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# Conserved herpesvirus protein kinases

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## Abstract

Conserved herpesviral protein kinases (CHPKs) are a group of enzymes conserved throughout all subfamilies of *Herpesviridae*. Members of this group are serine/threonine protein kinases that are likely to play a conserved role in viral infection by interacting with common host cellular and viral factors; however along with a conserved role, individual kinases may have unique functions in the context of viral infection in such a way that they are only partially replaceable even by close homologues. Recent studies demonstrated that CHPKs are crucial for viral infection and suggested their involvement in regulation of numerous processes at various infection steps (primary infection, nuclear egress, tegumentation), although the mechanisms of this regulation remain unknown. Notwithstanding, recent advances in discovery of new CHPK targets, and studies of CHPK knockout phenotypes have raised their attractiveness as targets for antiviral therapy. A number of compounds have been shown to inhibit the activity of human cytomegalovirus (HCMV)-encoded UL97 protein kinase and exhibit a pronounced antiviral effect, although the same compounds are inactive against Epstein-Barr Virus (EBV)-encoded protein kinase BGLF4, illustrating the fact that low homology between the members of this group complicates development of compounds targeting the whole group, and suggesting that individualized, structure-based inhibitor design will be more effective. Determination of CHPK structures will greatly facilitate this task.

### Keywords

herpesviruses; protein kinases; serine/threonine kinases; protein kinase inhibitors; antivirals compounds

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## 1. INTRODUCTION

Herpesviruses are among the most persistent of all pathogens owing to the fact that they co-evolved with their hosts for a long period of time and are relatively harmless in immunocompetent hosts. As opportunistic agents, herpesviruses [in particular Epstein-Barr Virus (EBV), Kaposi Sarcoma Herpesvirus (KSHV) and human cytomegalovirus (HCMV)] cause severe diseases with significant morbidity and mortality in immunocompromised patients. The population of such patients grows steadily due to AIDS, organ transplantation, cancer, and aging. Since the successful 1982 introduction of the anti-herpetic agent acyclovir, a number of anti-herpesvirus drugs have been and are currently approved [1]. However, most of these compounds, either directly or indirectly, target viral DNA polymerase (reviewed in [2]) and prolonged use of these antivirals is limited due to emergence of resistant (and cross-resistant) mutants as well as severe side effects. Hence, the combination of these factors emphasizes the need for improved therapies to treat herpesviruses-related complications, particularly by exploring new viral targets. Conserved herpesvirus-encoded protein kinases (CHPKs) are an attractive target for antiviral therapy due to (i) their role in viral infection, and (ii) their uniqueness, that is, very low sequence homology with cellular counterparts, and even among themselves, may allow development of highly selective inhibitors. In addition, development of inhibitors of protein kinases involved in the life cycle of a virus is not only of potential therapeutic interest, but can also furnish useful information about the pathways of viral gene expression and replication.

## 2. HERPESVIRUSES

Herpesviruses are enveloped viruses with virion size over 100 nm. The linear double-stranded DNA genomes (125 – 240 kb) encode up to 200 open reading frames, including a repertoire of enzymes for viral DNA replication as well as protein modifications (such as phosphorylation). DNA replication and capsid assembly occur in the nucleus. All members of *Herpesviridae* have a biphasic infection cycle consisting of latent and replicative (lytic) phases. Latency is characterized by limited gene expression, lack of virion production, and, in the case of gammaherpesviruses, is associated with immortalization and transformation of infected cells. In contrast, most of viral genes are expressed in a cascade manner during the lytic cycle, including viral reactivation, and large numbers of infectious virus particles are released [3]. Primary infection results in lifelong persistence of the virus in the host. Eight herpesviruses infect humans: alphaherpesviruses - herpes simplex virus 1 and 2 (HSV-1 and -2), and varicella-zoster virus (VZV); betaherpesviruses - human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 and -7); and gammaherpesviruses - Epstein-Barr virus (EBV) and Kaposi Sarcoma associated herpesvirus (KSHV). The differences between different subfamilies include host range, type of cells they are able to infect, and the length of the replication cycle.

## 3. CONSERVED HERPESVIRUS PROTEIN KINASES

All avian and mammalian herpesviruses encode protein kinases. A subset of these enzymes, exemplified by the HSV UL13 gene product, is conserved throughout all subfamilies of *Herpesviridae* [4,5] and will be referred to as conserved herpesvirus protein kinases (CHPKs). HSV UL13 homologues encoded by other human herpesviruses include: VZV ORF47 [6], EBV BGLF4 [7,8], HCMV UL97 [9-11], HHV-6 U69 [12], and KSHV ORF36 [13]. Putative substrates of CHPKs identified to date are summarized in Table 1, and characteristics of the individual CHPKs, as well as their common features, are discussed below.

### 3.1. Herpes simplex virus UL13

Studies of HSV mutants, in which UL13 is deleted, yielded conflicting data. While earlier studies demonstrated dispensability of UL13 for viral replication (at least in cell culture) [14, 15], later studies claimed that such mutants exhibit impaired replication in a cell type-dependent manner [16], with reduced expression levels of immediate-early ICP0 protein and a subset of late proteins, including UL26, UL26.5, UL38, UL41 and US11 [17]. A number of viral and cellular UL13 putative targets have been identified (Table 1); however, the biological significance of UL13-mediated phosphorylation remains unclear. Kato et al. [18] recently suggested UL13 may be involved in regulation of nuclear egress, based on the fact that another HSV protein kinase, US3, which regulates nuclear egress of alphaherpesviruses [19,20], is a physiological substrate for UL13, and that UL13 deletion resulted in aberrant localization of HSV egress factors UL31 and UL34 [18]. The UL13 protein has also been implicated in promoting tegument dissociation [21].

Daikoku et al. [22] purified HSV-2 UL13 from infected cells. The kinase activity of the purified protein was optimal at pH-9.0 and in the absence of NaCl. Casein, phosvitin, and, to some extent, histone, but not protamine, were efficiently phosphorylated. Phosphoamino acid analysis of phosphorylated casein revealed that UL13 phosphorylates serine and threonine residues, but not tyrosine. The UL13 kinase activity was resistant to treatment with heparin and CK I-7, inhibitors of protein kinases CK2 and CK1 respectively, but sensitive to the bioflavonoid quercetin.

### 3.2. Varicella-Zoster virus ORF47

VZV ORF47 protein is related to HSV UL13 [5]. The ORF47 protein autophosphorylates and phosphorylates the major immediate-early transactivator, IE62 [23-25], as well as IE63 [23], gE [26], ORF9 [27], and ORF32 proteins [28]. VZV gE is essential for replication [29] and requires ORF47 phosphorylation to mediate cell fusion and TGN trafficking for virion assembly [26]. The ORF47 protein is dispensable for VZV replication in vitro, as shown in studies of ROka47S, a recombinant virus described by Heineman and Cohen [30], in which ORF47 transcription was blocked by a stop codon mutation. In contrast, the ORF47 protein is essential for VZV infection of differentiated human T-cells, skin xenografts in the SCID-hu model of VZV infection in vivo [31], and immature dendritic cells [32]. Rahaus et al. [33] suggested that both VZV ORF47 and ORF66 protein kinases play key roles in activating the PI3/Akt/GSK-3α/β pathway, which plays an essential role in viral infection.

*In vitro* activity of ORF47 has been studied with the protein immunoprecipitated from VZV-infected cells [6], or recombinant protein expressed in mammalian cells [23]. It preferentially phosphorylates serine residues, utilizing both ATP and GTP as phosphate donors, and its activity is optimal in the presence of $Mn^{2+}$ (rather than $Mg^{2+}$) and 50 – 250 mM KCl [6]. Kenyon et al. [23] reported up to an 18-fold increase in autophosphorylation of ORF47 in the presence of polyamines. Conflicting data are reported as to whether ORF47 contributes to nucleoside analogue metabolism: Koyano et al. [34] studied growth inhibition by nucleoside analogues of COS cells expressing VZV-TK or ORF47, and their results suggested that ACV and BV-araU were phosphorylated by TK only, whereas GCV, OXT-G and cOXT-G were phosphorylated by both TK and ORF47 (or by cellular factors activated by ORF47). In contrast, Suzutani et al. [35], studying sensitivity of viruses deficient in TK or ORF47 genes (or combination of both) to anti-herpesvirus nucleoside analogues, showed no changes in sensitivity in the absence of ORF47 and suggested that this kinase is not involved in phosphorylation of the tested compounds.

### 3.3. Human cytomegalovirus UL97

HCMV UL97 is probably the most widely studied CHPK, both in the biochemical sense and in the context of viral infection. UL97 is the only protein in the CHPK group for which the nuclear localization signal has been identified [36], and it is also a component of the tegument in mature virions [37,38]. Studies of HCMV mutants lacking most of the UL97 gene demonstrated their severe replication deficiency in fibroblast infection, and thus confirmed the importance of the UL97 gene product for viral replication [39]. Replication of these mutants could be partially restored by expression of rat cytomegalovirus (RCMV) UL97 protein or EBV BGLF4, but not by HSV UL13 [40]. The involvement of UL97 was implicated in regulation of viral DNA synthesis [41-43], modification of cellular transcriptional and translational factors [44,45], and viral nuclear egress [43,46,47]. The connection between UL97 and viral DNA synthesis is thought to occur through its interaction with, and phosphorylation of, the HCMV DNA polymerase processivity factor UL44 [41,42], and the region responsible for this interaction has been mapped between aa 366 and aa 459 [42]. Both proteins co-localized in the viral replication centers, and treatment with inhibitors of viral DNA synthesis (cidofovir, CDV) or UL97 activity (NGIC-I and Gö6976) prevented this co-localization. However, as in the case of EBV BGLF4 and EA-D [8,48], no direct evidence has been obtained yet to connect the phosphorylation status of UL44 with its functions in viral DNA replication. UL97 has also been reported to phosphorylate elongation factor 1 delta [45] and RNA polymerase II carboxyl-terminal domain [44], but again, the physiological relevance of these modifications remains unclear. The best-substantiated claim connects UL97 with viral nuclear egress. Three research groups have independently presented evidence for accumulation of viral capsids in the nucleus in the absence of UL97 protein [43,46] and started to uncover the mechanism by which UL97, through interaction with cellular p32 protein, destabilizes nuclear lamina [47]. Recently, UL97 has been implicated in regulation of tegumentation, since its deletion resulted in aberrant aggregation of tegument proteins such as pp65 and ppUL25 [49], and changed subcellular distribution of the viral structural protein assembly sites [50].

Biochemical studies have been reported with UL97 protein expressed in heterologous systems, followed by affinity purification or immunoprecipitation. These studies demonstrated the ability of UL97 to autophosphorylate [11] and to phosphorylate certain exogenous protein substrates [51,52] as well as two nucleoside homologues, GCV and ACV [53], although UL97 does not show homology with known nucleoside kinases [36]. Phosphorylation occurred in the presence of either $Mg^{2+}$ or $Mn^{2+}$, and both ATP and GTP could be used as phosphate donors in protein phosphorylation. Unusually, the optimal activity of pUL97 was observed at 1.5M NaCl and pH-9.5 [11]. Baek et al., mapped the autophosphorylation sites within the UL97 N-terminal region and demonstrated the importance of specific amino acids (arginine or lysine) in the P+5 position from the phosphorylated residue for the efficiency of histone 2B phosphorylation. The authors speculated that HCMV UL97 might regulate gene expression by histone phosphorylation and chromatin condensation [52]. The relation between UL97 autophosphorylation and efficient phosphorylation of exogenous substrates (both protein and GCV) remains unclear, with a number of conflicting reports that either support the necessity of autophosphorylation for allophosphorylation activity [42,54] or describe autophosphorylation mutants that retain high levels of allophosphorylation activity [51].

### 3.4. Human herpesvirus 6 U69

HHV-6-encoded U69 is a homologue of HCMV UL97 [55]. The purified protein autophosphorylates and phosphorylates casein and histone (substrates that typically phosphorylated by serine/threonine protein kinases), but not endolase (a typical substrate for tyrosine protein kinases). Phosphorylation occurs predominantly on serine residues, with both ATP and GTP utilized as phosphate donors. The reaction requires both $Mg^{2+}$ and $Mn^{2+}$, and

reaches optimal kinetics at physiological pH and low NaCl concentration. In the same study, U69-expressing baculoviruses exhibited higher susceptibility to GCV in plaque reduction experiments [12] suggesting that its product is involved in GCV phosphorylation. These data were further confirmed by GCV metabolic studies in a recombinant vaccinia virus system in which both U69 and UL97 were expressed. Analyses of metabolites showed increased GCV phosphorylation in the presence of both proteins, but the level of this phosphorylation was approximately 10-fold lower in cells expressing U69 [56]. Nevertheless, U69 seemed to play a role in GCV phosphorylation, since GCV-resistant HHV-6 strains carry an M318V substitution in U69 [57,58], which corresponds to M460I and M460V substitutions in UL97, one of the most frequently observed mutations conferring GCV resistance in clinical HCMV isolates.

Similar to other CHPKs, HHV-6 U69 localized in nucleus [56], its importance for the viral infection as well as physiologically relevant targets have never been studied. Based on homology with UL97, one can predict that U69 will be crucial for viral infection, but this needs to be verified experimentally.

### 3.5. Epstein-Barr Virus BGLF4

EBV BGLF4 protein exhibits early expression kinetics with high levels throughout the EBV lytic phase [59]. It is detected mainly in the nuclei of EBV-infected cells [59,60]. The NLS, although not clearly defined, is localized on the C-terminus of the protein [59]. We have recently reported [61] that the EBV mutant phenotype, in which BGLF4 protein level was knocked down by RNAi, exhibited properties similar to a HCMV UL97 knockout mutant [39], in particular by retention of nucleocapsids in the nucleus. Moreover, we have shown that the knockdown of BGLF4 abolished the expression of BFLF2 (a homologue of HSV UL31), a viral factor that is directly involved in nuclear egress [62] thus demonstrating that BGLF4 is involved in regulation of nuclear egress. Lee et al. [63] reported that transient expression of EBV BGLF4 protein induces unscheduled chromosome condensation, nuclear lamina disassembly, and stress fiber rearrangements, independently of cellular DNA replication and cyclin-dependent protein kinase 2 activity. BGLF4 interacts with condensin complexes, the major components in mitotic chromosome assembly, and induces condensin phosphorylation at CDK1 consensus motifs. BGLF4 also stimulates the decatenation activity of topoisomerase II, suggesting that it may induce chromosome condensation through condensin and topoisomerase II activation. The authors speculate that gammaherpesvirus kinases may induce multiple premature mitotic events to provide more extrachromosomal space for viral DNA replication and successful egress of nucleocapsid from the nucleus. Finally, BGLF4 has been reported to phosphorylate components of the cellular replication origin binding MCM4-MCM6-MCM7 complex [64], and, since such a phosphorylation inhibits helicase activity of MCM4, Kudoh et al. hypothesize that BGLF4 may play a role in blocking cellular DNA replication during EBV lytic infection. The EBV BGLF4 protein is a part of the tegument [60,65] and has been shown to dissociate from it in a phosphorylation-dependent manner [65]. Although it phosphorylates a number of viral and cellular targets (Table 1), the biological relevance of EBV-BGLF4-mediated phosphorylation (except for its part in viral nuclear egress) remains hypothetical, and even when this phosphorylation has been linked to reduction of transcriptional activity for EBNA2 and EBNA-LP [66,67], its consequences in the context of viral infection have not been explored.

Recombinant EBV BGLF4 has been expressed in insect cells [68,69]. The purified protein autophosphorylates and phosphorylates histone and myelin basic protein [68,69], utilizes both ATP and GTP as phosphate donors, and requires both $Mn^{2+}$ and $Mg^{2+}$, pH-7.4 and 300 mM KCl for optimal activity [7].

In contrast to betaherpesviruses, gammaherpesviruses encode functional thymidine kinase (TK) [70-72]. When expressed in EBV-negative cells, EBV TK conferred moderate sensitivity to GCV, BVDU, ACV and PCV; in contrast, BGLF4 conferred sensitivity to GCV only [73]. Neither has been shown to directly phosphorylate GCV in vitro ([74] and Gershburg and Pagano, unpublished data).

### 3.6. Kaposi Sarcoma-associated herpesvirus (KSHV) ORF36

KSHV ORF36 protein is a serine protein kinase that is localized in the nucleus [13]. In vitro protein kinase assays indicated that this viral protein is autophosphorylated and that the lysine residue in the catalytic kinase subdomain II was essential for enzymatic activity [13]. ORF36 is transcribed as two polycistronic transcripts that are initiated from promoters that are active in the early stage of the viral life cycle and inducible by hypoxic conditions [75]. Their kinetics have been reported both as late [76,77] or early/early-late as verified by the treatment with CDV, an inhibitor of viral DNA synthesis [78]. ORF36 was found in replication/transcription complexes in infected cells and packaged in mature virions [79]. The ORF36 protein has been shown to phosphorylate components of the c-Jun N-terminal protein kinase signal transduction pathway, which in turn activated c-Jun in the activating protein 1 transcription complex [80]. Recently, Izumiya et al. [79] showed that ORF36 interacts with and phosphorylates the transcriptional regulator K-bZIP at Thr111, and both proteins are recruited to selected viral promoters as well as the Ori-Lyt region. This threonine residue of K-bZIP is also the target of the cyclin-dependent kinase CDK1. K-bZIP activity is also modified by sumoylation [81], and phosphorylation at Thr111 seem to change levels of sumoylation. The authors of the study propose a model whereby ORF36 switches K-bZIP from being a strong repressor of K-Rta, which targets immediate-early genes, to a transactivator that synergizes with K-Rta to activate early and late viral gene expression [79]. Coincidently, EBV BZLF1 gene product, the homologue of K-bZIP, is also modified by sumoylation [82] and phosphorylation [83-85]; however, the significance of sumoylation of Zta on EBV transcription or of an interplay between the sumoylation and phosphorylation remain to be studied.

In vitro kinase activity of the KSHV ORF36 has been studied on partially purified GST-fused protein expressed in mammalian cells. The kinase autophosphorylates on serine residues and prefers $Mn^{2+}$ over $Mg^{2+}$ at pH-7.5 and no salt [13].

Similar to EBV, KSHV encodes a thymidine kinase, ORF21 [74], and both ORF21 and ORF36 have been shown to confer sensitivity to GCV [86] with ORF36 being more efficient. In contrast to EBV [73], both proteins did not confer significant sensitivity to PCV and BVDU [86].

### 3.7. Common features and biological role(s) of CHPKs

CHPKs were identified based on the motifs diagnostic of conserved regions within the catalytic domains of protein kinases [4,5]. Noteworthy are that the sequences of CHPKs encoded by beta- and gammaherpesviruses diverge from those of cellular counterparts sufficiently to raise the question of whether they are in fact protein kinases [4]. Nevertheless, despite low sequence homology within the group [40], CHPKs represent a group of protein kinases with some distinct common features and potentially conserved biological role(s) in viral infection. First, the CHPKs are commonly packaged into virions as component of the tegument [21,37,38,65] implying their involvement in the formation, maintenance, and/or disassembly of virion structures through the phosphorylation of tegument components [21,27,65,79]. Second, the CHPKs localize in the nuclei of infected cells [36,56,59,60], which, along with shown interaction with the viral DNA Pol processivity factor [7,8,13,36,41,42,48,79], indicates their involvement in viral DNA replication. Third, all CHPKs autophosphorylate and phosphorylate a common substrate - cellular translation factor EF-1δ [45,69,87,88]. An interesting feature of

the interaction between UL13 homologues and EF-1δ is that both cellular protein kinase cdc2 and UL13 homologues phosphorylate the same EF-1δ amino acid residue [45]. These observations suggest that UL13 homologues may share a function that mimics the cellular cdc2 protein kinase [89]. Fourth, in at least two studies CHPKs were able to complement each other functionally: HCMV UL97 partly substituted for HSV UL13 [90], and EBV BGLF4 complemented the replication of delta-UL97 HCMV [40]. Importantly, this complementation is not always reciprocal - HSV UL13 was unable to complement HCMV UL97 [40]. This phenomenon could be explained by the fact that alphaherpesviruses encode an additional protein kinase (US3 gene product homologues) and therefore the two protein kinases carry out different biological functions, whereas beta- and gammaherpesviruses encode only one multifunctional protein kinase. In fact, beta- and gammaherpesviruses-encoded protein kinases may represent dual-specificity kinases, which are able to phosphorylate both protein and certain nucleoside targets. The latter assertion has only been verified for purified UL97, which phosphorylated both GCV and ACV in vitro [53], although other CHPKs conferred sensitivity to GCV when expressed recombinantly [9,10,12,56-58,73,86,91,92] or when their expression is induced in infected cells [93]. Otherwise, the ability of CHPKs to confer sensitivity to certain nucleotides could be explained by induction of cellular enzymes that metabolize nucleosides; however this hypothesis has never been tested. Fifth, CHPKs can utilize both ATP and GTP as a phosphate donor and prefer $Mn^{2+}$ to $Mg^{2+}$ for optimal activity; salt concentration and pH vary.

Romacker et al. [40] recently reported structural studies of several CHPKs using a molecular modeling approach with cellular Cdk2 as a template. The modeling was possible for HCMV UL97 and RCMV R97, but not for HSV UL13 and EBV BGLF4. Moreover, the authors acknowledged that the accuracy of the resulting models is limited by low sequence homology between target and template and indicate that the modeling was rather intended to confirm overall fold than to address structural details [40]. Hence, the conclusions of this report reiterate the need for experimental determination of CHPKs structure.

## 4. CHPK inhibitors and their antiviral activities

With recognizing CHPKs as potential targets for antiviral drug development [94], a number of compounds that exhibit both anti-CHPK and antiviral activity have been identified.

### 4.1. Maribavir

5,6-dichloro-2-(isopropylamino)-1,ß-L-ribofuranosyl-1-*H*-benzimidazole (1263W94 or maribavir, MBV) [95,96] (Fig. 1) is a potent and selective inhibitor of HCMV and EBV replication [97-99], but is inactive against HSV-1 and -2, VZV, HHV-6 and -7, and KSHV [98]. MBV showed significant antiviral potency in vitro against different HCMV strains, including strains resistant to GCV (mutations at 460, 520, 594 in pUL97), foscarnet (Thr700Ala in the viral polymerase) and BDCRB (Asp344Glu and Ala355Val in pUL89) [97,100,101]. MBV inhibited viral DNA synthesis, however this effect was not mediated by inhibition of the viral DNA polymerase, but rather by a novel mechanism. Several lines of evidence imply that UL97 protein kinase is a target for MBV. First, MBV-resistance of HCMV has been mapped in UL97 at amino acid positions L397R [97], V353A and T409M [102]; all three mutations located upstream of UL97 mutations linked to GCV resistance, closer to kinase domains that are associated with ATP-binding and phosphotransfer. Second, MBV treatment exhibited a phenotype similar to the UL97-knockout [46]. Third, MBV inhibited UL97 kinase activity in vitro [52]. However, several MBV-resistant HCMV strains were isolated recently [103,104] that carry mutations in pUL27, whereas the UL97 gene remained intact. Since EBV is also inhibited by MBV, we have tested the effects of the compound on EBV BGLF4 and found that the kinase was absolutely insensitive to MBV treatment both in vitro [68] and in cell culture [8]. Therefore, the mechanism of action of MBV remains elusive. Despite that, MBV is the

only compound with potential connections to CHPKs that is currently in clinical development by ViroPharma for the prevention of cytomegalovirus (CMV) infections in transplant patients. It has been tested in several phase I/II studies by both GlaxoSmithKline and ViroPharma, in which the drug demonstrated antiviral activity, oral bioavailability and an acceptable safety and tolerability profile [105-109]. A phase III study with MBV as a prophylactic agent in CMV seropositive patients undergoing allogeneic stem cell transplants has been initiated, and another phase III trial in solid organ transplant patients is planned. MBV was granted fast-track status by the US FDA in February 2006 for the prevention of CMV infection in allogeneic bone marrow and solid organ transplant patients, and received orphan drug status in the US for the prevention of CMV viraemia and disease in at-risk populations. The patents covering MBV are held by GlaxoSmithKline and expire in 2015.

### 4.2. Indolocarbazoles

Slater et al. [110] showed the inhibitory effect of Arcyriaflavin A as well as its synthetic analogues (symmetrical indolocarbazoles) on HCMV infection in cultured cells. In follow-up studies Zimmermann et al. [111] and Marschall et al. [91,112] attributed this inhibitory effect to direct targeting and inhibition of the pUL97 kinase activity. The compounds were effective against GCV-sensitive and -resistant HCMV strains, and selected compounds in nanomolar concentrations could reduce virus yield by three orders of magnitude [111]. Marschall et al. [112] also showed that the indolocarbazoles Gö6976 and NGIC-I (Fig. 1) do block the kinase activity in vitro. Reasoning that UL97 inhibitors may be efficient on EBV BGLF4, we have studied inhibitory effects of selected indolocarbazoles in vitro and in cell culture [68]. To our surprise, only one compound, K252a (Fig. 1), inhibited BGLF4 autophosphorylation in vitro; importantly both MBV and NGIC-I, which failed to inhibit BGLF4 activity, efficiently inhibited viral DNA replication at non-toxic concentrations [68]. These results illustrate a potential problem with development of inhibitors targeting CHPKs as a group. Low sequence homology, which likely translates into marked differences in structure, and makes the development of such inhibitors difficult if not impossible.

### 4.3. Quinazolines

In a recent report, Herget et al. [113] described a class of quinazolines as novel pUL97 inhibitors and provided evidence that selected quinazolines qualify for use in the development of anti-HCMV drugs: (i) the quinazolines selected for this study (Ax 7376, Ax 7396, Ax 7543) (Fig. 1) were highly potent and selective inhibitors of UL97 protein kinase activity in vitro (of 17 protein kinases tested, only the UL97 and EGFR protein kinases were efficiently inhibited); (ii) they significantly reduced sensitivity of UL97-expressing cells to GCV; (iii) the kinetics of HCMV inhibition and failure to inhibit replication of an HCMV mutant from which UL97 was deleted argue that pUL97 is the target responsible for the anti-HCMV activities of the quinazolines; (iv) clinical isolates of HCMV possess a quinazoline-sensitive phenotype even after they have acquired GCV and CDV resistance-conferring mutations in the UL97 gene; (v) the emergence of viral resistance to quinazolines was not observed at the frequency of resistance to GCV, as analyzed in long-term treatment experiments. It is noteworthy that the quinazolines selected for this study are structurally related to the drug gefitinib (Iressa; ZD1839), a well-characterized inhibitor of EGFR kinase approved for therapy of non-small cell lung cancer [114].

### 4.4. Anti-UL97/anti-HCMV high-throughput screening

Mett et al. [115] screened a library of 5000 compounds deduced from known protein kinase inhibitors and covering 60 different scaffolds. The compounds were tested for their ability to inhibit protein kinase activity of the purified GST-UL97 in vitro, whereas their cytotoxicity and capability to reduce UL97-mediated GCV toxicity were measured by the in-cell activity

assays [112]. The study identified 93 compounds that were nontoxic, GCV-protective UL97 inhibitors, and antiviral effects of 26 compounds from this group were quantified in HCMV/GFP-based infection and plaque reduction assays. Seventeen compounds out of 26 tested demonstrated pronounced antiviral effects; some of them, such as benzimidazole Ax 6438 (Fig. 1) or quinazolines (described above), may serve as promising leads for further drug development.

## 5. CONCLUSIONS AND PERSPECTIVES

Conserved herpesvirus-encoded protein kinases are crucial for viral infection and represent attractive and novel targets for antiviral therapy. Although the biological significance of CHPKs is unquestioned, and a considerable number of putative substrates of CHPKs have been identified (Table 1), the biological relevance of their phosphorylation remains obscure. Studies of alphaherpesvirus protein kinases are complicated by the fact that these viruses encode at least one more protein kinase [116] and another protein with potential kinase activity [117]. Therefore early studies with mutant viruses lacking CHPK corresponding genes suggested their dispensability for viral infection [14,15]. In contrast, studies of both beta- and gammaherpesviruses demonstrated the severe deficiency of such mutants [39,61] and involvement of CHPKs in various steps of viral infection. The analyses are even more complicated due to the high efficiency of viral genomes, that is, their overlapping and bi-directional genes and transcriptional elements. Therefore, deletion of large fragments may unintendedly result in disruption of more than one viral function. One can envision two ways to address this issue: design of finer, point mutants that will affect kinase activity only - for instance, substitution of an invariable lysine in CHPKs subdomain II abolishes kinase activity and can be used to study the roles of CHPK-mediated phosphorylation in viral infection (such as in [16]); alternatively, development of CHPK-selective inhibitors, which, in addition to their therapeutic potential, will serve as tools in future studies of these protein kinases. The latter approach however requires extensive knowledge of enzyme structure and will greatly benefit from additional studies aimed to determine experimentally structures of CHPKs. Therefore future studies are warranted (i) to verify biologically relevant CHPK targets, (ii) to determine the structure of CHPKs, and (iii) to develop selective CHPK inhibitors, which may lead to effective antiviral drugs.

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## ABBREVIATIONS

CHPK, conserved herpesviral protein kinase
HSV, herpes simplex virus
VZV, varicellazoster virus
HCMV, human cytomegalovirus
EBV, Epstein-Barr virus
KSHV, Kaposi sarcoma associated virus
HHV, human herpesvirus
AIDS, acquired immunodeficiency syndrom
ICP0, infected-cell protein 0
SCID, Severe Combined Immunodeficiency
GCV, ganciclovir (2-amino-9-(1,3-dihydroxypropan-2-yloxymethyl)-3H-purin-6-one)
OXT-G, oxetanocin G (2-amino-9-[(2R,3R,4R)-3,4-bis(hydroxymethyl)oxetan-2-yl]-3H-purin-6-one)
ACV, acyclovir (2-amino-9-(2-hydroxyethoxymethyl)-3H-purin-6-one)

BV-araU, bravavir (5-[(E)-2-bromoethenyl]-1-[(2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidine-2,4-dione)
CDV, cidofovir ([(2S)-1-(4-amino-2-oxo-pyrimidin-1-yl)-3-hydroxypropan-2-yl] oxymethylphosphonic acid)
BVDU, brivudin (5-[(E)-2-bromoethenyl]-1-[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl) oxolan-2-yl]pyrimidine-2,4-dione)
PCV, penciclovir (2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-3H-purin-6-one)
MBV, maribavir ((2S)-2-[5,6-dichloro-2-(propan-2-ylamino)benzoimidazol-1-yl]-5-(hydroxymethyl)oxolane-3,4-diol)
NLS, nuclear localization signal
RNAi, RNA interference
EBNA2, Epstein-Barr nuclear antigen 2
EBNA-LP, Epstein-Barr nuclear antigen leader peptide
GST, Glutathione-S-transferase
BDCRB, (2R,3R,4S,5R)-2-(2-bromo-5,6-dichlorobenzoimidazol-1-yl)-5-(hydroxymethyl) oxolane-3,4-diol
CDK1, cyclin-dependent kinase 1
JNK, c-Jun N-terminal kinase
MCM, minichromosome maintenance proteins

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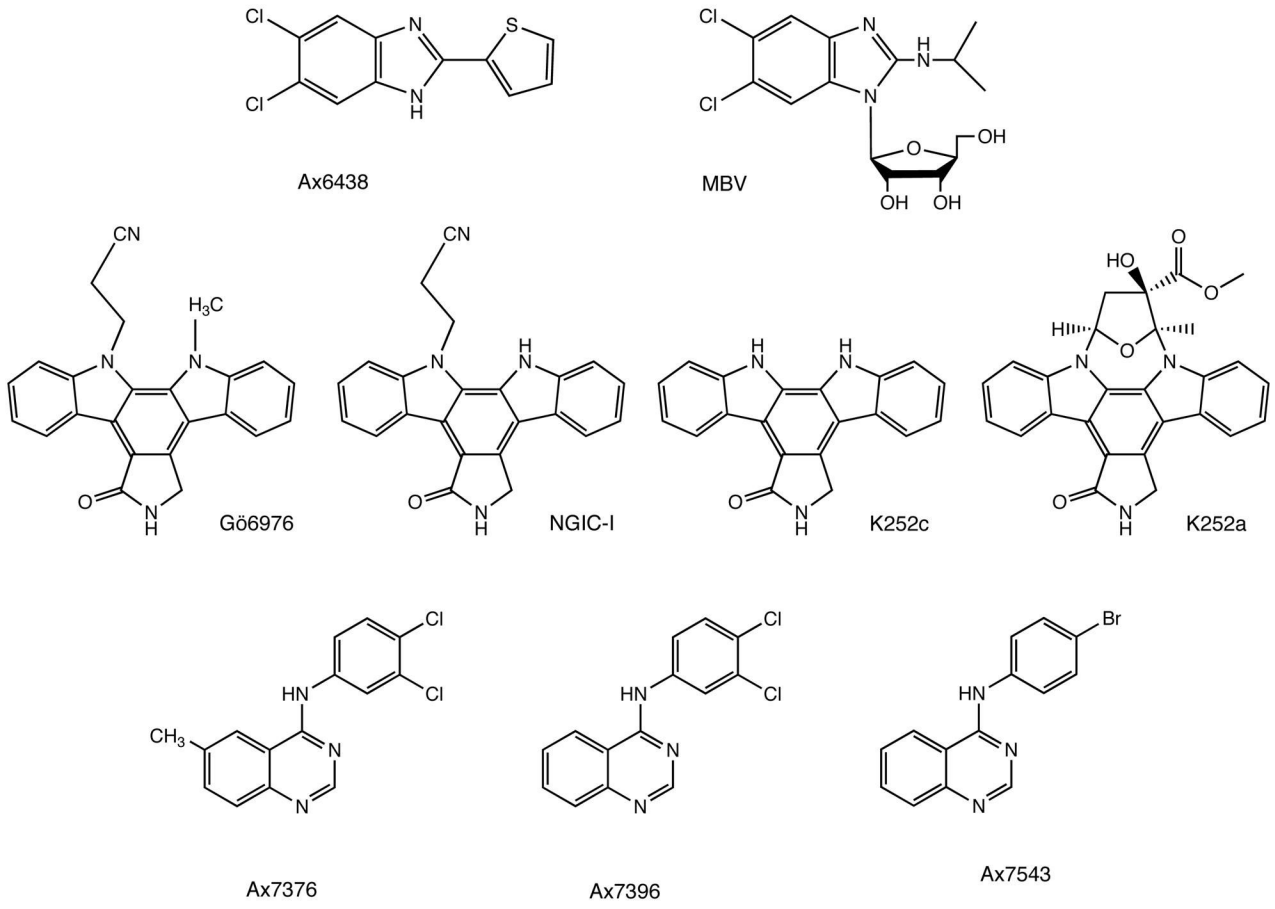


**Figure 1.**
Anti-CHPK inhibitors: benzimidazoles – Ax6438 and MBV; indolocarbazoles - Gö6976, NGIC-I, K252C and K252A; quinozolines – Ax7376, Ax7396 and Ax7543. All listed compounds inhibited HCMV UL97 protein kinase both *in vitro* and in cell culture [91, 111-113,115]; K252A also inhibited EBV BGLF4 protein kinase *in vitro* [68].

**Table 1**

Viral and cellular targets of herpesvirus-encoded protein kinases

| CHPK[§] | Substrates | |
|---|---|---|
| | **Viral** | **Cellular** |
| HSV-1 UL13 | gE/gI [118], ICP0 [119], ICP22/Us1.5 [120], VP22 [15], US3 [18] | CKIIß [45], EF-1δ [88], p60 [121], RNA Pol II [122] |
| VZV ORF47 | gE [26], ORF32 [28], ORF62 [25], ORF63 [23], ORF9 [27] | |
| HCMV UL97[*/**] | UL44 [41,42] | EF-1δ [87], p32 and lamin A/C [47] |
| HHV-6 U69[*] | U69 [12] | |
| EBV BGLF4[*] | EA-D [7,8], EBNA-LP [66], Z [65], EBNA2 [67], BZLF1 [65] | CKIIß [45], EF-1δ [69], condensin [63], MCM4 [64] |
| KSHV ORF36[*] | K-bZIP [79] | JNK [80] |

§ -all CHPKs autophosphorylate.

* -These protein kinases have been reported to confer ganciclovir (GCV) sensitivity [9,10,12,56-58,73,86,91,92].

** -HCMV UL97 has the ability to phosphorylate GCV and ACV in vitro [53].