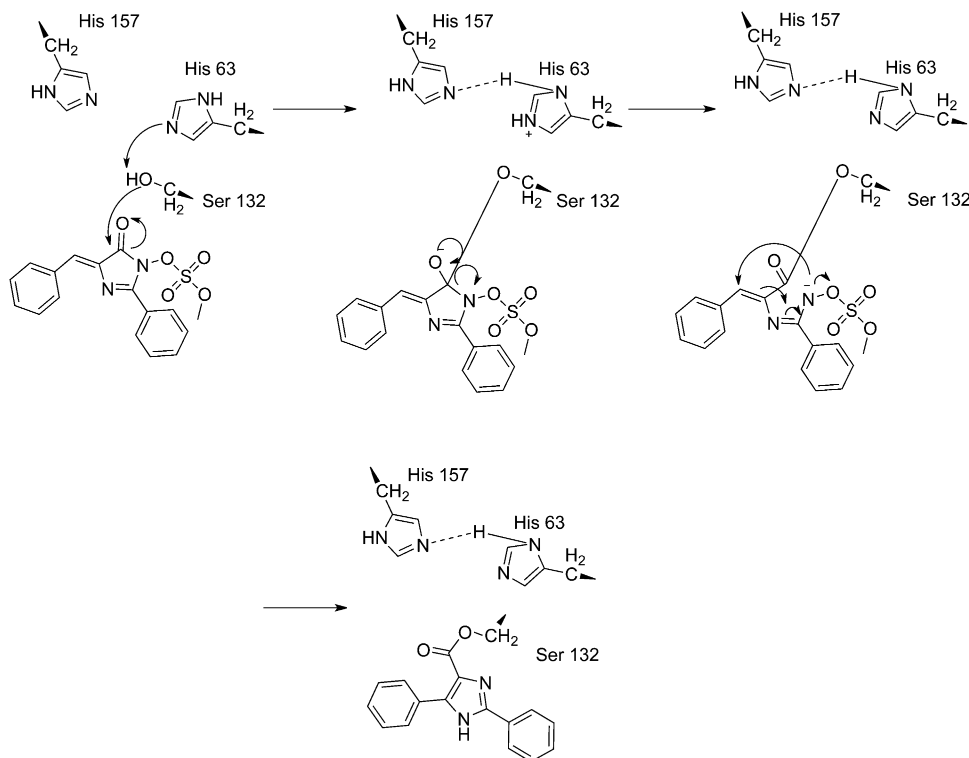
**6.4.5. Azetidine Inhibitors.** Several papers have aimed to develop efficacious noncovalent active-site inhibitors against HCMV protease that move away from substrate-based $\beta$-lactam compounds.[167] The first examples of the switch from covalent inhibitor to noncovalent inhibitor against HCMV protease were published in 2004. The authors first hypothesize that derivatives at the C4 position with carbonyl functionality (**64**) will introduce an interaction with Arg165 and Arg166, which function to stabilize the oxyanion hole.[158b,167a] Interestingly, the absolute stereochemistry of the covalent inhibitors had little effect on potency in either the AD-169 or Davies strain. The first compounds showed a narrow effective concentration range, however, and all displayed significant cellular toxicity. Removing the electrophile from the $\beta$-lactam led to the discovery of azetidine **65**, which is hypothesized to be the first noncovalent competitive inhibitor of the HCMV protease.[167a] The compound displays an excellent cellular $EC_{50}$, comparable to ganciclovir (**1**), but cellular toxicity remains an issue. These compounds were not assayed against isolated protease, so it is difficult to assess any relationship between biochemical and cellular potencies.

Subsequent efforts to more fully understand the structure–activity relationships describe modifications to the lactam nitrogen substituents as well as the C4 carbonyl substitutions. The authors explore conformational rigidity and stereochemical consequences of lactam and azetidine compounds and extend the cellular assays to include VZV.[167b,c] However, the SAR is complicated. The benzyl carbamate **65** remains the preferential substituent over urea derivatives, and the simpler *tert*-butyl esters are preferred over amino acid coupled esters and amides at the C4 carbonyl position.[167b,c] Overall, the balance between potency and toxicity remains an issue for these compounds and further work on the mechanism of action is warranted.

**6.4.6. Benzoxazinone and Thieno[2,3-*d*]oxazinone Mechanism-Based Inhibitors.** Scientists at SmithKline Beecham (now GlaxoSmithKline) and Searle (now a part of Pfizer) took an alternative approach focused on benzoxazinones typified by **66** and related compounds which are mechanism-based serine protease inhibitors, particularly of human leukocyte elastase.[168] In 1996 Jarvest et al. from SmithKline Beecham developed benzoxazinones that inhibit HSV-1 protease. The authors began their optimization efforts by synthesizing derivatives that contain the alanine from the HSV-1 VNA/S cleavage recognition sequence.[168a] The initial efforts verified that inhibition takes place through an acyl-enzyme intermediate, stereochemistry around the amino acid moieties is relevant, specific SAR exists at various positions of the benzoxazinone, and the aqueous half-life could be optimized. The optimized compound from the initial work contained an isopropyl amine in the 2- position of the benzoxazinone and a Cbz-Ala-NH in the 7- position (**67**). This inhibitor had an $IC_{50}$ of 5 $\mu$M on HSV-1 protease and an aqueous half-life of 171 h.[168a] Similar inhibitors designed from the known elastase inhibitors were concurrently developed against HCMV protease.[168b] The most potent benzoxazinone inhibitor (**68**) of HCMV protease had an $IC_{50}$ of 0.22 $\mu$M; however, it was not selective (e.g., $IC_{50}$ against chymotrypsin of 0.065 $\mu$M). Selective inhibitors were about 10-fold less potent.[168b]

Optimization of related thienoxazinone compounds typified by **69** as a follow-up study by the scientists at SmithKline Beecham yielded nanomolar inhibitors of HSV-1, HSV-2, and HCMV proteases.[168c] SAR in the series varied between the proteases, and selectivity remained challenging. A follow-up report described reoriented thienophenes as in **70**. The simplest compound in the study, an unsubstituted cinnamoyl group at the R position of **70** inhibits HSV-2, VZV, and HCMV proteases with 300, 38, and 500 nM $IC_{50}$ values, respectively. The authors reported reasonable cytotoxicity data but do not report selectivity over other relevant proteases. A second lead that used a 2-bromo-5-methyl thiophene in place of the benzyl group of the initial cinnamoyl hit inhibits HSV-2, VZV, and HCMV proteases with 270, 8, and 210 nM $IC_{50}$s, respectively.[168d] Both lead compounds showed no inhibition of elastase and trypsin up to 100 $\mu$M and inhibited HSV-2 protease in cells with ~8 $\mu$M potency—though the assay was not described and the citation which had been submitted for publication was never published, leaving the assay design and interpretation unclear.[168c,d] It was later reported by these same authors that additional unpublished data revealed that these inhibitors did not show antiviral activity in a yield reduction assay.[169] Without publication of the cell culture assays and data it is difficult to make sense of these ostensibly conflicting results

Scheme 2. Potential Mechanism of Covalent Protease Inhibition by Imidazolones




(though the nanomolar $IC_{50}$s in enzymatic assays remain interesting).

**6.4.7. Spirocyclopropyl Oxazolones and the Benzylidine *N*-Sulphonyloxyimidazolones Mechanism-Based Inhibitors.** The scientists from SmithKline Beecham also identified two new classes of serine protease inhibitors, the spirocyclopropyl oxazolones (**71**) and N-substituted benzylidine imidazolones (**72**).[170] The SAR shows excellent selectivity over the canonical mammalian serine proteases elastase, trypsin, and chymotrypsin for the oxazolones. Several of the oxazolone analogs with an exodouble bond or acyclic aliphatic chain replacing the cyclopropyl moiety highlight the importance of the cyclopropyl group which is hypothesized to properly orient the optimal phenyl substituent (i.e., $R_3$ = Ph, **71**). The most potent spirocyclopropyl oxazolone had $IC_{50}$ values of 500 nM and 200 nM against HSV-2 and HCMV proteases, respectively, while showing modest inhibition at elastase, trypsin, and chymotrypsin at 100 $\mu$M. Interestingly, the reported imidazolones were generally less potent, showing only minimal activity of HSV-2 protease. These compounds were initially hypothesized to inhibit the enzymes through the previously described nucleophilic attack/elimination/Lössen rearrangement reported by Groutas et al. for the structurally similar succinamide inhibitors of elastase.[171] However, mechanistic studies of the imidazalones did not observe the expected acyclic product, but rather isolated an imidazole leading to the proposed mechanism in Scheme 2.[170] A sulfonate of the imidazolone with an ethenylbenzene in place of the phenylcyclopropyl moiety was also active against HCMV protease with an $IC_{50}$ of 0.4 $\mu$M, while showing little inhibition of HSV-2 protease.[170]

### 6.5. Human Herpesvirus Protease Dimerization and Activation

Dimerization is an essential step in the activation of all human herpesvirus proteases.[150c,172] The dimer interface is composed largely of helix 5−helix 5 intermonomeric interactions, with additional binding surface contributed by helix 1 of each monomer. On average these bury 2000 Å$^2$ surface area. Unlike HIV protease, each monomer contains its own Ser-His-His catalytic triad, and these active sites are functional and independent of one another. Titration of inactive catalytic serine-to-alanine mutants into wild type monomeric enzyme drives dimerization and activates the wild type monomer of the heterodimeric species.[173] Two key studies provide a mechanism by which dimerization is allosterically linked to activation at the catalytic site and support the notion that this mechanism is conserved across HHV proteases. Batra et al. demonstrate by mutagenesis and structure determination that mutation of the dimer interface of HCMV protease can result in a dimerization-competent protein that is nonetheless inactive due to a rearrangement of the conserved residues that help form the oxyanion hole. A single point mutation, S225Y, results in a dimer where conserved residues Arg165 and Arg166 are disordered. Arg165 is part of the oxyanion hole, and Arg166 contributes to its stabilization. This loss of the oxyanion hole explains the roughly 1700-fold reduction in $k_{cat}$ for this mutant. Interestingly, the catalytic triad (which resides on the core $\beta$-barrel of these proteases) is unperturbed in the S225Y variant. In support of this model, the $K_M$ of substrate binding and the disassociation constant ($K_d$) of dimerization are unchanged as well. As a first hint of allosteric networks throughout these proteases, the binding of an activated peptidomimetic inhibitor caused a structural change in the variant protease that reverted it to a wild type conformation.[151b] The structure of the monomer for these variants, however, remained elusive. Pray et

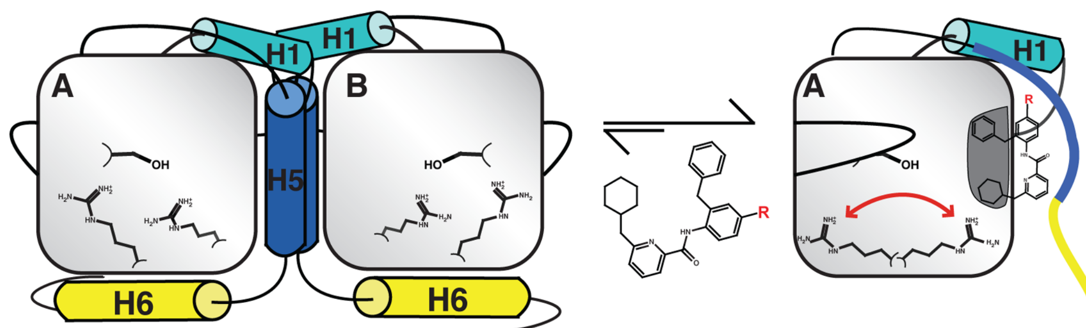



**Figure 20.** Proposed mechanism of dimer disruption and allosteric inhibition of herpesvirus proteases. Inhibitor binding in the core of the protein precludes folding of the C-terminal helices, preventing both dimerization and proper formation of the oxyanion holes formed by two conserved arginines. The catalytic serine is unperturbed, but the substrate binding site may be occluded. Reprinted from ref 176. Copyright 2014 American Chemical Society.

al. interrogated conformational changes between monomer and dimer using circular dichroism and tryptophan fluorescence. These data confirmed an increase in helical content upon dimerization leading to the hypothesis that dimerization orders the two c-terminal helices and that this results in activation.[174] Indeed, subsequent studies validated that hypothesis. $^{13}C$ HSQC NMR chemical shift index measurements and NMR hydrogen/deuterium exchange experiments show the two C-terminal helices of KSHV protease are ordered in the dimer and largely disordered in the monomer. Furthermore, an engineered disulfide bond between the C-terminal helix and oxyanion loop acts as a redox switch that can activate the enzyme. Even the obligate monomeric enzyme, KSHV protease M197D, can be activated in this way showing that the monomer is catalytically active when in the appropriate conformation.[175]

How then is dimerization regulated? The most obvious answer is through changes in concentration of the enzyme. This is consistent with the current model of HHV protease activation, wherein it is concentrated into the nucleus and ultimately the immature capsid as a fusion to the capsid assembly protein. As was suggested by the work of Batra et al., communication between the substrate binding site and the dimer interface also regulates dimerization and thus activity. This was illustrated by the use of an optimized hexapeptide diphenylphosphonate inhibitor of KSHV protease. In an initially puzzling result observed by Marnett et al., addition of this covalent inhibitor up to roughly 0.4 mol equiv relative to protease concentration actually increased enzyme activity, followed by a decrease at higher inhibitor concentrations. An NMR assay using a selectively labeled methionine at the dimer interface revealed that inhibitor addition promoted dimerization. Size exclusion chromatography confirmed this, and circular dichroism indicated an increase in helical content upon dimerization. Thus, in the absence of inhibitor the majority of enzyme is monomeric and inactive. As inhibitor is increased up to roughly 0.5 mol equiv a population of stabilized dimers in which only a single monomer is inhibited emerges, leaving the other monomer in a highly active conformation and able to process substrate. Further increase in the inhibitor concentration leads to an increasing population of enzyme for which both monomers are inhibited and overall enzyme activity is reduced. This study solidified a model in which enzymatic activity is linked to quaternary structure and an allosteric link exists between the dimer interface and the substrate binding site. Interestingly, these data also suggest that substoichiometric active-site inhibition of HHV proteases may lead to overall activation, a potential contributor to the challenges realized by those developing active-site inhibitors of these unusual enzymes.[176] The cocrystal structure of KSHV protease and the hexapeptide phosphonate inhibitor supports these findings.[152d]

The crystallization of an HHV protease monomer for the first time further supported this model of a significant structural change between monomer and dimer and loss of the oxyanion hole as a mechanism by which dimerization regulates activity (Figure 20). Truncation of the two C-terminal helices from KSHV protease established an obligate monomeric HHV protease. In the presence of a small-molecule protein−protein interaction inhibitor, discussed in section 6.6, Lee et al. crystallized this monomeric KSHV protease. Consistent with the findings from HCMV protease, monomeric KSHV protease had a disordered oxyanion hole with no structural perturbation of the catalytic triad. In the case of KSHV protease, however, the substrate binding pocket was also occluded by a loop (residues 17−21, Figure 18).[177]

### 6.6. Dimer Disruption as an Alternative to Active-Site Inhibitors

Although a great deal of work has been put toward developing active-site inhibitors of the HHV proteases, compounds targeting the protease have yet to advance to the clinic. An alternative approach that could potentially overcome limitations inherent to targeting the shallow and dynamic active site is identifying small molecules that prevent dimerization, and thus inhibit one or more of the HHV proteases. Proof of concept for this approach was first achieved using a peptide instead of small molecules. Interfacial helix 5 of KSHV protease was grafted onto the avian pancreatic polypeptide (APP). The avian pancreatic polypeptide scaffold provided stability for the interfacial helix, whereas a peptide composed of the helix 5 residues alone is unstructured and shows no inhibition within the solubility limit of the peptide. This grafted helix 5 APP inhibited KSHV protease with a 300 $\mu$M $IC_{50}$. Size exclusion chromatography was used to confirm that addition of the grafted helix 5 peptide results in loss of the dimer species, and a 7-amino-4-carbamoylmethyl coumarin (ACC) fluorogenic substrate was used to monitor inhibition of protease activity.[173b] While providing proof of concept, this peptide inhibitor lacked potency and would not be suitable for showing antiviral efficacy in cell culture due to its likely being unable to cross the cell membrane. To pursue a small-molecule inhibitor of KSHV protease dimerization a 183-compound helical

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mimetic library was screened using an optimized fluorogenic ACC substrate. From an initial hit, a series of 4-benzoylaminobenzoic acid compounds were synthesized, and a 3 $\mu$M inhibitor named dimer disruptor 2 (DD2, **73**) was identified.[178] Again, size exclusion chromatography was used to show a loss of the dimeric state of KSHV protease with increasing concentrations of inhibitor. Two-dimensional protein NMR was also used to confirm dimer disruption and map binding of the compound. Met197, a residue in helix 5, was selectively $^{13}C$ labeled, and a $^{13}C$-HSQC was used to probe dimerization state. The monomer−dimer equilibrium showed slow exchange kinetics on the NMR time scale revealing distinct monomer and dimer peaks that could be used to monitor the monomer and dimer populations in solution. Addition of DD2 resulted in a complete loss of the dimer peak and a chemical shift perturbation of the monomer peak. Uniform $^{15}N$ labeling and $^{15}N$ HSQC showed significant peak broadening for Trp109 suggesting DD2 binds at or near Trp109, an aromatic hotspot at the dimer interface.[178] Truncation of helix 6 and all but a single turn of helix 5 enabled crystallization of the monomer bound to DD2. This crystal structure confirmed that the small molecule bound at the dimer interface in a transient hydrophobic pocket formed by rotation of the tryptophan indole side chain causing the structural changes diagramed in Figure 20.[177] While this approach validated the dimer interface as a druggable site on KSHV protease, solubility and permeability of DD2 appear to have prevented its use in cell culture. Therefore, KSHV protease, as of yet, has not been validated pharmacologically though it is presumed that inhibition of the enzyme would prevent viral replication as has been shown for other HHV proteases.[178] While this lack of cell culture activity is disappointing, disrupting dimerization to allosterically inactivate the herpesviruses still is a promising approach, as is the DD2 scaffold. Recently, a combination of kinetics, NMR, and crystallography revealed that DD2 and two carboxylate bioisostere analogs inhibit not only KSHV protease, but also HCMV, EBV, and to a lesser extent HSV-2 proteases.[179] These data suggest that elaboration of the DD2 scaffold could give rise to potent cell-permeable allosteric inhibitors of HHV proteases. Such a compound would be one of few protein−protein interaction inhibitors in an infectious disease setting and an exciting advance in allosteric modulation of traditionally challenging targets.

## 7. CONCLUSION

Enormous progress has been made in the field of herpesvirus drug discovery, though approved treatments are still limited to a single viral target. Herpesviruses such as Kaposi's sarcoma-associated herpesvirus and Epstein−Barr Virus have no approved antiviral treatments—despite causing severe disease. Cytomegalovirus infection has no approved treatment in the congenital setting, and treatments for the organ transplant and HIV/AIDS settings suffer from resistance and dose-limiting toxicities.

Encouragingly, many scientists in both academia and industry are stepping up to the challenge. Small molecules against multiple herpesviruses with targets throughout the viral replication cycle are being developed. Many of these have activity in cell culture; some are making it to clinical trials. These inhibitors span the gamut in both target and mechanism: competitive kinase inhibitors, prodrug DNA polymerase inhibitors, allosteric protease inhibitors, and host−protein inhibitors, to name only a few. The field is clearly on the move. As we increase our understanding of herpesvirus biology and improve our ability to target "undruggable" proteins, even the lauded goal of targeting latency may become a reality.

The complexity of herpesviruses presents both challenges and opportunities for drug discovery. These viruses encode more than 100 proteins, many with unclear biology. Small open reading frames and noncoding RNA are abundant and varied, with little known about their function.[22,180] These challenges also represent opportunities. Indeed, every one of these macromolecules provides a chance to both better understand and prevent human disease through chemical intervention.

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### Notes

The authors declare no competing financial interest.

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Timothy M. Acker obtained his Bachelor of Science degree in biochemistry from San Francisco State University. He attended Emory University for his Ph.D. in biomedical sciences from the Molecular and Systems Pharmacology Program with an emphasis in chemical biology

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Charles S. Craik is a Professor in the Departments of Pharmaceutical Chemistry, Cellular & Molecular Pharmacology, and Biochemistry & Biophysics at the University of California, San Francisco. He is also the founder and director of the Chemistry and Chemical Biology Graduate Program. He received his Ph.D. in chemistry from Columbia University in New York and carried out his postdoctoral research at UCSF with Dr. William Rutter. He joined the UCSF faculty in 1985 where his research interests focus on defining the roles and the mechanisms of enzymes in complex biological processes and on developing technologies to facilitate these studies. He is also founder of Catalyst Biosciences, a biotechnology company focused on therapeutic proteases. The current research in the Craik lab focuses on the chemical biology of proteolytic enzymes and their natural inhibitors. A particular emphasis of his work is on identifying the roles and regulating the activity of proteases and degradative enzyme complexes associated with infectious diseases, cancer, and development. These studies are providing a better understanding of both the chemical make-up and the biological importance of these critical proteins to aid in the rapid detection, moinitoring, and control of infectious disease and cancer.

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