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Potential of protein kinase inhibitors for treating herpesvirus associated disease

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Abstract

Herpesviruses are ubiquitous human pathogens that establish life-long persistent infections. Clinical manifestations range from mild self-limiting outbreaks such as childhood rashes and cold sores to the more severe and life threatening outcomes of disseminated infection, encephalitis and cancer. Nucleoside analog drugs that target viral DNA replication provide the primary means of treatment. However, extended use of these drugs can result in selection for drug resistant strains, particularly in immunocompromised patients. In this review, we will present recent observations about the participation of cellular protein kinases in herpesvirus biology and discuss the potential for targeting these protein kinases as well as the herpesvirus encoded protein kinases as an anti-herpesvirus therapeutic strategy.

Keywords

herpesvirus; protein kinase; phosphorylation; protein kinase inhibitor; antiviral drug development

Herpesviruses and human disease

Herpesviruses are large double-stranded DNA viruses with genomes ranging in size from 125 kb to 245 kb. There are three sub-families; the alpha-, beta- and gamma-herpesviruses. The eight human herpesviruses discovered to date are the alphaherpesviruses herpes simplex 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV); the betaherpesviruses human cytomegalovirus (HCMV), human herpesviruses six (HHV-6) and human herpesvirus 7 (HHV-7); and the gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi sarcoma associated herpesvirus (KSHV) [1] (Table 1). Herpesviruses establish life-long infections in their host. Diseases associated with these viruses can arise upon primary infection (e.g. infectious mononucleosis and chickenpox), upon reactivation of the virus from an established latent infection (e.g. shingles and cold sores) or, in the case of gammaherpesvirus associated cancers, as a consequence of the action of viral latency proteins in concert with co-factors such as genetic alterations in the infected cell or suppression of the host immune system (Table 1). The predominant drugs used to treat herpesvirus infections are nucleoside analog prodrugs that are activated inside the cell by the

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herpesvirus encoded thymidine kinase or serine/threonine protein kinases (Table 2). The viral serine/threonine protein kinases have a dual specificity, and can phosphorylate nucleoside analogs as well as cellular and viral proteins. Since these viral protein and thymidine kinases are expressed only during the herpesvirus lytic replicative cycle, the nucleoside analog drugs are not effective against latent herpesvirus infections or against the cancers associated with a latent herpesvirus infection. Drug-resistant herpesvirus strains evolve in response to the selective pressure applied by nucleoside analog drug treatment, and these resistant herpesviruses carry mutations in the viral thymidine kinase, viral protein kinase and, less frequently, viral polymerase. Given the limited number of alternative treatment options, there is interest in exploring new classes of therapeutic agents.

Cellular protein kinases that impact the herpesvirus life cycle

There is an abundance of evidence that host cellular protein kinases, and the downstream pathways that they control, play a critical role in herpesvirus infections. Cellular protein kinases may therefore represent novel antiviral therapeutic agents. An inherent attraction of such a strategy is that inhibitors for a variety of cellular protein kinases are already available and are being studied in clinical trials targeting other diseases; more than 2,900 trials using protein kinase inhibitors are listed in <http://clinicaltrials.gov>. However, the targeting of a cellular protein kinase with a broad tissue expression does require that there is a distinct therapeutic window between the impact of the protein kinase inhibitor on the herpesvirus infected cells and any deleterious effects on uninfected cells. Suppression of virus replication by a number of small molecule inhibitors of cellular protein kinases has been demonstrated in culture-based assays and several cell protein kinase inhibitors have already been incorporated into clinical trials examining their efficacy for the treatment of cancers that include herpesvirus associated malignancies. The protein kinases studied to date and the antiviral effects of their inhibition are described below.

Cyclin-dependent kinases (CDKs)

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases that regulate the cell cycle, apoptosis, gene expression and differentiation [2]. The human genome encodes 13 putative CDKs and 25 putative cyclins, the regulatory subunits of the CDKs [3]. Dysregulation of cell cycle control by CDKs is often associated with cancer, a circumstance that has stimulated the search for pharmacological inhibitors of these protein kinases [4]. CDK inhibitors display antiviral activity *in vitro* at concentrations that have been found safe in clinical trials against cancer.

Roscovitine, which inhibits CDKs 1, 2, 5 and 7, was first shown to inhibit HCMV DNA replication and production of progeny virus at 10 μ M concentration in cultured human lung fibroblasts [5] and at 25-50 μ M in human foreskin fibroblasts [6]. The CDK inhibitors olomoucine and flavopiridol have similar effects on HCMV replication [5, 6]. CDK activity is required at multiple steps in HCMV infection, including the expression, modification and localization of viral proteins [7].

HSV replication is inhibited by the CDK inhibitors roscovitine, purvalanol and olomoucine [8, 9]. No roscovitine- or olomoucine-resistant mutants were isolated in that study indicating that targeting the cellular protein kinase raised the barrier for the development of anti-herpesvirus drug resistance [8]. Transcription of HSV immediate-early and early genes, HSV DNA replication, and reactivation from latency were inhibited by roscovitine [10, 11]. Olomoucine II, a subsequent derivative of roscovitine, also inhibits the replication of both HSV and HCMV [12].

In 2004, two reports showed that CDK inhibitors inhibit EBV lytic reactivation [13] and VZV infection [14]. Roscovitine and another CDK inhibitor, purvalanol A, inhibited the expression of EBV immediate-early and early genes, expression of the VZV major transactivator IE62 as well as VZV DNA synthesis [14, 15]. Roscovitine also has the potential to interfere with maintenance of latent infection. Roscovitine affected the phosphorylation and subcellular localization of the EBV EBNA1 protein, which is required for replication of the latent episomal form of the EBV genome [16]. In the case of VZV, roscovitine altered the phosphorylation and sub-cellular localization of IE63 which has a role during initial infection and in latency maintenance [17].

Indirubin-3'-monoxime, a derivative of the Chinese medicine indirubin, exhibits strong CDK inhibition [18]. Indirubin-3'-monoxime inhibits the production of HCMV progeny virus and is synergistic when combined with the HCMV UL97 protein kinase inhibitor maribavir/1263W94 [19].

There was no detectable cytotoxicity on non-infected cells treated with the CDK inhibitors described above at doses that were effective against viral infection. Roscovitine, olomoucine, olomoucine II and purvalanol A inhibit only a subset of CDKs and are therefore oligo-specific CDK inhibitors. Flavopiridol and indirubin-3'-monoxime are pan-specific CDK inhibitors that target all CDKs without preference. In addition, flavopiridol and indirubin-3'-monoxime also inhibit GSK3 β with 50% inhibition concentrations (IC₅₀) comparable to those of CDKs. The IC₅₀ of roscovitine, olomoucine, purvalanol A, flavopiridol, and indirubin-3'-monoxime against a panel of protein kinases is summarized in the review of Schang *et al.* [20].

Flavopiridol (alvocidib), the first CDK inhibitor to enter the clinic, has been used in Phase I/II clinical trials to treat patients with relapsed, refractory lymphoma. A subset of these patients had EBV-associated lymphoma (Table 3). Roscovitine (seliciclib) has also recently been tested in clinical trials for EBV-associated nasopharyngeal carcinoma in Singapore [21].

Ephrin receptor tyrosine kinase A2 (EphA2)

The ephrin (Eph) receptor tyrosine kinase family comprises at least fourteen receptors and nine ligands. Eph receptor tyrosine kinases, together with their membrane anchored ligands, ephrins, transduce bidirectional signals at sites of cell-cell contact and affect multiple oncogenic signaling pathways [22]. EphA2 functions as a cellular receptor for KSHV and EphA2-mediated signaling is critical for KSHV infection [23, 24]. The KSHV glycoproteins H and L (gH-gL) bind to EphA2 triggering EphA2 phosphorylation and endocytosis and KSHV entry into the cell. Dasatinib, an ATP-competitive tyrosine kinase inhibitor that inhibits multiple tyrosine kinases including EphA2, BCR-ABL, Src, c-Kit and platelet-derived growth factor receptor (PDGFR) with IC₅₀ of 0.5 nM-80 nM, significantly reduces KSHV infection when cells are pre-treated prior to exposure to virus. EphA2 inhibitors therefore have promise as anti-KSHV agents. Dasatinib has been used in a Phase I clinical trial to treat patients with lymphoma, including EBV-associated lymphoma (Table 3).

PDGFR and vascular endothelial growth factor (VEGF) receptor tyrosine kinases

Two types of PDGFRs have been identified; alpha and beta. PDGFRs regulate cell proliferation and differentiation and dysregulated signaling occurs in a variety of cancers [25]. PDGFR-alpha plays a critical role in HCMV infection [26]. HCMV glycoprotein B binds to PDGFR-alpha and promotes tyrosine phosphorylation of the receptor. Blockade of PDGFR with anti-PDGFR-alpha antibody or targeted inhibition of its protein kinase activity with gleevec (imatinib mesylate), a small molecule inhibitor of multiple tyrosine kinases

including PDGFR, BCR-ABL and c-Kit with IC_{50} of 100 nM - 600 nM, inhibited HCMV entry into cells and HCMV gene expression in human epithelial, endothelial and fibroblast cells [26]. Sorafenib (BAY43-9006), an inhibitor of multiple tyrosine and serine/threonine protein kinases including PDGFR, VEGFR and Raf (IC_{50} of 6 nM-90 nM), was found to inhibit HCMV major immediate-early promoter activity and HCMV immediate-early protein expression with therapeutic indices [TI; TI = 50% of cell viability (CC_{50})/ IC_{50}] of 6-15 in five different cell lines [27].

The mammalian VEGFR family consists of three receptors VEGFR1 (also known as FLT1), VEGFR2 (also known as KDR) and VEGFR3 (also known as FLT4). In response to ligand binding, the VEGFR tyrosine kinases activate a network of downstream signaling pathways involved in vasculature formation and angiogenesis [28]. Gleevec (imatinib mesylate), Sorafenib (BAY 43-9006) and SU5416 (a VEGFR tyrosine kinase inhibitor) have been used in Phase I/II clinical trials treating KSHV associated cancer (Table 3) [29].

Brunton's tyrosine kinase (Btk)

Brunton's tyrosine kinase (Btk), a non-receptor type cytoplasmic tyrosine kinase and member of the Tec family of protein kinases, plays a critical role in B cell development, differentiation and signaling. Mutations in the Btk gene result in X-linked agammaglobulinemia. In the B cell antigen receptor (BCR) signaling pathway, BCR aggregation activates the protein tyrosine kinases Lyn and Syk, which in turn activate Btk. Phosphatidylinositol (3,4,5)-triphosphate (PIP3) binding to the PH domain of Btk induces Btk to phosphorylate phospholipase C, which in turn hydrolyzes phosphatidylinositol diphosphate (PIP2) into two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG) [30].

The EBV latent membrane protein 2A (LMP2A) has been implicated in the modulation of BCR signaling through its interactions with the downstream protein tyrosine kinases Syk, Lyn and Btk. In this way, LMP2A alters B cell development and enhances B cell survival [31]. Targeting Btk therefore has potential for the treatment of EBV associated cancers. PCI-32765 (a potent and highly selective Btk inhibitor with IC_{50} of 0.5 nM) has entered Phase III trials for patients with chronic lymphocytic leukemia and B cell lymphoma, including EBV-associated lymphoma (Table 3) [32].

Aurora A protein kinase

Aurora A is a serine/threonine protein kinase that has a key role in mitosis. Dysregulation of Aurora A protein kinase has been implicated as an oncogenic driver in several cancers, including EBV and KSHV associated tumors [33, 34]. A recent study found that the KSHV encoded latency associated nuclear antigen LANA enhances Aurora A expression and that knockdown of Aurora A induces cell death in KSHV-positive primary effusion lymphoma cells [33]. MLN8327, a highly selective Aurora A inhibitor with IC_{50} of 1.2 nM, is in Phase I/II trials for treating relapsed or refractory B cell lymphomas, including EBV-associated lymphoma (Table 3).

Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is the catalytic subunit of the mTORC1 and mTORC2 complexes. mTOR is a serine/threonine protein kinase that plays an important role in mRNA translation. Rapamycin blocks mTORC1 protein kinase activity by binding to its intracellular receptor FKBP12 with IC_{50} of ~0.1 nM. The rapamycin-FKBP12-mTOR complex prevents the association of mTOR and Raptor, an essential component of the mTORC1 complex. Viruses manipulate the cellular translational machinery to support virus replication. The HCMV-mediated mTOR activation that is induced late in infection is

essential for efficient HCMV protein synthesis and virus production in human macrophages as shown by the suppressed replication seen in rapamycin treated cells [35]. In a murine model of EBV-associated Burkitt lymphoma, rapamycin significantly decreased tumor growth, splenomegaly and metastasis of tumor cells to the bone marrow of tumor recipients, possibly by blocking the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway mediated by the EBV encoded latent membrane protein 2A (LMP2A) [36, 37].

Treatment of cells with Torin 1, a new class of mTOR inhibitor with IC_{50} of 2-10 nM that directly targets the catalytic site of the mTOR protein kinase, inhibits the phosphorylation of rapamycin sensitive and rapamycin resistant mTOR targets. Torin 1 markedly inhibits the replication of HCMV, HSV-1 and murine gamma herpesvirus 68 (MHV68) and the inhibition of virus replication is dose dependent and does not result from cellular toxicity [38]. Most excitingly, rapamycin and its derivatives, everolimus and CCI-779 (temsirolimus), have been used in Phase II clinical trials to treat EBV- and KSHV-associated cancers (Table 3) [39]. Everolimus is also in a Phase II clinical trial to treat HCMV disease in renal transplant patients (Table 4).

Ataxia telangiectasia mutated (ATM)

ATM is a serine/threonine protein kinase that is central to the DNA damage response. ATM is recruited and activated in the presence of DNA double-strand breaks. ATM then phosphorylates several key downstream proteins to initiate the DNA damage checkpoint, leading to cell cycle arrest and DNA repair or, if damage is too severe, to apoptosis [40]. The ATM-induced DNA damage response has been incorporated into multiple aspects of the viral replication cycle [41]. KU55933 (a potent and specific ATM inhibitor with IC_{50} of 13 nM) and caffeine (an inhibitor of phosphoinositide 3-like kinases including ATM) have been shown to inhibit the replication of EBV, HCMV and HSV (Table 4). KU55933 shows low toxicity to cells at the doses used to inhibit viral replication. Inhibition of the ATM downstream effector Chk2 by Chk2 inhibitor II also inhibits HSV replication (Table 4).

AMP-activated protein kinase (AMPK)

The cellular AMPK consists of three subunits: AMPK α , AMPK β and AMPK γ . AMPK senses the energy status of the cell and has an important role in metabolism, both in energy producing catabolic processes and energy consuming anabolic processes. Consequently, it is not surprising that viruses have evolved to manipulate AMPK to foster their replication. Two recent studies demonstrated that HCMV infection induces AMPK activity with the resulting activation of downstream pathways [42, 43]. Inhibition of AMPK by AMPK knockdown or a non-specific small molecule inhibitor, Compound C, reduced the production of infectious HCMV virions. Compound C treatment is not toxic to MRC-5 fibroblasts up to the concentration of 5 μ M that was used to inhibit HCMV replication. Inhibition of the calcium-calmodulin-dependent kinase kinase (CaMKK), a protein kinase known to activate AMPK, by a highly selective, potent, ATP-competitive inhibitor STO-609 also blocked HCMV mediated AMPK activation and HCMV virus production [42-44] (Table 4).

Other cellular protein kinases

Casein kinase 2 (CK2) participates in EBV and HSV replication by phosphorylating viral proteins to facilitate their activities and by modifying cell protein activities in ways that facilitate virus replication and cell survival. In EBV, CK2 phosphorylation of the immediate-early lytic regulators Zta and Mta is important for their activities while EBNA1 recruitment of CK2 to promyelocytic leukemia (PML) nuclear bodies leads to PML dispersal [45-47]. Inhibition of CK2 synergizes with interferon beta in blocking HSV-1 replication without changing cell viability in mock-infected cells [48] (Table 4).

The p90 ribosomal S6 kinases (RSKs) are activated during KSHV primary infection as well as during reactivation from latency. Treatment of cells with an RSK inhibitor at a non-cytotoxic dose, suppresses KSHV lytic gene expression and progeny virion production [49] (Table 4). The RSK inhibitor BRD 7389 also induces a decrease in KSHV LANA protein levels and a loss of primary effusion lymphoma cell viability, suggesting that RSKs also have a role in the maintenance of KSHV latency [50].

c-Jun N-terminal kinase (JNK) is a mitogen-activated protein kinase (MAPK) family member that phosphorylates and activates the transcription factor c-Jun. The broad-spectrum JNK inhibitor SP600125 blocks c-Jun phosphorylation and VZV replication at a concentration that does not have visible effects on cell viability [51].

Goswami *et al.* [52] tested 80 protein kinase inhibitors for their effect on EBV lytic reactivation and found that U-0126, wortmannin and BAY11-7082, inhibitors of MEK, PI3K and IKK (inhibitor of $\text{I}\kappa\text{B}$ kinase), respectively, inhibited EBV replication with a high TI of 13. Kenpaullone (inhibitor of GSK-3 β and several CDKs) and K252A (a pan-specific protein kinase inhibitor that also inhibits the EBV protein kinase BGLF4) have a medium TI of 8 and 6, respectively. In addition, the PI3K selective inhibitors LY294002 and wortmannin inhibit HCMV-induced cell signaling and viral replication, however, the cytotoxicity effects were not examined (Table 4).

Protein kinase inhibitors have a propensity to inhibit multiple targets. Recently, a comprehensive study of protein kinase inhibitor selectivity was performed using 38 protein kinase inhibitors across a panel of 317 human protein kinases. Flavopiridol, sorafenib and dasatinib were found to bind a large number of kinases at concentrations required for potent inhibition of their primary targets. In contrast, roscovitine and gleevec (imatinib mesylate) showed greater binding specificity at the concentrations required for potent inhibition of their primary targets [53].

To summarize, cellular protein kinases play an important role during the course of viral infection. Pharmacological inhibition of serine/threonine protein kinases (such as CDKs, mTOR, aurora A, ATM, Chk2, AMPK, CaMKK, MEK, PI3K, IKK, CK2, RSKs and JNK) or tyrosine kinases (such as EphA2, PDGFR and VEGFR) suppresses herpesvirus infection in cell culture. Furthermore, an mTOR inhibitor has been found to be effective against HCMV disease in renal transplant patients.

Herpesvirus protein kinases

In addition to cellular protein kinases, the protein kinases of the herpesviruses themselves are also attractive drug targets. Herpesviruses express two types of serine/threonine protein kinases: the conserved herpesvirus protein kinases (CHPKs) encoded by all herpesviruses, namely HSV UL13, VZV ORF47, HCMV UL97, HHV-6 U69, EBV BGLF4 and KSHV ORF36 and the protein kinases encoded only by the alphaherpesviruses, namely HSV US3 and VZV ORF66 [54]. The CHPKs have a substrate specificity that overlaps with the cellular cyclin dependent kinase CDK1 [55], CDK2, and partially CDK4 and 6. However, unlike the CDKs, the CHPKs do not require a cyclin partner for activity. The substrate specificity of the alphaherpesvirus US3-like protein kinases is similar to that of cellular protein kinase A (PKA) [56]. The precise substrate recognition motif remains incompletely defined for both the CHPKs and the US3-like protein kinases. These viral protein kinases participate at multiple steps in the herpesvirus life cycle.

The alphaherpesvirus protein kinase US3 encoded by HSV-1/2 blocks host cell apoptosis by targeting BCL2-associated agonist of cell death (BAD) and programmed cell death protein 4 (PDCD4) [57, 58]. US3 also activates mTORC1 by directly phosphorylating tuberous

sclerosis complex 2 (TSC2) and hence stimulating translation and virus replication [59]. VZV protein kinase ORF66 is required for efficient virus replication in primary human corneal stromal fibroblast cells [60]. ORF66 contributes to the chromatin remodeling that is required for VZV infection by inducing hyperphosphorylation of histone deacetylase (HDAC) 1/2 [61].

EBV BGLF4 and KSHV ORF36 manipulate cell factors to promote viral gene expression [62, 63]. EBV BGLF4 and HCMV UL97 mimic CDK activity to regulate cell cycle progression and create a pseudo S-phase environment for viral DNA replication [64-66]. HSV UL13, VZV ORF47, HCMV UL97, EBV BGLF4 and KSHV ORF36 interfere with the host innate immune response by inhibiting the function of interferon regulatory factor 3 [67-69]. HCMV UL97, EBV BGLF4, HSV UL13 and KSHV ORF36 play an important role in virus nuclear egress by phosphorylating lamin A/C to disassemble the nuclear lamina [70-73]. CHPKs also facilitate viral DNA replication by inducing a host DNA damage response through TIP60-mediated activation of ATM [62, 74, 75]. The importance of this pathway for herpesvirus replication is shown by the dramatic suppression of EBV and HCMV replication seen in cells treated with an ATM inhibitor [62, 76, 77].

The potential for targeting the CHPKs as an antiviral strategy has led to attempts to identify inhibitors of their protein kinase activity and potential protein kinase inhibitors are reviewed in Gershburg *et al.* [54]. The HCMV UL97 protein kinase inhibitor maribavir/1263W94 is the only herpesvirus protein kinase inhibitor to reach Phase III clinical trials (ClinicalTrials.gov Identifier: NCT00411645) [78]. In this study, maribavir did not prevent HCMV disease in a setting in which drug treatment was initiated subsequent to stem cell transplantation. One possible explanation is that the dose of maribavir used in these trials was surprisingly low (100 mg twice daily). Recently new Phase II clinical trials that test the higher doses of 400, 800 and 1,200 mg twice daily have been initiated (ClinicalTrials.gov Identifier: NCT01611974).

Concluding remarks

The human kinome comprises 518 putative protein kinases [79]. RNA interference (RNAi) screening suggests that over 100 cellular protein kinases influence the growth of HCMV. Knockdown of 77 of those protein kinases inhibited HCMV replication [42]. Extrapolating these numbers to the other herpesviruses leads to the prediction that approximately 10% of human protein kinases could potentially be targeted to treat herpesvirus associated diseases. Herpesviruses themselves each encode a conserved protein kinase that, along with the alphaherpesvirus specific protein kinases, also represent attractive drug targets. These viral and cellular protein kinases participate at multiple stages of the viral life cycle from virus entry to viral gene expression, viral DNA replication, nuclear egress and progeny virus release (Figure 1). However, in most cases, the exact mechanisms by which these protein kinases regulate the viral life cycle are poorly understood. Furthermore, the same protein kinase might play different roles in different cell backgrounds or different stages of viral infection. For example, the ATM/Chk2-mediated DNA damage response is a robust host antiviral response that occurs upon primary EBV infection and limits the establishment of EBV latency [80]. However, ATM activity is critical for EBV lytic gene expression, DNA replication and reactivation [62, 76]. Therefore, the differential contribution of protein kinases in primary infection, lytic reactivation or viral latency needs to be considered.

New methods are required to pinpoint the key protein kinases critical for the different stages of the viral life cycle, and physiologically relevant animal models will be needed to evaluate the antiviral efficacy of targeting these protein kinases with small molecule inhibitors. Potential approaches and strategies include:

RNAi screening of the human kinome

High-throughput RNAi screening has been successfully applied to study the contribution of protein kinases on HCMV infection and KSHV reactivation [42, 81]. The same strategy can be applied to study the function of host protein kinases in other herpesvirus infections. The disadvantages of RNAi screening are that the strategy is expensive and labor-intensive. Further, effective protein kinase knock-down usually requires more than 24 hours of transfection with the appropriate siRNAs, a factor that can result in secondary down-regulation of other protein kinases and complicate interpretation of the results.

Protein kinase inhibitor screening

With the commercial availability of protein kinase inhibitor libraries (containing around 200 protein kinase inhibitors), it is also feasible to perform protein kinase inhibitor screens to identify those with anti-herpesvirus potential. However, such screens are complicated by the choice of infection system as different cell backgrounds may influence the observed effectiveness, treatment concentration or toxicity. Additionally, off-target effects should also be kept in mind since protein kinase inhibitors frequently affect the activity of multiple kinases when used at the concentrations required for potent inhibition of their primary targets.

Quantitative mass spectrometry analysis

In order to identify the key cellular protein kinases critical for the viral life cycle, it will be necessary to globally understand the phosphorylation events that occur upon viral infection, upon lytic reactivation or during the establishment of latency. Stable isotope labeling by amino acids in cell culture (SILAC) coupled with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis has been widely used to dissect cellular signaling pathways [82]. This technology can be applied to investigate the host signaling pathways manipulated by herpesviruses. Multiplex SILAC based strategies will further provide the temporal analysis of host protein phosphorylation during the course of viral infection, which will facilitate the identification of key protein kinases critical at different stages. Target inhibition by RNAi knockdown or specific protein kinase inhibitors will allow the function of these potentially key protein kinases in the viral life cycle to be elucidated and will provide a rationale for anti-viral drug development.

Pre-clinical animal models

Laboratory mice are good models for HSV-1/2 replication and pathogenesis [83]. Human dorsal root ganglia (DRGs) xenografts in immunodeficient mice provide a good model for evaluating HSV-1 and VZV neuropathogenesis [84, 85]. The efficacy of small molecule inhibitors against HSV-1/2 and VZV can also be tested using these mouse models [86]. Nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice can be stably engrafted with human CD34+ hematopoietic stem cells. These humanized mice develop a largely intact human immune response and have been used to study EBV and HCMV infections [87, 88]. The humanized mouse model will also be valuable in evaluating protein kinase inhibitors for the treatment of herpesvirus infections. Wide application of the humanized mouse model is challenging because of the specialized manipulations needed for their creation.

Lytic induction combined with nucleoside analogs for treatment of herpesvirus-associated cancer

A novel strategy for treatment of herpesvirus-associated cancer involves the induction of the viral lytic phase combined with nucleoside analogs. Lytic induction allows the expression of the herpesvirus encoded thymidine kinases and protein kinases. These viral thymidine and

protein kinases are required for the phosphorylation of nucleoside analogs, such as ganciclovir and [¹³¹I]2'-fluoro-2'-deoxy-beta-D-5-iodouracil-arabinofuranoside ([¹³¹I]FIAU). The cytotoxic property of phosphorylated ganciclovir is utilized to kill the tumor cells that express viral thymidine or protein kinases [89]. The phosphorylation of [¹³¹I]FIAU leads to its specific enrichment in tumor tissue and subsequently the [¹³¹I]FIAU kills lytically induced and surrounding tumor cells by beta emissions [90].

In summary, as we discussed above, a number of protein kinase inhibitors have already been given to patients in a cancer clinical trial setting. As more information becomes available, it will become important to stratify the patients according to their infection status to determine whether the outcome of protein kinase inhibitor treatment is more or less beneficial for the herpesvirus-associated diseases. Advances in high-throughput screening technologies combined with bioinformatics will provide the detailed information base needed to integrate the complexity of protein kinase signaling networks into herpesvirus biology to identify those protein kinases whose inhibition has the greatest therapeutic potential. With these applications, the humanized mouse model and clinical evaluation, more protein kinase inhibitors may be incorporated into the repertoire of anti-herpesvirus agents. The success of mTOR inhibitors in reducing HCMV disease in transplant patients [91, 92] will further encourage the application of protein kinase inhibitors for the treatment of herpesvirus infections.

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Highlights

- Cellular protein kinases are anti-herpesvirus drug targets.
- Herpesvirus protein kinases are anti-herpesvirus drug targets.
- Directions for exploring protein kinases in antiviral therapy are given.

Figure 1. Protein kinases involved in the herpesvirus life cycle

1. Viral glycoproteins bind to host cell receptors on the cell membrane (tyrosine kinases PDGFR and EphA2 are the receptors for HCMV and KSHV, respectively). 2. Viral envelope fuses with the cell membrane and releases the nucleocapsid into the cytoplasm. 3. The nucleocapsid associates with cytoskeletal elements and translocates to the nucleus where the nucleocapsid interacts with nuclear pores and releases the viral genome into the nucleus. 4. The viral DNA is transcribed into early mRNAs which are then transported to the cytoplasm for early protein synthesis. 5. These early proteins are imported into the nucleus and promote viral DNA replication. 6. The viral DNA is then transcribed into the late mRNAs which are responsible for late viral protein synthesis (capsid and envelope proteins). [a-d. The viral envelope proteins (pink, green and blue circles) are processed in the endoplasmic reticulum (ER) and Golgi complex]. 7. The capsid proteins are imported into the nucleus and then encapsidate the newly replicated genomes. 8-10. These capsids egress from the nucleus and bud into the exocytotic vesicles. 11. The virion containing vesicles migrate to and fuse with the cell membrane. 12. Infectious viruses are then released from the cell [1]. Viral (black circle) and cellular (red circle) kinases discussed in this review are shown in the cell compartment most likely to be relevant to their impact on the viral life cycle (steps 2-9). The viral and cellular kinases involved in steps a-d and 10-12 have yet to be identified.

Table 1

Human herpesvirus classification

Subfamily	Formal name	Common name	Pathophysiology
Alpha(α)	Human herpesvirus 1 (HHV-1)	Herpes simplex virus 1 (HSV-1)	Gingivitis, cold sores, genital herpes, encephalitis, ocular infections, neonatal infections, herpes gladiatoris, disseminated infections in neonates or immunocompromised individuals
	Human herpesvirus 2 (HHV-2)	Herpes simplex virus 2 (HSV-2)	Cold sores, genital herpes, encephalitis, ocular infections, neonatal infections, herpes gladiatoris, disseminated infections in neonates or immunocompromised individuals
	Human herpesvirus 3 (HHV-3)	Varicella zoster virus (VZV)	Chickenpox, shingles
Beta(β)	Human herpesvirus 5 (HHV-5)	Human cytomegalovirus (HCMV)	Birth defects, infectious mononucleosis-like syndrome, retinitis, colitis, pneumonitis, esophagitis, encephalitis, infections in transplant patients
	Human herpesvirus 6 (HHV-6)		Exanthem subitum (roseola infantum or sixth disease)
	Human herpesvirus 7 (HHV-7)		Exanthem subitum (roseola infantum or sixth disease), lower incidence than HHV-6
Gamma(γ)	Human herpesvirus 4 (HHV-4)	Epstein-Barr virus (EBV)	Infectious mononucleosis, Burkitt's lymphoma, central nervous system lymphoma in AIDS patients, Hodgkin's disease, post-transplant lymphoproliferative syndrome (PTLD), nasopharyngeal carcinoma, HIV-associated hairy leukoplakia, NK/T cell lymphoma
	Human herpesvirus 8 (HHV-8)	Kaposi's sarcoma-associated herpesvirus (KSHV)	Kaposi's sarcoma, primary effusion lymphoma, multicentric Castlemann's disease

Table 2

Drugs for treatment of herpesvirus infections

Classes	Name	Mechanism of action
Nucleoside analogs	Acyclovir/valacyclovir Ganciclovir/valganciclovir Penciclovir/famciclovir Vidarabine Idoxuridine Trifluridine Edoxudine Brivudine Cytarabine Cidofovir	DNA synthesis inhibitor, upon phosphorylation by the herpesvirus encoded thymidine kinases and/or the conserved herpesvirus protein kinases these drugs competitively inhibit the incorporation of dNTPs by the viral DNA polymerase resulting in the termination of elongation of viral DNA
Pyrophosphate analog	Foscarnet	DNA synthesis inhibitor, selectively interferes with pyrophosphate binding by the viral DNA polymerase
Oligonucleotide	Fomivirsen	21 nucleotide sequence complementary to the immediate early region 2 of HCMV mRNA
Saturated fatty alcohol	Docosanol	Potentially inhibits fusion of the viral envelope with the plasma membrane of the host cell [93]
Adamantane analog	Tromantadine ^a	Inhibits penetration of the virus

^aTromantadine or Tromantadine hydrochloride is reported as an ingredient of Viru-Merz in the following countries: Peru, Austria, Bulgaria, Costa Rica, Czech Republic, Dominican Republic, El Salvador, Germany, Guatemala, Honduras, Hungary, Indonesia, Israel, Latvia, Malaysia, Malta, Nicaragua, Panama, Poland, Portugal and Slovakia.

Table 3

Protein kinase inhibitors in clinical trials that include patients with herpesvirus associated cancers

Drug	Primary targets	Virus	Status/ClinicalTrials.gov Identifier
Flavopiridol (alvocidib)	Cyclin-dependent kinases (CDKs)	EBV	Phase I/II NCT00112723 NCT00445341
Roscovotine (seliciclib)	CDKs	EBV	[21]
Dasatinib	Inhibits multiple tyrosine kinases [Break point cluster - abelson tyrosine kinase (BCR-ABL), Src, c-Kit, Ephrin receptor tyrosine kinase A2 (EphA2)]	EBV	Phase I NCT00608361
Gleevec (imatinib mesylate)	Inhibits multiple tyrosine kinases [BCR-ABL, c-Kit, Platelet-derived growth factor receptor (PDGFR)]	KSHV	Phase II NCT00090987
Sorafenib (BAY 43-9006)	Vascular endothelial growth factor receptor (VEGFR), PDGFR and Raf kinases	KSHV	Phase I NCT00287495
SU5416	VEGFR tyrosine kinase	KSHV	Phase II NCT00002226 NCT00005931
PCI-32765	Bruton's tyrosine kinase (Btk)	EBV	Phase II/III NCT01109069
MLN8237	Aurora A kinase	EBV	Phase I/II NCT00807495 NCT01397825
Rapamycin (sirolimus)	Mammalian target of rapamycin (mTOR)	KSHV	Phase I NCT00450320
Everolimus	mTOR	EBV	Phase I/II NCT00918333 NCT01075321
CCI-779 (temsirolimus)	mTOR	EBV	Phase II NCT00290472 NCT01076543

Table 4

Cellular protein kinase inhibitors tested for anti-herpesvirus efficacy

Drug	Primary targets (IC ₅₀ , nM) ^a	Virus	Status ^b	Refs
Roscovitine	CDKs (200-700)	EBV, HSV, VZV and HCMV	Phase I for advanced solid tumors	[5-8, 10, 11, 13-17, 94, 95]
Purvalanol A	CDKs (4-75)	EBV, HSV and VZV	Research	[9, 13, 15]
Olomoucine II	CDKs (20)	HCMV and HSV	Research	[12]
Indirubin-3'-monoxime	CDKs (5-500), GSK3 β (22)	HCMV	Research	[19]
Dasatinib	Inhibits multiple tyrosine kinases [BCR-ABL (0.6), Src (0.8), c-Kit (79), EphA2(17)]	KSHV	Imatinib-resistant chronic myeloid leukemia	[23, 24]
Gleevec (imatinib mesylate)	Inhibits multiple tyrosine kinases [BCR-ABL (600), c-Kit (100), PDGFR (100)]	HCMV	Chronic myeloid leukemia, gastrointestinal stromal tumors	[26]
Sorafenib (BAY 43-9006)	(90), PDGFR (57) and Raf kinases (6-22)	HCMV	Renal cell carcinoma	[27]
Everolimus	mTOR (1.6-2.4)	HCMV ^c	Advanced kidney cancer, prevention of organ rejection after renal transplant, breast cancer, pancreatic cancer	[35, 91, 92]
Torin1	mTOR (2-10)	HCMV and HSV	Research	[38]
KU55933	Ataxia telangiectasia mutated (ATM) (13)	EBV, HCMV and HSV	Research	[62, 76, 77, 96]
Chk2 inhibitor II	Chk2 (15)	HSV	Research	[96]
Compound C	AMP-activated protein kinase (AMPK) (235), VEGFR (25), BMPR-I (148)	HCMV	Research	[42, 43]
STO-609	Calmodulin-dependent kinase kinase (CaMKK) (100-300)	HCMV	Research	[42-44]
U-0126	MEK1/2 (58-72)	EBV and HCMV	Research	[52, 97]
Wortmannin	Phosphatidylinositol-3-kinase (PI3K) (5)	EBV	Research	[52]
LY294002	PI3K (500-1,000)	HCMV	Research	[98]
BAY 11-7082	Inhibitor of κ B kinase (IKK) (10,000)	EBV	Research	[52]
Kenpauillone	GSK3 β (230), LcK (470), CDKs (400-850)	EBV	Research	[52]
K252A	Multiple protein kinases	EBV	Research	[52]
TBB	Casein kinase 2 (CK2) (900-1,600)	EBV and HSV-1	Research	[45-48]
DMAT	CK2 (140)	EBV and HSV-1	Research	[45-48]
TMCB	CK2 (250)	EBV and HSV-1	Research	[45-48]
BI-D1870	p90 ribosomal S6 kinases (RSKs) (31)	KSHV	Research	[49]
SP600125	c-Jun N-terminal kinase (JNK) (40-90)	VZV	Research	[51]

^aSources: www.selleckchem.com; www.millipore.com; [20].^bSources: www.clinicaltrials.gov; www.fda.gov; www.selleckchem.com; www.millipore.com.^cClinicalTrials.gov Identifier: NCT00828503.