Antiviral strategies targeting herpesviruses

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ABSTRACT

Herpesviruses, known as large DNA viruses, have a wide host range. In addition to human beings, cattle, and horses, even carp can be hosts for herpesvirus infection. Herpesviruses are pathogens possessing elaborate mechanisms that regulate host cell components for its replication, assembly and generating mature virus particles that can infect humans and most animals, usually causing multiple and lifelong infections. In addition, several human diseases, such as genital or mouth herpes, mononucleosis, and Burkitt lymphoma, are usually associated with herpesvirus infection. Blocking the steps of viral infection, such as entry, replication and assembly, may be an effective way for many different herpes viruses and their related diseases. Therefore, we aim to describe antiviral agents that are able to prevent herpesvirus entry, replication and assembly in host cells. We summarize antiviral strategies, including certain small molecular drugs, RNA interference and CRISPR/Cas9 system-based antiviral approaches, which represent promising approaches.

1. Introduction

Herpesviruses consist of enveloped double-stranded DNA and primarily affect the skin, mucous membranes, and nerve tissues of hosts, often resulting in severe morbidity and mortality.¹ A wide range of hosts can be infected by herpesviruses, including mammals, primates, amphibians, poultry, and carp. To date, more than 200 distinct herpesvirus species have been identified.² This old viral lineage is diverse, with multiple naming schemes: 1) according to the hosts of certain isolated viruses, such as avian herpesvirus, monkey herpesvirus, and others; 2) depending on the diseases resulting from herpesvirus infection, such as herpes simplex virus and frog kidney adenocarcinoma herpes virus; 3) by the person(s) who discovered it, like the Epstein-Barr virus, Lucke herpes virus, Marek herpes virus, and others. Nevertheless, on the basis of their distinct biological and genetic structure, herpesviruses are divided into three groups of alpha (α), beta (β) and gamma (γ). Especially, herpes simplex virus (HSV) type 1 and 2, varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein–Barr virus (EBV) which are members of the Alphaherpesvirinae, Betaherpesvirinae, Gamma-herpesvirinae subfamily, caused the most health concerns, thus have received extensive attention.

2. Herpesvirus infection

Herpesvirus infection is a complex process (Fig. 1) that includes virus attachment to the host cell, virus-host membrane fusion and internalization of the virus particles, viral genome replication, capsid assembly and virion release. The infection begins when a large number of virions are attached to heparan sulfate (HS), located on host cell surfaces by gB and/or gC, promoting gD or gE binding to relevant receptors, it usually in the form of proteoglycans, known as heparan sulfate proteoglycans (HSPG). Interaction between gB and heparan sulfate (HS) is required for HSV-1 surfing. The binding of these viral envelope proteins and host membrane receptors induces membrane fusion, resulting in internalization of the envelope and capsid into the host cytoplasm. Interestingly, several reports have found complete virions in the host cytoplasm, indicating that herpesvirus may infect the host by inducing cell endocytosis. Moreover, herpesvirus can enter the body in other ways. For instance, Nicola et al. found that HSV infection occurs partly through PH-dependent endocytosis in certain types of host cells.³ In addition, many herpesviruses have also been reported to enter cells through a phagocytosis-like mechanism, such as HSV-1, HHV-8 and CMV.⁴

After passing through the host membrane, capsid containing viral genomic DNA is transferred by the host cell movement protein along the microtubules to the host nucleus. Subsequently, the viral genomic DNA

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comes out of the capsid via a portal and enters host cell nucleus through the nuclear pore to initiate lytic-cycle infection. Immediate early (IE) proteins are first produced to promote the expression of early proteins (E proteins). Some of early proteins are critical for inducing viral DNA replication in replication compartments (RCs). Next, late gene of capsid proteins are transcribed, and the synthesized viral genomes are packaged into nucleocapsids. By budding, the incomplete virions leave the host nucleus to the cytoplasm through inner and outer nuclear layers and acquire most of tegument proteins in there. Then, those incomplete virions are wrapped by secondary envelopment on the Golgi membrane.

Fig. 1. Model of the herpesvirus life cycle. All members of the Herpesviridae share a common structure, a mature virion composed of these four parts: genomic DNA, capsid, tegument, and envelope with glycoproteins. The innermost layer is viral DNA which located in an icosahedral protein cage called the capsid surrounded by a tegument proteins layer. The outermost layer is the envelope containing multiple glycoproteins. Infection is initiated when viral envelope glycoproteins bind to the specific types of cell surface receptors include HVEM, nectin-1/2, PDGFα, CD21 and 3-O-S HS etc. Post-binding events: viral particles enter into cells mainly through direct fusion with cell membrane or via an endocytic pathway. Next, viral capsid is dismantled and viral DNA enters host cell nucleus through nuclear pore. Within the nucleus, viral IE and E genes will be transcribed mediating the replication of viral genomic DNA. After 24h infection, transcript of the late (L) gene occurs and the produce capsid proteins so that a new nucleocapsid will assemble in host cell nucleus and guiding viral DNA to enter the capsid. After these immature particles pass through the nuclear membrane, they acquire an envelope. A secondary envelopment occurs in host cytoplasm to help virus forming mature virions, finally the virions leave the host cell via exocytosis.
These specialized transitional vesicles take all virus particles to the cell plasma membrane and release them via exocytosis. All herpesviruses can establish lifelong latency in the nuclei of cells, and then distribute to its daughter cells. During the latent period, there is almost no obvious viral replication or viral gene expression as a strategy to successfully evade the immune system, thereby causing chronic infection throughout the whole life of their hosts. This brief description about viral replication applies to most, herpesviruses.

### 3. Antiviral strategies for treating herpesviruses

As a common contagious virus, although most herpes viruses infections are asymptomatic, some herpesvirus usually cause serious disease. Drugs against herpesvirus infections in the clinic are mainly of two types: nucleoside analogs such as acyclovir targeting DNA polymerase/thymidine kinase and helicase-primease inhibitors such as amantadine (ASP2151), with both types having the ability to reduce or stop the replication of the viral genome. However, these therapeutic applications often induce drug resistance, making it necessary to gain a deeper understanding of the viral replication mechanism for use in exploring more suitable antiviral targets.

With the continuous development of biotechnology, new approaches to suppress herpesvirus at the gene level have been developed; for instance, RNA interference (RNAi) be could represented as a powerful tool to silence gene expression at the mRNA level and has demonstrated promise in combating several herpesviruses, such as HSV-1, HHV-6B and CyHV-3.1-4 At the DNA level, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspersed short palindromic repeat (CRISPR)-associated protein 9 (Cas9) make editing viral DNA genomes feasible. The specific editing of viral DNA may lead to better effects during the acute and latent periods with reduced virus-induced toxicity and a strong host immune response. Taken together, both gene-specific siRNA and DNA can in theory repress viral attachment to host cells, membrane fusion and internalization of viral particles, DNA replication, virion assembly and release. In this section, we mainly discuss antiviral approaches that impact on multiple aspects of virus such as entry, replication and assembly and mechanisms by which these treatments have enhanced efficacy, reduced viral resistance to treatment and potential for treatment failure.

#### 3.1. Antiviral strategies for herpesvirus infection at the protein, DNA and RNA levels

##### 3.1.1. Suppressing herpesvirus attachments and invasion by targeting viral proteins

Attachment to host cells is an important step to successfully establish herpesvirus infection, and one strategy is to directly block viral attachment factors such as an ideal and classic antiviral approach involves directly blocking viral attachment factors such as HSV gD and gC at the protein level. Bushra et al. showed that retrocyclin 2 can directly bind to the HSV-2 gB2 with high affinity and thereby prevent HSV-2 from entering target cells. A mandelic acid condensation polymer named SAMMA is also effective against HSV-2 and targets gB2, reducing both viral attachment and invasion. Agents that prevent the virus from entering or spreading by forming a stable complex with gB, include PRO 2000, polystyrene sulfonate, cellulose sulfate, and polyethylene hydroquinone sulfonate. Among these, PRO 2000 has been tested in clinical phase I trials and has displayed good safety and tolerability. In addition, when using anti-gC, it can target the cell-binding domain of gC protein and neutralize the virus infectivity in human immortalized keratinocytes (HaCat). Additionally, gD, gH and gL also play a key role during the related processes. Subash et al. isolated a series of RNA aptamers that can inhibit viral entry by specifically binding to the HSV-1 gD protein. A 45-nt-long DNA aptamer also showed high affinity for HSV-1 gD, with an impact on virus entry and replication in vivo and in vitro. Thus, these studies have demonstrated that it is feasible to inhibit viral entry by targeting HSV-1 entry glycoproteins.

Varicella zoster virus (VZV) is different from other α-herpesviruses, the gE is critical for binding to the specific entry receptors of host cells since it does not have a gD. During VZV infection, gE is associated with gH targeting of specific regions in the host cell. However, there are few current reports showing VZV infection by blocking gE.

Similar to VZV, the human cytomegalovirus (HCMV) has no gD, the gB and gH/L complex play an important role in invasion. During the initial interaction, gB can bind not only cell surface heparan sulfate (HS) but also interact with epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor-α (PDGFRα), BST/tetherin and various integrins to promote HCMV entry, but the potential of these targets are still unclear and needs to be studied further.

The Epstein-Barr virus (EBV) gp350/220 first binds to B-cell receptor CD21. Studies have shown that soluble recombinant CD21 can inhibit EBV infection in vitro, indicating that soluble CD21 may have potential as an effective agent for treating human EBV infection. In addition, EphA2 is essential for virus entry, as it triggers fusion upon virus attachment to epithelial cells. However, few works have been published investigating whether the inhibitor of EphA2 can suppress EBV infection.

##### 3.1.2. Suppressing herpesvirus genome replication at the protein level

The results from genetic analysis show substantial similarity between HSV-1-and 2-induced diseases. Acyclovir (ACV) and related nucleoside analogs have been used to treat infection and suppress HSV transmission. These drugs can be phosphorylated by TK and form triphosphates through host enzymes, which compete with deoxyguanosine triphosphate to inhibit HSV-encoded DNA polymerase and selectively inhibit viral DNA replication with low host cell toxicity. Notably, inhibitors targeting viral DNA polymerase can suppress only viruses at the outbreak stage with rapid replication but do not inhibit viruses in the latent phase. Thus, drug-resistant HSV can occur during clinical therapy, especially in immunocompromised patients. Genotypic identification of these drug-resistant strains revealed that the mutations are located either in the UL23 gene, which encodes the activating/phosphorylating TK enzyme, or in the UL30 gene, which encodes the viral DNA polymerase. However, these aforementioned inhibitors are not suitable for TK-deficient HSV or VZV strains, as acyclic nucleoside analogs require phosphorylation by the virus-encoded TK. Interrupting the interaction of cofactors within herpesvirus DNA replication may also suppress herpesvirus infection; for example, the small molecule BPs is capable of depressing HSV-1 replication by interfering with the UL30/UL42 complex formation in vitro.

Similar to the effects on suppressing HSV, suppressing viral DNA polymerase is effective against CMV, with inhibitors such as foscarnet, cidofovir and ganciclovir. Targeting CMV terminase is also an attractive strategy, the CMV terminase consists of the products of UL89 and UL56 gene. Letermovir is the new CMV terminase inhibitor that has been approved by the FDA for prevention of CMV in allogeneic hematopoietic stem cell transplantation (allo-HSCT) recipients. However, there is appearance of a benzimidazole ribonucleoside-resistant CMV strain with mutated UL89. By high-throughput screening, Arianna et al. obtained five compounds that can specifically inhibit the UL54/UL44 interaction and then hamper the replication of CMV. The successful inhibition of these functional complexes provides potential new anti-CMV agents; that is, agents may be designed to inhibit not only the target protein itself but also the formation of functional complexes.

In fact, most DNA polymerase inhibitors, such as penciclovir and foscarnet, are able to suppress genome replication of all three herpesvirus subfamilies. Similarly, protein kinase enzyme inhibitors cyclopropavir and maribavir (1263W94) are also effective antiviral drugs against EBV. However, ACV can block EBV replication in lytically infected cells but does not have the same effect in latently infected cells.
In contrast to nucleoside analogs, glycyrrhizic acid (GL) has been confirmed to impact on the early step of the EBV replication cycle.\(^{27}\)

### 3.1.3. Inhibition of herpesvirus replication at the DNA and RNA levels

Virus-host interactions are dynamic and complex processes, and interfering with virus receptor genes to block virus binding at the DNA level is an alternative method. With the development of specific genome editing technologies, such as ZFNs, TALENs and the CRISPR/Cas9 system, targeting certain genes to inhibit viral infections appears feasible. However, ZFNs and TALENs have not been widely applied as ideal methods in the treatment of herpesvirus since the cost and complexity of designing these custom-built endonucleases are limiting factors. CRISPR-Cas, found in bacteria and archaea, is an adaptive (acquired) system that can target foreign sequences and then silence or modify these genetic elements in hosts. Furthermore, in this system, multiple single guide RNA (sgRNAs) can be used simultaneously for the specific editing of different genes.\(^{38}\) As a double stranded DNA, the herpesvirus genome is easily edited by CRISPR/Cas9. To identify the most effective target sites for limiting HSV productive and latent infections, Ferdy et al. used CRISPR/Cas9 to disrupt a series of essential viral genes, including seven involved in DNA replication: UL30, UL42, UL9, UL5, UL8, UL52, and UL29; two genes encoding tegument proteins, namely, UL36 and UL37; the terminase UL15, which is related to DNA packaging; and UL27, which encodes capsid-associated gB and UL54, a multifunctional regulator of gene expression. The results suggest that the CRISPR/Cas9 system is able to impair HSV-1 replication in Vero cells by targeting all the aforementioned genes.\(^{20}\) Additionally, Xu et al. demonstrated that the CRISPR/Cas9-induced UL7 mutation, encoding a tegument protein, reduced HSV-1 replication capacity.\(^{30}\) Editing ICP0, ICP4 or ICP27 by CRISPR/Cas9 can also effectively reduce HSV-1 infection.\(^{31}\) Similar to other herpesviruses, the treatment of HCMV infection by CRISPR/Cas9 is feasible. Ferdy et al. designed sgRNAs targeting seven essential genes: viral DNA polymerase UL54, the polymerase accessory factor UL44, the single-stranded DNA-binding protein UL57, the primase UL70, the DNA helicase UL105, the major capsid protein UL86, and UL84, and almost completely inhibited viral replication by one or more sgRNAs. In his study, modification of the nonessential genes US6, US7, and US11 did not impact HCMV replication, because of the longer replication cycle of HCMV, resulting in the virus having sufficient time to repair the double-strand breaks (DSBs) of the viral genome.\(^{22}\) Indeed, a study has indicated that \(\pm 75%\) of DSBs can be repaired within 24 h in MRC5 cells.\(^{23}\) In addition, the selection pressure from CRISPR/Cas9 may drive viral evolution to produce mutants. Therefore, to minimize the escape of the virus to the greatest extent possible, targeting the viral genome using a single sgRNA was insufficient; at least two sgRNAs need to be used simultaneously to target multiple genes or different loci of one gene. The incurability of EBV infection is mainly due to viral latency in B lymphocytes. In vitro studies have shown that the CRISPR/Cas9 system can successfully eliminate latent EBV in cells. For example, Wang et al. reported that utilizing CRISPR/Cas9 technology generated a small deletion in EBV nuclear antigen 1 (EBNA1), EBV nuclear antigen 3C (EBNA3C) and latent membrane protein-1 genes (LMP1), which are essential for the regulation of latent genome replication and host cell transformation, resulting in a dramatic decrease in viral load in a Burkitt lymphoma cell line.\(^{34}\) Thereafter, Ferdy et al. designed sgRNAs targeting EBV EBNA1 and EBV origin of replication (OriP), and the results indicated that CRISPR/Cas9 could effectively reduce the EBV viral load in Akata cells.\(^{28}\) Subsequently, Yuen et al. confirmed that the CRISPR/Cas9 system targeting 3 different genomic elements, namely EBNA1, OriP and W repeats can inhibit EBV DNA accumulation in nasopharyngeal carcinoma cells.\(^{25}\)

At the RNA level, synthetic siRNA is a potential treatment strategy for viral genome replication. For HSV-1, Fang et al. knocked down the immediate-early (IE) gene ICP4, a major regulatory gene involved in lytic infection, and viral infection was observed in HCE cells, human trabecular meshwork cells (HTMs) and Vero cells.\(^{26}\) To test whether RNAi technology can be used as a method to prevent HSV-2 infection, Guo et al. showed that UL54-specific siRNA can significantly reduce the HSV-2 virus titer between 12 and 24 h.\(^{37}\) In vitro studies have shown that siRNAs can effectively inhibit HCMV infection by targeting the viral DNA polymerase enzyme pUL54, kinase pUL97, and immediate-early genes UL123 and UL122. Because treatment with siUL54B, siUL97A and siUL122B led to a 52.9%, 49.2% and 58.3% reduction in the number of infected cells, respectively.\(^{38}\) EBNA1, the EBV latency replication factor, is uniquely expressed in all EBV-positive proliferating cells. Currently, many reports have shown that inhibition of EBNA1 mRNA can cause loss of EBV episomal maintenance.\(^{39,40}\) In addition, Epstein-Barr virus latent membrane protein 1 (EBV-LMP1) is also useful for affecting EBV infection, Yu et al. constructed a vector encoding a short hairpin RNA (shRNA) against LMP1, an integral membrane protein gene, and which induced apoptosis in EBV-positive lymphoma cells.\(^{41}\)

### 4. Conclusion and future directions

Currently, clinical therapy against herpesviruses does not cure the infection, it only reduces virus replication. Here, we summarize various antiviral strategies in herpesvirus treatment (Supplementary Table 1). Although CRISPR/Cas9 and similar systems seem to be very promising for eradicating herpesviruses at the latent stage, it is possible that CRISPR/Cas9-resistant variants could arise due to survival selective pressure, which may limit the application of specific DNA-editing technologies. Several bottlenecks limit the use of siRNA, low transfection efficiency and original siRNA instability make totally silencing the target genes difficult. High levels of agent-facilitated transfection may lead to a host interferon overresponse and off-target side-effects. In addition to traditional siRNA, modified antisense oligonucleotides (AODs) are able to effectively suppress these important viral genes with suitable stability and low toxicity. For example, fomiviren, an antisense thiodeoxygenucleotide molecular drug targeting CMV immediate-early mRNA, has been approved by the FDA.\(^{42}\) In addition, recent studies have shown that DNA tetrahedrons and carbon nanotubes, which are recently developed nanophase biomaterials, have the ability to deliver exogenous biomolecules, such as siRNA, to target cells with enhanced stability.\(^{43,44}\)

In theory, directly suppressing viral functional proteins is the efficient strategy since the polypeptide sequence is relatively conservative, compared to that of viral DNA or RNA, which are diverse among strains. Several novel methods for screening new small molecule inhibitors targeting known target proteins have been developed, such as virtual screening (VS), high-throughput screening (HTS), and high-content screening (HCS). By calculating the electrostatic field, hydrophobic field, and hydrogen bond distribution of the binding sites in silico, VS is used for screening a large number of conditional compounds for subsequent detection in a short time.

Early studies have shown that several chemical inhibitors are able to induce the degradation of target proteins. For example, canertinib (CI-1033) is an effective TK inhibitor that can also promote ErbB-2 (HER2/ neu) degradation. Fulvestrant can inhibit the function of Era (estrogen receptor alpha) and induce its degradation\(^{38}\) by selectively degrading functional viral proteins, rather than attenuating their activity. Currently, proteolysis targeting chimera (PROTAC) technology offers an opportunity to degrade intracellular proteins by coupling small molecule inhibitors and E3 ubiquitin ligases. Compared with other antiviral strategies, a PROTAC has many advantages, including cell membrane permeability and high stability, and can be repeatedly used in cells. Similar to PROTAC technology, hydrophobic tagging of the HaloTag protein has also been employed to degrade target proteins.\(^{43}\) However, the current application of these technologies has been mainly focused on cancer research.

The discovery of new, effective targets associated with herpesvirus progression is a matter of urgency. The heparanase (HPSE), a host enzyme, is upregulated through NF-kB and translocated to the cell
surface upon HSV-1 infection for the removal of cell surface heparan sulfate (HS) to facilitate viral release, it may be a potent target for inhibiting viral release.17,18 In order to better understand the infection mechanism of the herpesvirus and develop more antiviral drug targets, more attention to host proteins is necessary. To improve screening efficiency, sensitive in vitro and in vivo evaluation systems are required; for instance, Rui et al. developed a cell culture-based green fluorescent protein reporter system for determining EBV drug sensitivity.19 Employing known effective small molecules to identify targets by immunoprecipitation, surface plasma resonance and other technologies are also important tools.

In this review, we highlight the current methodologies and strategies including small molecular drugs, RNA interference and CRISPR/Cas9 system-based antiviral approaches that impact on multiple aspects of virus such as entry, replication and assembly and hope to inform present and future drug development against herpes viruses.

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H.D., Z.W., D.Z., X.L., and Y.Z. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest
The authors declare no conflict of interest.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvec.2021.100047.

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